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REVIEW ARTICLE

INTERNATIONAL JOURNAL OF BIOMEDICINE

The Possible Role of Herpesviruses in the Pathogenesis of Coronary Atherosclerosis

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Abstract

Cardiovascular diseases are still the dominant cause of death worldwide. Coronary artery disease (CAD) is the most common type of heart disease and the leading cause of death for both men and women. Coronary atherosclerosis underlies multiple clinical manifestations ranging from asymptomatic to stable angina, acute coronary syndrome, MI, heart failure, and sudden cardiac death. The prerequisites for a closer study of the pathogenesis of the atherosclerotic process were the development of atherosclerotic vascular lesions at a younger age and the rapid progression of the process. Currently, it is generally accepted that CAD is a multifactorial disease. Attention is drawn to hereditary disorders of the receptor apparatus, endothelial dysfunction, and lipid metabolism disorders. In addition, latent viral infections are one of the etiopathogenetic factors in the development of atherosclerosis. A number of scientific studies have confirmed the relationship between infectious agents and the development of atherosclerotic vascular lesions. The viral etiology of the development and progression of atherosclerosis is the subject of debate among scientists around the world. (**International Journal of Biomedicine. 2021;11(4):391-396.**)

Key Words: herpesviruses • coronary atherosclerosis • Toll-like receptors • vascular endothelial cells

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Abbreviations

CAD, coronary artery disease; **CA**, coronary atherosclerosis; **CMV**, cytomegalovirus; **CRP**, C-reactive protein; **EBV**, Epstein-Barr virus; **FCs**, foam cells; **HSV**, herpes simplex virus; **HAV,** hepatitis A virus; **HBV**, hepatitis B virus; **HCV**, hepatitis C virus; **ox-LDL,** oxidized low-density lipoprotein; **MI**, myocardial infarction; **MMP**, matrix metalloproteinases; **SMCs**, smooth muscle cells; **TLRs**, Toll-like receptors; **TNF-α**, tumor necrosis factor-alpha; **VSMCs**, vascular smooth muscle cells; **VECs**, vascular endothelial cells.

Coronary atherosclerosis (CA) is one of the main causes of disability and premature death of people all over the world,[1] the development of which can take many years and not have any pronounced symptoms. The first signs of the problem appear in cases when the pathological process is pronounced. During the past decades, our understanding of the pathophysiology of CAD has undergone a remarkable evolution. There are numerous factors involved in the causation of atherosclerosis. Many studies have found a significant association between atherosclerosis and smoking, sedentary lifestyle, hypertension, atherogenic dyslipidemia, genetic factors, and life stress.[2-7] However, about 30% of patients with atherosclerosis lack identified risk factors, an observation indicating that additional factors predispose to atherosclerosis.

Injury to the vessel wall and the associated inflammatory response to injury are now generally recognized as the essential components of atherogenesis. Among candidates for triggers are ox-LDL and heat shock proteins. Another candidate trigger of both inflammatory and autoimmune responses may be an infection.[8]

Since the 19th century, it has been suspected that the development of atherosclerotic plaques and their rupture is associated with inflammation caused by infection. The idea that atherosclerosis results from injury to arterial walls traces back to Rudolph Virchow. In 1856, Virchow recognized the inflammatory nature of atherosclerotic plaques.[9] "In some, particularly violent cases the softening manifests itself even in the arteries, not as the consequence of a really fatty process, but as a direct product of inflammation."[10,11]

In the last decades, various studies were conducted on the association between CAD and infectious agents, including viruses.[12-15] In 1983, Fabricant CG et al.[16] showed that repeated experiments have established that infection with Marek's disease herpesvirus (MDV) leads to atherosclerosis in specific pathogen-free normocholesterolemic chickens.

Currently, many viruses are known to be associated with atherosclerosis. A number of herpes viruses have been identified in atherosclerotic coronary arteries.[17-19] These herpes viruses include CMV (human herpesvirus 5),[20,21] EBV(human herpesvirus 4)[22] and HSV(human herpesvirus 1 and 2).[23-25] In addition, the possible role of HAV,[26] HBV,[27] and HCV [28,29] in the development of CA was also shown.

Although the evidence is not entirely consistent, infections of vascular wall cells lead to inflammation and atherosclerosis. The mechanism of viral damage to the vascular endothelium is universal.[30] All viruses possess pathogen-associated molecular structures, such as lipopolysaccharides, released by viral RNA,[31] which interact with Toll-like receptors (TLRs) located on the cell membrane of immunocompetent cells. TLRs are major initiators of inflammation. Previous studies involving SNP analysis of TLRs demonstrated that TLRs are involved in the development and progression of diseases like atherosclerosis, cardiac dysfunction in sepsis and congestive heart failure.[30-32]

TLR2 promotes atherosclerosis in LDL receptor (LDLr)-deficient mice fed a high-fat diet (HFD).[33] In atherosclerosis-susceptible low-density lipoprotein receptor-deficient (Ldlr-/-) mice, complete deficiency of TLR2 led to a reduction in atherosclerosis.[33] In a study by Curtiss et al.,[34] deficiency of TLR1 or TLR6 did not diminish HFD-driven disease. When HFD-fed, LDLr-deficient mice were challenged with Pam3 or MALP2, specific exogenous ligands of TLR2/1 or TLR2/6, respectively, atherosclerotic lesions developed with remarkable intensity in the abdominal segment of the descending aorta.

Michelsen et al.[35] demonstrated that genetic deficiency of TLR4 was associated with a significant reduction of aortic plaque areas in atherosclerosis-prone, APOE-deficient mice, despite persistent hypercholesterolemia, implying an important role for the innate immune system in atherogenesis.

In a study by Ding Y,[36] TLR4 deficiency led to markedly decreased atherosclerosis in obese, TLR4 and LDL receptor double knockout mice. It has been observed that TLR3 has a protective effect on the vascular wall after mechanical and hypercholesterolemia-induced arterial injury. TLR3 plays a critical role in regulating the degradation of the extracellular matrix in lesions, in part by modulation of macrophage MMP-2 and -9 activities.[37]

Karper et al.[38] showed that blocking TLR7 and TLR9 reduced postinterventional vascular remodeling and foam cell accumulation in hypercholesterolemic APOE*3-Leiden mice.

Thus, the mechanisms of atherogenesis induced by TLRs include the dysfunction of vascular cells, the recruitment of macrophages and other immune cells to the site of vascular injury, the formation of foam cells, and the instability of plaques, while the anti-atherosclerotic effect of TLRs is more in line with its evolutionary conservative function.[39]

However, if the general mechanism of the viral contribution to the pathogenesis of CA seems clear, then the study of the specificity of the effect of a wide variety of viral load on the development of CA is at the early stages of research.[17]

For example, CMV is widely distributed, can infect blood vessel wall cells, and exhibit the persistence, latency, and recurrence of infection. The infection can indirectly influence the earliest lesions of atherogenesis consisting of intimal accumulations of FCs and T-lymphocytes intermixed with VSMCs.[40] Host defenses to extravascular infections are usually associated with the production of proinflammatory cytokines and cellular adhesion molecules, enhancing leukocyte adhesion.

Studies have linked CMV to three arterial diseases: primary atherosclerosis, post-angioplasty restenosis, and posttransplantation arteriosclerosis. The Atherosclerosis Risk in Communities Study showed that high levels of CMV antibodies were significantly associated with CAD incidents (RR=1.76; 95% CI: 1.00-3.11) during a 5-year follow-up period.[41] In contrast, the Cardiovascular Health Study demonstrated that the risk of MI and CAD death was associated with the presence of IgG antibodies to HSV-1 (OR=2.0, 95%CI: 1.1-3.6) but was not associated with the presence of IgG antibodies to CMV (OR=1.2, 95% CI: 0.7-1.9).[42] A study by Zhou et al. found that prior infection with CMV was a strong independent risk factor for restenosis after coronary atherectomy.[43]

Sambiase et al.[44] showed a direct role for the CMV in the pathogenesis of accelerated graft coronary atherosclerosis. DNA and/or protein of HSV, HIV, CMV, HCV, EBV, as well as influenza, have been found in atherosclerotic plaques.[13,45]

In experimental studies, it was found that CMV infection can induce severe endothelial dysfunction [46,47] and altered response to oxidized lipids in the sub-endothelium,[48] leading to atherosclerosis progression. Thus, replication of CMV in VECs and macrophages[49] triggers the release of proinflammatory cytokines, adhesion molecules, and MMP, as well as cellular death.[50-53] CMV appears to induce a proliferation of VSMCs by inhibiting the tumor suppression gene p53.[54,55] CMV and HSV can each have an atherogenic effect, including smooth muscle proliferation, increased expression of cytokines, chemokines, and increased uptake of low-density lipoprotein.[56]

Chanouzas et al.[57] showed that the host cellular immune response to CMV leads to the expansion of cytotoxic CD4+CD28null T-cells that express endothelial homing markers and are independently linked to increased arterial stiffness, a marker of cardiovascular mortality.

CD4+CD28null T-cells were CMV-specific and expressed a T-helper 1 (Th1) phenotype with high levels of interferon-gamma (IFN-γ) and TNF-α secretion. As known, IFN-γ and TNF-α cytokines mediate inflammation in blood vessel walls through disruption of endothelial junctions and induction of chemokine and adhesion molecule expression on vascular endothelium. This promotes the recruitment and adherence of lymphocytes and monocytes on the inflamed endothelium and facilitates leukocyte transmigration.[58]

Apostolou et al.[59] reported that acute infection with EBV was associated with atherogenic lipid changes. The results obtained by Gargouri et al.[60] suggest that lipid peroxidation, protein oxidation, and DNA fragmentation are generally induced during EBV lytic cycle induction.

Many studies have demonstrated possible links between HSV and native atherosclerosis.[24,61-64] A meta-analysis performed by Wu et al.[64] indicated that HSV-1 and HSV-2 infections could increase the risk of contracting atherosclerosis. HSV infection of endothelial cells increases endothelial cell synthesis of tissue factor, the rate of thrombin generation on the cell surface, and platelet adherence, while it decreases prostacyclin and thrombomodulin generation.[65-67]

In 1990, Key et al.[66] reported two procoagulant consequences of endothelial HSV infection: loss of surface thrombomodulin activity and induction of synthesis of tissue factor, which could contribute to the deposition of thrombi on atherosclerotic plaques and to the "coagulant-necrosis" state.

The nature of the cellular immune response to three human herpesviruses— HSV1, EBV, and CMV—has received considerable attention.[68-71] Some features of these responses suggest they may have significant modulatory effects on the immune system in totum. CMV DNA is present in atherosclerotic and normal vascular tissue, and the anti-CMV immune response may be increased in the context of severe disease.[72,73]

The mechanisms whereby CMV might promote atherosclerosis in vivo are not completely clear. Some studies suggest that chronic, unresolved inflammatory responses against latently infected cells distributed systemically or directly in the vessel wall may play a role.[74,75] Alternatively, immunomodulatory proteins secreted by CMV may alter the VSMC motility or macrophage activation within atherosclerotic lesions.[76,78] The latent CMV infection is periodically reactivated, resulting in a chronic immune response or inflammatory response that is damaging to the vascular endothelium and inner membrane, resulting in VSMC proliferation and mutation.[79] The replication of CMV in VECs is the key point of persistent viral infection, transmission, and disease onset, and is the most direct cause of endothelial dysfunction and apoptosis.

The formation of antibody immune complexes of CMV antigen deposited in the vascular wall in the atherosclerotic lesions can induce VECs, macrophages, FCs, VSMCs, and T-lymphocytes to express the monocyte proteins CCL-2,−3,−4, and −5 and macrophage colony-stimulating factor.[53,82] Moreover, they also stimulate macrophages to produce and release of interleukins IL-1,−6,−8,−10, and −12, TNF-α, and other inflammatory cell factors, which cause cellular and humoral immune responses, and accelerate the release of CRP to induce an inflammatory chain-reaction.[78] A study by Izadi et al.[80] showed that, due to periodic CMV activation in VSMCs caused by local immune responses and inflammation, VSMCs show degeneration and apoptosis caused by inflammatory substances, leading to instability in plaques, which are prone to rupture and bleeding, leading to acute coronary syndrome.

Many viral infections target hemostasis and coagulation, introducing either hemorrhagic or thrombotic complications.[81] There is strong evidence showing abnormal hemostasis linked to inflammation during viral infections in humans.[21,82-84]

Thus, the biological characteristics of CMV are consistent with the CA pathogenesis.[78] CMV infects VECs, leading to cellular injury and metabolic changes.[85] The activation of latent CMV infection can periodically affect VECs and cause high expression of platelet alpha-granule membrane protein-140, E-selectin, intercellular adhesion molecule-1, vascular cell adhesion molecules, coagulation factors, and other tissue factors.[78] Viruses from VECs then infect VSMCs, and the latent or persistent infection in VSMCs leads to proliferation and the accumulation of cholesterol and cholesterol esters. In infected cells, abnormal apoptotic changes may play an important role in CA pathogenesis.[86] The latent CMV infection is periodically reactivated, resulting in a chronic immune response or inflammatory response that is damaging to the vascular endothelium and inner membrane, resulting in VSMC proliferation and mutation. Inflammatory factors can stimulate monocytes to chemotaxis to the vascular wall and differentiate into macrophages, and form FCs through phagocytosis of modified lipoproteins, such as ox-LDL.[39] With the development of inflammation, activated leukocytes and VECs can release fibroblast growth-regulating factor, induce the phenotype change of VSMCs, migrate from the middle membrane through the inner elastic layer to the subintimal of arteries, and proliferate and express a large number of cytokines and adhesion factors.[39,62] In the late stage of atherosclerosis, inflammatory cytokines and MMP can degrade extracellular matrix proteins, which make the plaque easy to rupture. In addition, inflammatory cells secrete vascular growth factors, which can promote the formation of blood vessels in the plaque, and eventually lead to MI or stroke.[39,87] Large cohort studies indicate that seropositivity for CMV or HCV represents an independent risk marker for cardiovascular diseases.[88,89]

Conclusion

Over the past 30 years, numerous studies provide powerful evidence regarding the involvement of herpesviruses, especially CMV, in the formation of CA. The development of CA is closely related to inflammatory reactions and the immune response, endothelial injury, lipid deposition, metabolic disorders of VSMCs, and coagulation thrombosis. However, the role of viral infection in the development of CA needs further study, since many remaining issues need to be explored and resolved.

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Competing Interests

The authors declare that they have no competing interests.

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REVIEW ARTICLE

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Mechanisms for Cardiac Troponin Increase in Arterial Hypertension

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Abstract

Despite the fact that cardiac troponins (cTnI and cTnT) are cardiospecific, they can be elevated in many systemic and non-cardiac physiological and pathological conditions. The diagnostic value of cTnI and cTnT significantly depends on the method of their determination. Thus, previously used low- and moderate-sensitivity immunoassays detected only serious myocardial damage and did not determine troponins in patients suffering from certain chronic pathologies. High-sensitivity troponin assays can detect minor damage to cardiac muscle cells in many pathological conditions, and troponin levels have a high predictive value. Among the early pathological conditions requiring the attention of clinicians is arterial hypertension (AH), which is also accompanied by an increase in the levels of hsTn in serum and urine. Currently, mechanisms responsible for increased levels of cardiac troponins in the blood serum and urine in hypertension are not well covered in the scientific literature. This article discusses in detail the presumptive mechanisms that cause increased levels of cTnI and cTnT in AH.(**International Journal of Biomedicine. 2021;11(4):397-402.**)

Key Words: cardiac troponins • cardiovascular disease • arterial hypertension • glomerular filtration rate

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Abbreviations

AH, arterial hypertension; **AMI**, acute myocardial infarction; **CVD**, cardiovascular disease; **cTnT**, cardiac troponin T; **cTnI**, cardiac troponin I; **GFR**, glomerular filtration rate; **hsTn**, high-sensitivity troponin; **MMP2**, matrix metalloproteinase-2; **Tn**, troponin; **TnT**, troponin T.

The cardiac troponin (cTn) complex is a critical regulator of cardiac muscle contraction. cTn is composed of three distinct subunits named according to their functions: a highly conserved Ca^{2+} binding subunit (cTnC); an actomyosin ATPase inhibitory subunit (cTnI), and a tropomyosin binding subunit (cTnT).[1,2] The importance of cTn in the regulation of myocardial function is evidenced by the fact that mutations causing changes in the amino acid sequence in cTnI, cTnT, cTnC are accompanied by significant and life-threatening disorders of the contractile function of the cardiac muscle, and in particular, hereditary cardiomyopathies.[3-5] The amino acid composition of cTnC is similar to the amino acid composition of TnC in skeletal muscle fibers; therefore, this protein is not used as a biomarker of AMI. On the contrary, the amino acid composition of cTnI and cTnT is unique, giving them the necessary specificity, which is very important for use in AMI diagnosis.[6-8] In addition to the specific structure of the protein, the features of determination methods play an important role in laboratory diagnostics, which are constantly being improved and change our understanding of the biology and diagnostic value of many biomarkers, including cTnI and cTnT.[6-10] For example, the methods for determining cTnI and cTnT, originally developed by Cummins and Katus,[11-13] were characterized by low sensitivity and specificity, which was manifested by the relatively late detection of diagnostically

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significant concentrations in AMI patients and a significant number of nonspecific (cross) reactions of anti-cTnI and anti-cTnT antibodies with troponins released from damaged skeletal muscle fibers during rhabdomyolysis and/or exercise.[14-16]

The use of hs-cTn assays showed that the levels of cTnI and cTnT depend on a number of biological factors, including gender, age, and circadian characteristics.[17-20] The gender peculiarities of the levels of cTnI and cTnT are that the serum levels of the latter are significantly higher in men than in women, which is typical for almost all high-sensitivity immunoassays currently used. By analogy with another cardiac-specific enzyme (creatine kinase) and creatinine, a product of protein metabolism, the gender differences in troponin concentrations are due to a higher mass of striated muscles, including cardiac muscles, in males.[6,17] Age-related features of the levels of cTnI and cTnT are characterized by higher levels of troponins in elderly patients than in young patients and, most likely, are associated with the presence of chronic latent comorbid pathologies that cause subclinical lesions of cardiomyocytes.[6,21-23] Circadian features consist in the predominance of morning levels of troponins over evening ones, which is explained by the increased activity of a number of systems of the human body. The increased activity of these systems is an evolutionarily developed adaptive mechanism necessary for a healthy person for the period of wakefulness;[24] however, these systems also have a negative effect on myocardial cells.[25] It should be noted that a number of studies using high-sensitivity immunoassays have shown that cTnI and cTnT can be determined not only in blood serum, but also in urine and saliva,[26-31] and the levels of cTnI and cTnT significantly differ between the patient groups and healthy individuals. In the future, the indicated scientific data will allow the development of new diagnostic approaches. In particular, the idea of creating test strips to determine cTnI and cTnT in non-invasively obtained biological fluids has been proposed.[32]

High-sensitivity methods for determining cTnI and cTnT have demonstrated that cardiomyocytes are extremely sensitive to any kind of damage, and the concentration of cTnI and cTnT can increase in many pathological and physiological conditions (Table 1). Moreover, even in healthy patients, cTnI and cTnT are released from cardiomyocytes, but their concentration, as a rule, does not exceed the 99th percentile.[9] The mechanisms underlying the cTnI and cTnT release from cardiomyocytes and, accordingly, their increase in serum in healthy patients, have not been conclusively established.

Currently, there are a considerable number of review articles that discuss in detail the mechanisms for increasing cTnI and cTnT in many cardiac (myocarditis, cardiomyopathy, heart failure, arrhythmias)[32-35] and non-cardiac pathologies; (sepsis, exercise, renal failure, cancer, the use of chemotherapeutic drugs)[46-39] however, AH is rarely discussed in this respect. The purpose of this article was to review some hypothetical mechanisms for increasing cTnI and cTnT in human biological fluids.

Mechanisms responsible for increased levels of cTnI and cTnT in AH

Although the molecules of cardiac troponins, taking into account their molecular weight (cTnI-25 kDa, cTnT-37 kDa), are low-molecular-weight proteins; whole molecules cannot pass through the intact membrane of cardiomyocytes. However, like any protein molecules, cTnT and cTnI are extremely sensitive to the action of proteases, which can be activated under certain pathological conditions. According to the results of experimental studies, stretching of the cardiac muscle tissue, oxidative stress, and ischemia of cardiomyocytes lead to the activation of MMP2, which, in turn, cleaves the cTnI molecule into small peptide fragments that can pass through the cardiomyocyte membrane into the extracellular space and, further, into the blood.[40-42] Another intracellular enzyme that can cause the degradation of cTnI is calpain-1, whose activity

Table 1.

Pathological and physiological conditions causing an increase in the levels of cardiac troponins in addition to AMI, according to [8, 19] with changes and additions

The possible causes of elevated serum cTns in addition to AMI		
Myocardial damage in cardiac pathologies	Myocardial damage in non-cardiac and systemic pathological and physiological conditions	False positive cTn elevation
• Inflammatory heart diseases (endocarditis, myocarditis, pericarditis) • Cardiac arrhythmias (atrial fibrillation, supraventricular tachycardia and others) • Cardiomyopathies (all types) and heart failure • Cardiotoxic drugs (chemotherapeutic drugs for the treatment of cancer, adrenomimetics, cocaine, and others) • Cardiac surgery (CABG, catheter ablation, and others)	• Physiological conditions (heavy physical exertion, stressful situations, old age, male gender, morning time interval) • Sepsis • Chronic diseases (chronic renal failure, chronic obstructive pulmonary disease, diabetes mellitus, oncological diseases) • Pulmonary embolism • Disorders of the central nervous system stroke, subarachnoid hemorrhage) • COVID-19	• Heterophilic antibodies • Rheumatoid factor • Biotin • Hemolysis • Fibrin clots in the blood serum • Cross (nonspecific) reactions of diagnostic antibodies with skeletal muscle troponin isoforms

increases with increasing load on the myocardium under experimental conditions.[43] Blocking calpain-1 with a specific inhibitor reduces the degradation of the troponin molecule. [44] Since under conditions of AH the load on the myocardium increases significantly, the fragmentation of troponins and the release of the smaller fragments from cardiomyocytes can be considered very reasonable.

In addition to proteolytic cleavage of troponins, the activated proteases are likely to induce proteolysis and cleavage of protein components of the cardiomyocyte membrane, facilitating the release of cytoplasmic proteins. In cardiomyocytes, approximately 5% of the total troponins are located outside the troponin complex directly in the cytoplasm (cytoplasmic or non-structural fraction). It is believed that the troponin proteins that make up this fraction are released first in pathological and physiological conditions. At the same time, given the relatively small volume of this fraction, the troponin levels in reversible myocardial damage, for example, during heavy physical exertion or stressful situations, do not exceed the 99th percentile by more than 3-5 times.[45-47] Considering the small degree of an increase in cardiac troponins in AH, it can be assumed that the key contributor to the increase is also made by the cytoplasmic fraction of troponins.[48-50]

Along with the intracellular proteolytic cleavage of troponins, the membrane permeability of cardiomyocytes also plays an important role, which can change under a number of physiological and pathological conditions. According to Hessel et al., myocardial overload leads to stretching of the heart muscle and activation of transmembrane glycoprotein receptors called integrins. These proteins function as mechanotransducers, increasing membrane permeability, and activating MMP-2 and calpain-1, which additionally enhance proteolytic degradation of troponins.[51] Thus, the cleavage of troponins into small fragments and an increase in the permeability of the cardiomyocyte membrane create the necessary conditions for the release of the cytoplasmic pool of troponins, which leads to increasing the levels of cardiac troponins in AH.

Apoptosis of cardiomyocytes

Apoptosis of cardiomyocytes is initiated by a number of mechanisms that may be associated with the development and progression of AH. According to the results of a study by Cheng et al.,[52] stretching the heart muscle increases oxidative stress and the expression of the Fas protein, which is one of the key inducers for programmed cell death. Another mechanism causing increased apoptosis of cardiomyocytes is the action of the adrenergic system, an increase in the activity of which is very characteristic for AH. Experimental studies have shown that the effect of beta-adrenergic receptor agonists (norepinephrine and isoproterenol) on cardiomyocytes is that they trigger intracellular apoptotic signals by cAMP-dependent and NF2-dependent mechanisms.[53-55] Programmed death of cardiomyocytes can lead to very significant increases in cTn levels, as demonstrated in a recent experimental study by Weil et al.[56] In that experiment, the researchers initiated apoptosis in the porcine myocardium by short-term overloading of the left ventricle with pressure. At the same time, after 30 minutes the levels of troponins already exceeded the upper limit of the norm, and after 1 and 24 hours the concentrations of TnT reached relatively high values (856±956 ng/l and 1.462±1.691 ng/l, respectively). At the same time, the researchers did not reveal any histological signs of cardiomyocyte necrosis, which indicates that the mechanism for apoptosis of myocardial cells was responsible for the increase in serum troponin levels.[56]

Features of cTn elimination through the glomerular filter: Influencing factors and possibilities of non-invasive diagnostics

In addition to the mechanisms for the release of troponin into the systemic circulation following myocardial cell injury, the mechanisms for troponin elimination from blood play an important role. Thus, in patients with no signs of CVD, but with signs of chronic renal failure, elevated troponin levels are often observed.[57,58] Moreover, in patients with a lower GFR, troponin levels are higher than in patients with a higher GFR. [58] However, direct evidence for the elimination of troponins through the glomerular filter, in particular studies confirming the presence of troponins in urine, has been lacking for a long time. In some studies, urinary troponin levels were detected only in isolated cases, and therefore this mechanism for troponin elimination was considered questionable.[59] Troponin molecules were considered relatively large and, according to some authors, could not pass through the glomerular filter.[59] However, several recent studies have shown the presence of troponin molecules in urine in patients with CVD. For example, in a study by Pervan et al.,[26] hsTnI was found in morning urine in patients with AH and normotensive individuals. However, in hypertensive patients, the mean hsTnI value was higher than in those with normal blood pressure. Since AH enhances GFR, it is probably this mechanism that determines the results obtained. Levels of troponins in urine are relatively low, which explains why moderate-sensitivity assays did not detect these concentrations. It should be noted that in the study by Pervan et al.,[26] a high-sensitivity troponin assay was used to determine hsTnI in urine. In a study by Chen et al.,[27] the authors observed significantly higher levels of urine hsTnI in patients with diabetes mellitus than in those without subsequent incident cardiovascular events. The multivariate logistic regression analysis using different models consistently showed that urine hsTnI >4.10 pg/mL was an independent factor predictive of incident cardiovascular events.

A possible explanation for how troponin molecules penetrate the glomerular filter is the proteolytic cleavage processes under the influence of a number of intra- and extracellular proteinases, most likely, splitting into small fragments that can penetrate into urine and saliva.[26-28,60] However, the processes of proteolytic cleavage of troponins inside cells and in blood serum are extremely poorly understood. Although researchers report several dozen fragments of various molecular weights and sizes, all the enzymes that are responsible for the cleavage of troponins and the formation of such a significant number of fragments are unknown.[61-63] At the same time, the results of the study by Katrukha et al. [61] suggest that the 29-kDa fragment of cTnT in AMI serum samples mainly appears due to the cleavage by thrombin during serum sample preparation. It is noteworthy

that under conditions of hypertension, thrombin is activated,[64] and, accordingly, the processes of proteolytic cleavage of troponins into small fragments are enhanced, and an increase in GFR promotes the elimination of formed small fragments through the glomerular filter into the urine. The identification of all factors influencing the proteolytic degradation of troponin molecules is essential for understanding this process and improving laboratory diagnostics, including the use of urine as a non-invasive biomaterial. The mechanisms described above for increasing the levels of cTnT and cTnI in human biological fluids in AH are summarized in Figure 1.

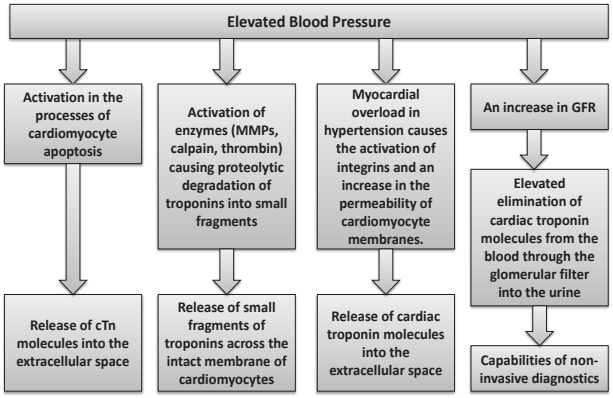

Fig. 1. *Mechanisms responsible for increased levels of cardiac troponins in AH.*

In conclusion, the increase in the levels of cardiac troponins in AH is based on the following mechanisms: activation of proteolytic cleavage of troponin molecules inside cardiomyocytes, the increased permeability of cardiomyocyte membranes, and the increased apoptotic processes, as well as the effect of AH on the filtration of troponin fragments through the glomerular filter into the urine. Measurement of urinary hsTn, collected easily and non-invasively, can be an acceptable biomarker with a high diagnostic value.

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ORIGINAL ARTICLE

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Assessment of Left Ventricle Myocardial Function in Hypertensive Patients Using Three-Dimensional Speckle-Tracking Echocardiography (3D-STE)

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Abstract

***Background*:** Increased myocardial fibrosis in hypertension leads to abnormalities in left ventricular diastolic function. 3D-speckle-tracking imaging (3D-STI) is a primary imaging modality used to detect early changes in the left ventricle (LV). The aim of this study was to assess the left ventricular myocardial function in hypertensive patients using 3D-speckle tracking imaging (3D-STI).

***Methods and Results*:** A case control, nonintervention, descriptive study was conducted in the Department of Ultrasound Diagnosis of Union Hospital of Tongji Medical College of Huazhong University of Science and Technology (Wuhan, Hubei, China). The study subjects included 64 patients with hypertension (HT) and, as control group, 44 normotensives. HT patients were divided into HT-I group (SBP of 130-139 mmHg or DBP of 80-89 mmHg, and HT-II group (SBP >140 mmHg or DBP >90 mmHg). In this study, LV geometry and function were assessed using conventional 2D- and 3D-echocardiography in a total of 108 consecutive subjects. LV volumes, global and regional strains were measured using 3D-STI. LV ejection fraction (LVEF) was in normal range in three groups, but in general, it slightly decreased in HT-II patients, compared with control and HT-I groups (62.5±2.1%, 68.0±2.2%, and 67.5±1.3%, respectively, *P*=0.00). Global systolic strain demonstrated a significant decrease in GLS, GCS, and GRS in the HT-II group, compared with control and HT-I groups. All regional strain parameters (longitudinal, circumferential, and radial) significantly decreased in HT-II patients, compared with control and HT-I groups.

Conclusion: A significant deterioration of global LV systolic functions is found in hypertensive patients with well-preserved LVEF, especially in patients with hypertension stage II.(**International Journal of Biomedicine. 2021;11(4):403-409.**)

Key Words: hypertension • left ventricle • speckle tracking • global strain • regional strain

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Abbreviations

3D-STE, three-dimensional speckle-tracking echocardiography; **3D-STI**, three-dimensional speckle-tracking imaging; **ASE**, American Society of Echocardiography; **BP**, blood pressure; **BSA**, body surface area; **DBP**, diastolic BP; **EDV**, end-diastolic velocity; **GLS**, global longitudinal strain; **GCS**, global circumferential strain; **GRS**, global radial strain; **HT**, hypertension; **IVSd**, interventricular septal thickness; **LAd**, left atrial dimension; **LV**, left ventricle; **LVEF**, left ventricular ejection fraction; **LVEDV**, left ventricular end-diastolic volume; **LVESV**, left ventricular end-systolic volume; **LDEDd**, left ventricular end-diastolic dimension; **LVH**, left ventricular hypertrophy; **LVM**, left ventricular mass; **LVMI**, LVM index; **LVSV**, left ventricular stroke volume; **PWT**, posterior wall thickness; **RWT**, relative wall thickness; **SBP**, systolic BP; **SR**, strain rate; **SSR**, systolic strain rate; **SV**, systolic velocity.

Introduction

According to the 2017 American College of Cardiology (ACC) and the American Heart Association (AHA) guideline, hypertension is defined as SBP ≥130 mmHg or DBP ≥80 mmHg.[1] The prevalence of hypertension rises dramatically with increasing age and is higher in blacks than in whites, Asians, and Hispanic Americans.[1,2]

Early detection of LV dysfunction before the development of LVH is a finding that requires extensive treatment to reduce morbidity and mortality due to cardiovascular disease; therefore, it has to be considered in the evaluation of global cardiovascular risk.[3]

Echocardiography is still the primary imaging modality for assessing cardiac function; The most common index of the myocardial systolic function used in routine clinical practice is LVEF, but conversely, in several studies, LVEF was found to be suboptimal for detecting early myocardial dysfunction, likely due to undetectable wall deformation.[4,5]

3D-STE provides an accurate determination of subclinical myocardial dysfunction and also demonstrates comprehensive information about the cardiac ventricle. In addition, the accuracy and reproducibility of 3D-STE in evaluating the function and structure of the LV has been previously demonstrated.[6,7]

We hypothesize that 3D-STI may provide a reproducible measurement for the evaluation of LV subclinical myocardial changes in patients with primary hypertension.

Materials and Methods

A case control, nonintervention, descriptive study was conducted in the Department of Ultrasound Diagnosis of Union Hospital of Tongji Medical College of Huazhong University of Science and Technology (Wuhan, Hubei, China) during a period from June 2017 to May 2018. In this study, LV geometry and function were assessed using conventional 2D- and 3D-echocardiography in a total of 108 consecutive subjects. The study subjects included 64 HT patients and, as control group, 44 normotensives. HT patients were divided into HT-I group (SBP of 130-139 mmHg or DBP of 80-89 mmHg, and HT-II group (SBP >140 mmHg or DBP >90 mmHg).

Inclusion criteria

Patients aged ≥27 years of different genders with a diagnosis of primary hypertension were included in hypertensive groups. The characteristic features of hypertension patients matched the diagnostic standards of the 2017 ACC/AHA guidelines.[8]

The control group included the participants with no hypertension or diabetes, no use of medication, no cardiac symptoms, and no abnormality detected in the heart in physical examination, electrocardiogram, and echocardiography results.

Exclusion criteria

Any patients with secondary causes of HT, coronary artery diseases, congenital heart disease, severe valvular stenosis or regurgitation, abnormal wall motion, heart failure, arrhythmia, pericardial effusion (moderate or massive) and lung disease were excluded.

Echocardiography image acquisition

The cardiac structure, chamber size, wall thickness, and cardiac function were evaluated by a conventional transthoracic echocardiogram, following the recommendations of the ASE.[9] A commercially available system (IE Elite, Philips Medical Systems, Andover, MA) with an S5-1 broadband phased-array transducer was used. A 3D echocardiographic examination was given to all subjects, using the same system and X5-1 transducer. Images were adjusted to assess the complete LV during a full-volume dataset within the four-chamber apical view. Datasets were acquired with an acquisition setting of four or seven heartbeats. End-expiratory breath holding was done whenever possible. At least four datasets were acquired for each subject, then for offline analysis, three datasets with the best image quality were selected.[10] Datasets that did not include the whole LV, had indefinite endocardial borders, or had obvious stitch artifacts were excluded.

Echocardiographic parameters

The cardiac chamber size, LVM, and LVEF were measured according to the ASE chamber quantification guidelines.[9] IVSd, LVEDd, PWT, and LAd were measured in the parasternal long-axis view. RWT was calculated by dividing myocardial thickness (IVST+PWT) by LVEDd. Trans-mitral peak velocity of early diastole (E) and late diastole (A) was measured by pulse wave Doppler. The ratio of E/A was calculated. For the semi-automatic analysis of LVM, QLAB 3D (Philips Medical Systems) quantification software algorithms were used.

3D speckle-tracking echocardiography (3D-STE)

Offline 3D-STE analysis was obtained with 4D LV-Analysis 3.0 (TomTec Imaging Systems, Unterschleissheim, Germany echocardiographic quantification software). The technique used for measurement is same as Zhang et al.: "Measurements were made using the data set with the best image quality, which was selected by consensus of the two readers. The frame rate of the volumetric image was 15 to 24 frames/sec. The 3D data sets were displayed as multiplanar reconstruction images corresponding to four tiles containing three standard LAX views and a short-axis view, which is orthogonal to the LAX views."[11] The offline analysis method was used. The following strain parameters were assessed during LV strain analysis: GLS (LV peak systolic global longitudinal strain), GRS (LV peak systolic global radial strain), GCS (LV peak systolic global circumferential strain). The three parameters were averaged over the 16 segments: "The peak value of each index was defined as its maximum absolute value with a positive or negative sign. Negative strain values reflect shortening, whereas positive strain values represent lengthening or thickening. All strain values are dimensionless and are expressed as percentages."[11,12]

Statistical analysis was performed using the IBM SPSS Statistics V22.0 (SPSS Inc., Chicago, IL, USA). The

normality of distribution of continuous variables was tested by one-sample Kolmogorov-Smirnov test. Continuous variables with normal distribution were presented as mean±standard deviation (SD). Multiple comparisons were performed with one-way ANOVA. Correlation coefficients (R^2) were calculated by linear regression analysis. A probability value of $P<0.05$ was considered statistically significant.

Results

Clinical characteristics

The demographic characteristics of two hypertensive groups (HT-I, HT-II) and the control group are summarized in Table 1. No significant differences in sex, height, and body surface area between the control, HT-I and HT-II groups were found.

Table 1.

Comparison between the study groups regarding demographic and clinical data

Variables	Control group (n=44)	HT-I group (n=20)	HT-II group (n=44)	*P*-value
Age, yrs	53.5 ±7.9	47.8±10.5*	56.3 ±11.6#	0.01
Gender M/F	30/14	12/8	30/14	0.79
SBP, mmHg	119.3±8.4	135.7±3.9*	153.5±24.7*#	0.00
DBP, mmHg	75.2±9.8	85.8±4.3*	93.5±19.1*	0.00
Height, cm	1.6±0.8	1.6±0.7	1.6±0.1	0.11
Weight, kg	62.7±10.4	67±9.6	69.9±9.4*	0.00
BSA, m^2	1.6±0.1	1.7±0.1	1.7±0.1	0.10

** Compared with control group, P <0.05; # compared with HT-I group, P <0.05.*

2D-echocardiographic analysis

The study found significant differences in RWT between HT groups and normotensive controls (0.4±0.1cm for HT- I group and HT-II respectively, 0.3±0.1cm for the control group, *P*=0.03). The parameters of diastolic function, such as E/A ratio, show significant differences between the control, HT-I and HT-II groups (1.4±0.9, 0.9±0.3, and 0.8±0.3, respectively, *P*=0.00). The LVEDd, PWT, LAd and LVM in the HT-II group were significantly higher than those of the control and HT-I groups (Table 2).

3D-STE analysis

The 3D-echocardiography found no significant differences between the control, HT-I and HT-II groups, regarding end-diastolic velocity and systolic velocity (Table 3). A significant difference was found in LVESV among groups: it was higher in the HT-II group than in the control and HT-I groups (42.8±10.7 mL, 35.7±6.7 mL, and 37.2±8.4 mL, respectively, *P*=0.00). Concerning LVEF, the study revealed that it was in normal range in three groups, but in general, it slightly decreased in HT-II patients, compared with control and HT-I groups (62.5±2.1%, 68.0±2.2%, and 67.5±1.3%, respectively, *P*=0.00). Global systolic strain demonstrated a significant decrease in GLS, GCS, and GRS in the HT-II group, compared with control and HT-I groups (-15.9±1.6%, -23.5±1.4%, and -20.2±0.9%, respectively, *P*=0.00; -33.7±1.8%, -37.4±1.7%, and -38.6±1.3%, respectively, *P*=0.00; and 41.0±2.1%, 51.1±1.7%, and 49.0±1.4%, respectively, *P*=0.00). Compared with controls, GLS and GRS decreased ($P<0.05$), but GCS in the HT-I group increased ($P<0.05$) (Table 3).

Table 2.

Comparison between the study groups by 2D-conventional echocardiography

Variables	Control group (n=44)	HT-I group (n=20)	HT-II group (n=44)	*P*-value
LAd, cm	2.9±0.3	3.5±0.4*	3.9±0.4*#	0.00
LVEDd, cm	4.5±0.3	4.6±0.4	4.8±0.3*#	0.00
IVSd, cm	0.9±0.1	1.0±0.1*	1.1±0.2*#	0.00
PWT, cm	0.8±0.1	0.9±0.2*	1.0±0.2*	0.00
RWT, cm	0.3±0.1	0.4±0.1*	0.4±0.1*	0.03
LVM, g	118.5±23.9	146.0±39.0*	202.7±42.8*#	0.00
E/A	1.4±0.9	0.9±0.3*	0.8 ±0.3*	0.00

*LAd, left atrium diameter; LVEDd, left ventricular end-diastolic dimension; IVSd, interventricular septum thickness; PWT, posterior wall thickness; RWT, relative wall thickness; LVM, left ventricular mass; * compared with control group, P <0.05; # compared with HT-I group, P <0.05.*

Table 3.

Comparison between the study groups by 3D-STE global strain parameters

Variables	Control group (n=44)	HT-I group (n=20)	HT-II group (n=44)	*P*-value
LVEDV, mL	111.3±18.3	113.8±26.6	113.7±25.4	0.87
LVESV, mL	35.7±6.7	37.2±8.4	42.8±10.7*#	0.00
LVSV, mL	75.6±12.2	76.6±18.3	70.9±15.0	0.21
LVEF, %	68.0±2.2	67.5±1.3	62.5±2.1*#	0.00
GCS, %	-37.4±1.7	-38.6±1.3*	-33.7±1.8*#	0.00
GLS, %	-23.5±1.4	-20.2±0.9*	-15.9±1.6*#	0.00
GRS, %	51.1±1.7	49.0±1.4*	41.0±2.1*#	0.00

EDV, end-diastolic volume; ESV, end-systolic volume; SV, stroke volume; LVEF, left ventricular ejection fraction; GLS, global longitudinal strain; GCS, global circumferential strain; GRS, global radial strain. compared with control group, P<0.05; #compared with HT-I group, P<0.05.*

In 3D-STE of regional strain parameters, significant differences were demonstrated in this study in the longitudinal, circumferential, and radial (basal, middle and apical strains) strains among three groups. All regional strain parameters (longitudinal, circumferential, and radial) significantly decreased in HT-II patients, compared with control and HT-I groups (Table 4, Fig.1-3).

Table 4.

Comparison between the study groups by 3D-STE regional strain parameters

Variables	Control group (n=44)	HT-I group (n=20)	HT-II group (n=44)	*P*-value
CS/B, %	-31.8±3.6	-32.2±3.1	-29.5±3.1*#	0.01
CS/M,%	-39.9±3.2	-41.5±2.1	-37.2±2.8*#	0.00
CS/A,%	-45.1±3.5	-47.1±4.0*	-39.1±4.7*#	0.00
RS/B,%	45.2±4.1	41.6±2.8*	38.5±3.1*#	0.00
RS/M,%	50.9±4.5	53.1±5.2	41.8±3.3*#	0.00
RSA,%	61.6±5.6	59.0±4.0*	47.0±5.5*#	0.00
LS/B,%	-23.2±3.4	-18.0±2.0*	-17.1±3.0*	0.00
LS/M,%	-21.1±3.3	-19.8±2.4	-13.9±2.7*#	0.00
LS/A,%	-27.9±4.7	-24.6±2.6*	-17.7±3.5*#	0.00

*CS/B, basal circumferential strain; CS/M, middle circumferential strain; CS/A, apical circumferential strain; RS/B, basal radial strain; RS/M, middle radial strain; RS/A apical radial strain; LS/B, basal longitudinal strain; L/M, middle longitudinal strain; LS/A, apical longitudinal strain. *Compared with control group, P<0.01; # compared with HT-I group, P<0.01.*

Regarding the HT-I group, compared with control group, the basal radial strain (RS/B), apical radial stain (RS/A), basal longitudinal strain (LS/B), and apical longitudinal strain (LS/A) decreased while the apical circumferential strain (CS/A) increased. Among the patients of the HT-II group, a significant negative linear relationship was found between LVEF and global strain parameters [LVEF and GLS (R^2=0.12, *P*<0.05), LVEF and GCS (R^2=0.34, *P*<0.05), and LVEF and GRS (R^2=0.27, *P*<0.05), GCS was more relevant to LVEF (Fig.4).

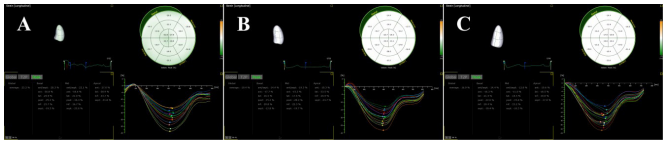

Fig.1. *The LV longitudinal strain of HT-I and HT-II patients was lower than that of normotensive individual. The LV longitudinal strain of HT-II patient was the lowest. A - normotensive individual; B - HT-I patient; C - HT-II patient. (These patients were all male patients of similar age).*

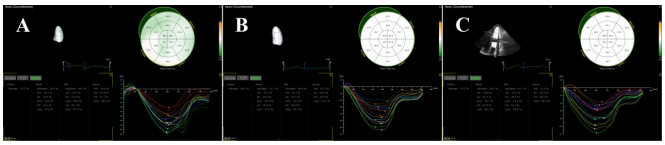

Fig.2. *The LV circumferential strain of HT-II patient was lower than that of normotensive individual and HT-I patient. The LV circumferential strain of normotensive individual and HT-I patient was similar. A - normotensive individual; B - HT-I patient; C - HT-II patient. (The same patients as in Figure 1).*

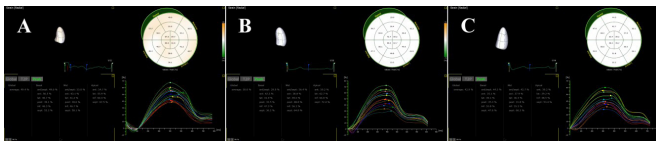

Fig.3. *The LV radial strain of HT-II patient was lower than that of normotensive individual and HT-I patient. The LV radial strain of normotensive individual and HT-I patient was similar. A - normotensive individual; B - HT-I patient; C - HT-II patient. (The same patients as in Figures 1 and 2).*

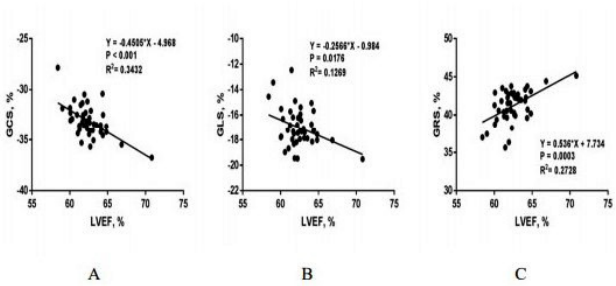

Fig.4. *Relationship of LVEF with global systolic function in patients of HT-II group. A - GCS; B -GLS; C - GRS.*

Discussion

The heart is a vital organ that sustains human life. In the late stage, systemic hypertension is one of the most frequent causes of heart failure.[5] Hypertension now accounts for the largest proportion of cardiovascular mortality worldwide. Cardiac hypertrophy occurs in hypertensive patients due to hemodynamic pressure overload, leading to increased wall thickness and LV remodeling. Echocardiography is the primary preferred imaging technique for assessing LV geometry and cardiac function for risk stratification.[13,14]

In this study, LV structure, mass, and E/A ratio were obtained from trans-mitral inflow Doppler. LV volumes, global and regional strains in longitudinal, circumferential, and radial directions were measured in primary diagnosed HT patients, using RT 3D-STE.

In 2D conventional echocardiography, in this study, the RWT was significantly increased in the hypertensive patients, compared to normotensive individuals; diastolic function parameters such as E/A ratio were significantly

different between the control group, HT-I, and HT-II groups (decreased gradually from the control group to HT-1 and HT-II), but LVEDd, PWT, LAd and LVM were significantly higher in the HT-II than in the HT-1 and control groups. Compared to the present study, Ikonomidis et al.[15] found that in 2D conventional echocardiography there was a non-significant difference in ejection fraction, LVEDV and LVESV between hypertensive and control groups; however, a significant difference was shown in LVMI, RWT, and the left atrial volume between the two groups; all these parameters were higher in a hypertensive group than in control group. Galderisi M. et al.[16] also demonstrate the same results: Hypertensive patients have higher LVM and wall thickness and a significant difference in diastolic parameters, compared to control, but no significant difference in ejection fraction in conventional 2D echocardiography. No significant differences were found in this study in 3D-echocardiography between control, HT-I, and HT-II groups regarding EDV and SV. LVESV was slightly more in the HT-II group than in the control and HT-I groups. LVEF significantly decreased in HT-II, compared to HT-I and control groups, but was in the normal range. Furthermore, GCS, GLS, GRS showed significant differences between HT groups and control (significantly decreased in HT-II compared to HT- I and control groups).

Longitudinal basal, middle strain, apical strain, circumferential basal, middle and apical strain, radial basal, middle, and apical strain were decreased in patients of the HT-II group, compared to control and HT-I groups. Regarding the HT-I group, compared with control, CS/A increased, RS/B, RS/A, LS/B, and LS/A decreased. Among the patients of the HT-II group, a significant negative correlation was found between EF and each parameter of GLS, and GCS, with GCS being more relevant to LVEF. According to Galderisi et al.,[16] there was no significant difference in LV volumes, EF, or sphericity index determined by RT 3DE between the hypertensive and control groups, but LVMI was more in the hypertensive groups than in the control group. GAS and GLS were significantly decreased in hypertensive patients, but GCS did not vary significantly between hypertensive and control patients; in general, our results were close to theirs, except for GCS, as this study shows a significant difference in the control, HT-I and HT-II groups.[16]

The results of this study are also similar to those of Yao et al.;[17] they found that GAS, GLS, GCS, and GRS in hypertensive patients were significantly decreased, as was LVEF, compared to controls. Our study clarified that GAS can detect early changes in LV global systolic function in hypertensive patients with normal ventricular geometry; in our study, GCS is more relevant to LVEF. Haque et al. found that the global left ventricular, longitudinal, systolic strain, radial strain and area strain by RT3DSTE were significantly reduced (P<0.001) in groups of hypertensive patients with presence or absence of LVH by 2D-echo-cardiography, compared to the control group.[18]

In contrast to other studies conducted using 2D-STE to assess global and regional strains in hypertensive patients, El-Noamany et al.[19] found that global peak systolic strain was significantly lower in the hypertensive group, compared to the control group. Global early diastolic SR was reduced in hypertensive patients, compared to controls (P=0.001). Meanwhile, global late diastolic SR was higher in hypertensives than in the control group (P=0.001). No significant differences in LVEF values and LV systolic function assessed by conventional echocardiography between hypertensives and control patients were noted. However, highly significant reductions in LV systolic strain and SR values were noted between control and hypertensive patients.[19] Hamed et al.[20] stated that the early diastolic SR was significantly decreased in hypertensive groups, compared with the control group, possibly due to increased myocardial fibrosis in hypertensive patients, leading to abnormalities in diastolic function and myocardial stiffness. GLS (global longitudinal strain) and SSR (systolic strain rate) were impaired in hypertensive patients, but the radial and circumferential strains showed insignificant differences between the control and hypertensive groups in a study done by Ikonomidis et al.[15] This is because the longitudinal subendocardial fibers of the myocardium are most vulnerable to adverse influences, such as hypo-perfusion and age-related interstitial fibrosis, so impaired longitudinal function, which is decreased in hypertensive disease, is an early sign of myocardial dysfunction.[21]

Conclusion

This study concludes that 3D-STE allows early diagnosis of abnormal LV myocardial systolic function, even in the subclinical stage. There was a significant reduction of myocardial systolic function in HT-II patients, compared with HT-I patients and the control group. The basal, middle, and apical segments strains significantly decreased in hypertensive patients, compared to the control group. GCS (global circumferential strain) may play a more important role in maintaining LVEF (left ventricular ejection fraction) than GLS (global longitudinal strain) and GRS (global radial strain).

Ethical Clearance

The ethical clearance was obtained from the Department of Ultrasound Diagnosis of Union Hospital of Tongji Medical College of Huazhong University of Science and Technology, Wuhan, Hubei, China. All the study participants were informed that all information would be used for research purposes only, then the result of the examination formed a part of this study.

All specific information relating to the participants' identities was protected, as was other medical data collected as routine case management. Participants were informed about the procedure and purpose of the study. Additional informed consent was obtained from all individual participants for whom identifying information is included in this article. Ultrasound and echocardiography scanning formed a part of routine management of the study cases.

Acknowledgements

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Competing Interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

Internal Medicine

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Neuropeptide Y and Asthma Clinical Course

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Abstract

***Background*:** The spectrum of clinical manifestations and pathogenetic mechanisms of bronchial asthma (BA) is very wide. Given the complex pathogenesis and syndromic nature of BA, it is not surprising that there is no single universal biomarker. The objective of this study was to evaluate levels of neuropeptide Y (NPY) and its association with levels of leptin, adiponectin, oxidative damage, antioxidant status, spirometry parameters, and asthma control in BA patients.

***Methods and Results*:** Overall, 140 patients [35(25%) men and 105(75%) women; mean age of 57.0±9.34 years] with moderate asthma participated in the study. According to body mass index, all patients were divided into three groups. The asthma diagnosis was based on the integral assessment of symptoms, medical history, health status, and spirometry values according to the Global Strategy for Asthma Management and Prevention. (GINA, 2017 REPORT). The Asthma Control Test (ACT) was used to assess asthma control. NPY was measured in blood serum in EIA.

The NPY level was significantly higher in overweight patients and patients with obesity than in patients with normal body weight. The NPY level significantly correlated with leptin (r=0.44; *P*<0.05), adiponectin (r=-0.24; *P*<0.05), ImanOx (r=-0.40; *P*<0.05), PerOx (r=0.58; *P*<0.05), ACT (r=-0.41; *P*<0.05), VC (r=-0.31; *P*<0.05), FEV1 (r=-0.41; *P*<0.05), FEF 25% (r=-0.26; *P*<0.05), FVC (r=-0.23; *P*<0.05), Tiffno index (r=-0.36; *P*<0.05), FEF 50% (r=-0.22; *P*<0.05), and PEF (r=-0.23; *P*<0.05)

Conclusion: The severity of the asthma clinical course is associated with different factors, including oxidative stress, levels of leptin, adiponectin and NPY. NPY seems to be associated with worse asthma control and higher levels of leptin and oxidative damage.(**International Journal of Biomedicine. 2021;11(4):410-413.**)

Key Words: asthma control • oxidative stress • neuropeptide Y • adipokines

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Abbreviations

ACT, Asthma Control Test; **BA**, bronchial asthma; **BMI**, body mass index; **BW**, body weight; **FEV_1**, forced expiratory volume in 1 sec; **FEF,** forced expiratory flow; **FVC,** forced vital capacity; **NPY**, neuropeptide Y; **OS**, oxidative stress; **PEF**, peak expiratory flow; **TAS,** total antioxidant status; **TOD,** total oxidative damage; **VC**, vital capacity; **WC**, waist circumference.

Introduction

Bronchial asthma (BA) is a disease that combines several syndromes characterized by inflammation, hyperresponsiveness, and intermittent airway obstruction.[1] The spectrum of clinical manifestations and pathogenetic mechanisms of BA is very wide.[2] In this regard, the approach to treatment methods cannot be standardized and applicable over a long period of time for all patients.[3] In addition to the generally accepted methods for an objective assessment of the clinical course of BA (detection of a viral infection and allergen), there are insufficiently known and not widely used methods. These methods are aimed at identifying other triggers that exacerbate the disease. In most

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cases, deterioration in asthma is associated with an increase in the inflammatory process in the airways.[4,5] Therefore, the measurement of markers of this inflammation will make it possible to clearly differentiate clinically similar phenotypes and to adequately adjust the treatment. Given the complex pathogenesis and syndromic nature of BA, it is not surprising that there is no single universal biomarker. Thus, the search for new biomarkers and factors affecting the course and control of asthma remains essential. One of the less-studied potential asthma biomarkers is neuropeptide Y (NPY). Several studies have reported that certain genotypes of NPY are associated with asthma and that Y1 receptors for NPY play an important role in allergic airway inflammation.[6] There is also evidence that during exacerbations of asthma, levels of NPY increase.[7]

The objective of this study was to evaluate levels of NPY and its association with levels of leptin, adiponectin, oxidative damage, antioxidant status, spirometry parameters, and asthma control in BA patients.

Materials and Methods

Overall, 140 patients [35(25%) men and 105(75%) women; mean age of 57.0±9.34 years] with moderate asthma participated in the study. According to body mass index, all patients were divided into three groups. Group 1 included 46 patients with normal body weight; Group 2 included 46 overweight patients; Group 3 included 48 patients with obesity.

The asthma diagnosis was based on the integral assessment of symptoms, medical history, health status, and spirometry values according to the Global Strategy for Asthma Management and Prevention (GINA, 2017 REPORT). All patients received standard asthma therapy.

Excluded criteria were asthma exacerbation, tuberculosis, chronic kidney disease, permanent atrial fibrillation, acute myocardial infarction and any other acute conditions, cancer, pregnancy and lactation, infectious diseases, mental illness.

The study was approved by the Ethics Committee of Voronezh State Medical University named after N.N. Burdenko. Written informed consent was obtained from each patient.

The Asthma Control Test (ACT) was used to assess asthma control. Lung function was assessed with spiroanalizer "Diamant-c" (Russia). Automatic ELISA analyzer Chemwell ELISA (Awareness Technology, USA) was used for testing the serum levels of leptin and adiponectin using reagent kits (MEDIAGNOST GMBH, GERMANY). NPY was measured in blood serum in EIA (BCM Diagnostics). The determination of TOD was carried out with use of a reagent kit to determine the degree of TOD to biological molecules (PerOx (TOS) (Oxidative Capacity)). We determined the overall antioxidant status by using reagents for determining TAS (ImAnOx (TAS) (Antioxidative Capacity)).

All data was evaluated with STATGRAPHICS Plus 5.1. The normality of the distribution of continuous variables was tested by the one-sample Kolmogorov-Smirnov test. For data with normal distribution, inter-group comparisons were performed using Student's t-test. Group comparisons with respect to categorical variables are performed using the chi-square test. A probability value of $P<0.05$ was considered statistically significant. The Bonferroni correction (0.05/3= 0.017) was used for multiple comparisons adjustments.

Results

The studied groups did not differ significantly in a number of social and demographic parameters and, therefore, could be used for a comparative assessment. Group 3 patients had the highest leptin levels while Group 1 patients had the highest adiponectin levels. The leptin level was significantly higher in Group 3 than in Group 1 ($P<0.0001$), while the adiponectin level was significantly higher in Group 1 than in Group 3 ($P<0.0001$). The TAS value was significantly higher in Group 1 than in Groups 2 and 3 ($P<0.0001$). The value of TOD in Group 1 was significantly lower than in Group 3 ($P<0.0001$) (Table 1). The NPY level was significantly lower in Group 1 than in Groups 2 and 3 ($P<0.0001$). Disease control was not achieved in any of the three groups, but there was a statistically significant difference in ACT scores between Groups 1 and Groups 2 and 3 ($P<0.017$) (Table 2).

Table 1.

Adipokines levels and oxidant/antioxidant status in the study groups

Variable	Group 1 (n=46)	Group 2 (n=46)	Group 3 (n=48)	Statistics
Leptin, ng/ml	9.44±4.32	12.21±4.01	22.16±8.53	P_{1-2}=0.0020 P_{2-3}<0.0001 P_{1-3}<0.0001
Adiponectin, µg/ml	21.83±5.65	19.16±6.02	16.32±4.30	P_{1-2}=0.0309 P_{2-3}=0.0097 P_{1-3}<0.0001
TOD, µmol/l	832.19±321.13	1031.27±291.36	1455.48±630.53	P_{1-2}=0.0025 P_{2-3}=0.0001 P_{1-3}<0.0001
TAS, µmol/l	524.08±93.29	285.68±46.71	257.25±59.47	P_{1-2}<0.0001 P_{2-3}=0.0118 P_{1-3}<0.0001

Table 2.

ACT score and NPY levels in the study groups

Variable	Group 1 n=46	Group 2 n=46	Group 3 n=48	Statistics
NPY, ng/ml	0.30±0.12	0.47±0.10	0.71±0.20	P_{1-2}<0.0001 P_{2-3}<0.0001 P_{1-3}<0.0001
ACT, points	18.33±3.74	15.61±4.45	13.60±5.01	P_{1-2}=0.0021 P_{2-3}=0.0429 P_{1-3}<0.0001

The NPY level significantly correlated with leptin (r=0.44; $P<0.05$), adiponectin (r=-0.24; $P<0.05$), ImanOx (r=-0.40; $P<0.05$), PerOx (r=0.58; $P<0.05$), ACT (r=-0.41; $P<0.05$), VC (r=-0.31; $P<0.05$), FEV1 (r=-0.41; $P<0.05$), FEF 25% (r=-0.26; $P<0.05$), forced vital capacity (FVC) (r=-0.23;

P<0.05), Tiffno index (r=-0.36; *P*<0.05), FEF 50% (r=-0.22; *P*<0.05), and PEF (r=-0.23; *P*<0.05)

Discussion

In recent years, special attention has been paid to NPY, due to the extensive effects in the human body carried out through the Y1, Y2, Y3, Y4, Y5, Y6 receptors, namely, participation in regulating appetite, cardiovascular system, stress, cognitive processes.[8-12] The NPY value for the respiratory system is also being investigated. Li et al.,[6] in their experimental study in mice, showed that NPY produced by the airway epithelium causes smooth muscle contraction, contributing to airway hyperresponsiveness. According to an experimental study by Thangaratnarajah et al., NPY affects the intrauterine formation of alveoli in mice and regulates the proliferation and migration of myofibroblasts.[13] Macia et al.[14] also showed that NPY, the release of which is greatly enhanced during stress, exacerbates allergic airway inflammation in mice via its Y1 receptor. Their data indicate that the development of allergic airway inflammation was associated with increased NPY expression in the lungs and that the absence of NPY-mediated signaling in mice, due to the absence of NPY or its Y1 receptor in mice, led to a significant reduction in inflammation. Makinde et al.[15] also studied the role of NPY and its receptors for allergic inflammation in the airways using mice. Expression of NPY was localized in macrophage-like cells in the peribronchial and perivascular regions of the lung tissue. Receptors Y1 and Y5 were expressed by both structural and inflammatory cells of lung tissue. Thus, the authors concluded that NPY produced by activated macrophage-like cells can participate in regulating cytokine production and cellular activity of immune cells.[15] A small number of clinical studies have also investigated the relationship between NPY and asthma. Lu et al.[16] investigated the relationship between the five most common genotypes of the *NPY* gene and the presence of asthma. The study included 126 patients with asthma without concomitant chronic pathology and 182 healthy volunteers who constituted the control group (aged 21-35 years). The study revealed that the CT genotype of rs5574 and the GT genotype of rs17149106 are interrelated with the presence of asthma. Lu et al.[17] also studied patients with an established diagnosis of asthma at the time of exacerbation, patients (n=51) with a stable course of asthma aged 21-35 years, as well as a control group (n=69) corresponding to the patients in gender and age. The data obtained by the authors allowed them to conclude that the presence of psychological stress in patients with asthma leads to an increase in the response of type 2 T-helpers, and this effect may be mediated by the NPY level in the blood.[17] Cardell et al.[18] conducted a comparative study of the levels of several neuropeptides (vasoactive intestinal peptide, substance P, neuropeptide Y, calcitonin-gene-linked peptide) in the blood of patients with asthma during an exacerbation in comparison with the control group. The NPY level did not correlate in any way with the reversibility of obstruction; however, NPY level was increased during exacerbation, compared to healthy subjects.[18] Despite the small number of studies investigating the relationship between NPY and asthma, there are results indicating a negative effect of NPY on the clinical course of asthma and airway inflammation. In our study, NPY was associated with higher oxidative damage, higher leptin levels, worse asthma control and lower adiponectin and antioxidant levels. The importance of leptin and OS for the asthma course has been shown in numerous studies, as they lead to a worse clinical course of the disease.[19-22] The antioxidant system and adiponectin were shown to have protective effects in asthma.[23,24]

In conclusion, the severity of the asthma clinical course is associated with different factors, including OS, levels of leptin, adiponectin and NPY. NPY seems to be associated with worse asthma control and higher levels of leptin and oxidative damage.

Competing Interests

The authors declare that they have no competing interests.

Disclaimers

The views expressed in this article are the author's own and do not reflect the official position of the institution.

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ORIGINAL ARTICLE

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Complications of the Early Neonatal Period in Children from Mothers with Gestational Diabetes Mellitus

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Abstract

Background: Changes in the course of gestational diabetes mellitus at the present stage determine the emergence of a certain spectrum of completely new problems associated with the health status of newborns from mothers with gestational diabetes mellitus. The aim of the study was to investigate early neonatal complications in newborns from mothers with gestational diabetes mellitus.

Methods and Results: The study included 404 pregnant women (gestational age of 37.0–41.0 weeks) with gestational diabetes mellitus. All patients with gestational diabetes mellitus were divided into 2 groups. Group 1 included 188 patients receiving insulin therapy; Group 2 included 216 patients receiving a well-balanced diet. The control group (Group 3) consisted of 68 pregnant women without disorders of carbohydrate metabolism. In Group 1, macrosomia occurred in 44(23.4%) newborns, in Group 2 - in 48(22.0%) newborns; in newborns from mothers of the control group, the frequency of macrosomia was only in 7.35% of newborns (P=0.01). Morpho-functional immaturity of newborns had the highest frequency of occurrence, despite the fact that all children were born on time; 80(42.6%) newborns from mothers of Group 1 and 77(35.6%) newborns from mothers of Group 2 had signs of morpho-functional immaturity.

Conclusion: Diabetic fetopathy in newborns from mothers with gestational diabetes mellitus is manifested by morpho-functional immaturity of organs and systems developing in unfavorable hyperglycemic conditions. (**International Journal of Biomedicine. 2021;11(4):414-417.**)

Key Words: gestational diabetes mellitus • hypoglycemia • diabetic fetopathy • macrosomia

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Abbreviations

GDM, gestational diabetes mellitus; **CNS**, central nervous system; **DF**, diabetic fetopathy; **RDS**, respiratory distress syndrome.

Introduction

Changes in the course of gestational diabetes mellitus (GDM) at the present stage determine the emergence of a certain spectrum of completely new problems associated with the health status of newborns from mothers with GDM. This change was influenced by, first,, the obesity pandemic and the prevalence of pregestational insulin resistance, which is responsible for the earlier onset of GDM; and, second, the emergence of clear, uniform diagnostic criteria adopted by major international medical associations.

The most common neonatal complication in GDM is diabetic fetopathy (DF).[1-4] However, in the world literature, there are practically no data on its most significant manifestations—the main problems of the early neonatal period. The frequency of DF in the era of new, more accurate criteria for the diagnosis of GDM by IADPSG (International Association of Diabetes and Pregnancy Study Groups) has

significantly decreased.[5,6] Previously, prevailing severe forms of DF, accompanied by visceromegaly, severe damage to the central nervous system (CNS), and multiple organ failure, determined the high level of perinatal mortality in GDM.[3,7] The introduction of strict, uniform diagnostic criteria and national hyperglycemia screening programs have positively influenced the levels of perinatal morbidity and mortality.

However, the problem of complications in newborns from mothers with GDM is not limited to the "traditional" symptom complexes of DF. According to modern data, the structure of DF is currently dominated by functional disorders caused by the immaturity of body systems formed against the background of existing pregestational disorders of carbohydrate and lipid metabolism.[1,4,6,8] These include neural tube defects, CNS depression, minimal heart defects, hepatic steatosis, and intestinal dysbiosis in newborns.[2,4,9]

The aim of the study was to investigate early neonatal complications in newborns from mothers with GDM.

Materials and Methods

The study included 404 pregnant women (gestational age of 37.0–41.0 weeks) with GDM and 68 without disorders of carbohydrate metabolism, who *gave birth* from the second quarter of 2018 to the third quarter of 2020 in the maternity ward of the City Clinical Hospital No. 29 named after N.E. Bauman. This was a prospective case-control study.

All patients with GDM were divided into 2 groups. Group 1 included 188 patients receiving insulin therapy; Group 2 included 216 patients receiving a well-balanced diet. The control group (Group 3) consisted of 68 pregnant women without disorders of carbohydrate metabolism.

The study was conducted in accordance with the ethical principles of the WMA Declaration of Helsinki (1964, ed. 2013) and was approved by the Ethics Committee of the RUDN University. Written informed consent was obtained from all participants.

Inclusion criteria were full-term pregnancy, cephalic presentation, and singleton pregnancy. Exclusion criteria were multiple pregnancy, premature birth, and breech presentation of the fetus. The diagnosis of GDM was based on the clinical recommendations of the MH of RF.[8]

The condition of newborns was assessed in the first minutes of life, in the first day of life and in the early neonatal period. Assessment of the state of a newborn in the early neonatal period began with an examination in the delivery room by a neonatologist, and included: 1) measurement of anthropometric parameters (weight, height, head circumference, chest circumference); 2) the presence or absence of phenotypic signs of DF, such as macrosomia, pitting edema, disproportionate physique, morpho-functional immaturity, moonlike face, cardiomegaly, splenomegaly, hepatomegaly, hypertrichosis, hyperbilirubinemia, hypoglycemia, neonatal CNS depression, RDS; 3) assessment of the Apgar score (at 1 minute and 5 minutes after birth); 4) the need for respiratory support and its duration, the duration of hospital stay; 5) perinatal brain damage.

Macrosomia was defined as a birth weight greater than or equal to 4000g.

The criteria for morpho-functional immaturity included pronounced lanugo on the body, soft auricles, softened skull bones, a short neck, undescended testicles in the scrotum in boys, and uncovered labia minora with the labia majora in girls. The blood glucose concentration in newborns was measured 1 or more times a day, depending on the condition of the newborn, using a Glucometer "Accu-Chek Active New"(Switzerland) and visual test strips "Accu-Chek." Hypoglycemia was defined as blood glucose concentration <2.6 mmol/L at any time after birth.

Statistical analysis was performed using the Statistica 8.0 software package (StatSoft Inc, USA). Baseline characteristics were summarized as frequencies and percentages for categorical variables and as means and standard error of the mean (SEM) for continuous variables. Multiple comparisons were performed with one-way ANOVA and Tukey's HSD Post-hoc Test. Group comparisons with respect to categorical variables are performed using the chi-square test or, alternatively, Fisher's exact test. A value of $P<0.05$ was considered statistically significant.

Results

The 1-minute and 5-minute Apgar scores in newborns from mothers of Groups 1 and 2 were moderately lower than in newborns from mothers of the control group. Consequently, newborns from women with GDM showed lower adaptive abilities in the first minutes after birth (Table 1).

Table 1.

The Apgar score in the study groups

Groups	The 1-minute Apgar score	The 5-minute Apgar score
Group 1 (n=188)	8.2±0.7	8.8±0.7
Group 2 (n=216)	8.4±0.6	9.0±0.9
Group 3 (n=68)	8.7±0.4	9.4±0.5
P-value	0.9158	0.9267

The average birth weight in newborns from women with GDM was moderately higher than in newborns from mothers of the control group. These data were due to the higher frequency of macrosomia in the GDM groups. Thus, in Group 1, macrosomia occurred in 44(23.4%) newborns, in Group 2 - in 48(22.0%) newborns. In newborns from mothers of the control group, the frequency of macrosomia was only in 7.35% of newborns ($P=0.01$) (Table 2).

In newborns from GDM mothers, complications such as hypoglycemia, symptoms of neonatal CNS depression, hepatomegaly, morpho-functional immaturity, and RDS were significantly more frequent (Table 3). Morpho-functional immaturity of newborns had the highest frequency of occurrence, despite the fact that all children were born on

time; 80(42.6%) newborns from mothers of Group 1 and 77(35.6%) newborns from mothers of Group 2 had signs of morpho-functional immaturity. In second place, in terms of frequency of occurrence, was hepatomegaly, which occurred in 60(31.9%) newborns from mothers of Group 1 and 44(20.3%) newborns from mothers of Group 2. Approximately the same frequency was observed for such complications of the early neonatal period as hypoglycemia and neonatal CNS depression. RDS was the least common, accounting for 10.1% of newborns from mothers of Group 1 and 6.9% of newborns from mothers of Group 2.

Table 2.

Anthropometric indicators of newborns

Groups	Birth weight M±SEM	Macrosomia n(%)
Group 1 (n=188)	3624.9±588.9	44(23.4%)
Group 2 (n=216)	3627.9±542.0	48(22.2%)
Group 3 (n=68)	3463.8±354.2	5(7.35)
P-value	0.9864	0.0138

Table 3.

Neonatal complications in newborns

Groups	Hypoglycemia	CNS depression	Hepatomegaly	MFI	RDS
Group 1 (n=188)	40(21.2%)	55(29.3%)	60(31.9%)	80(42.6%)	19(10.1%)
Group 2 (n=216)	45(20.8%)	45(20.8%)	44(20.3%)	77(35.6%)	15(6.9%)
Group 3 (n=68)	4(4.6%)	3(4.4%)	2(2.9%)	4(5.8%)	0
P-value	0.0125	0.0001	0.0000	0.0000	0.0248

MFI - Morpho-functional immaturity

Diabetic cardiomyopathy was significantly more common in children from mothers of Group 1 (Table 4). However, it should be noted that the incidence of this complication was quite low - 4.2%. In addition, DF was significantly more common in newborns from women of Group 1, which confirms the fact that the course of insulin-treated GDM was more severe.

Diabetic cardiomyopathy is one of the most severe manifestations of the uncompensated course of GDM in mothers.[3,10] The data on the incidence of cardiomyopathy, according to the literature, vary significantly: from 6.7% to 43.0% of newborns from GDM mothers.[4-6,8,10] Palmieri et al.[11] revealed echo signs of cardiomyopathy in 50.8% of fetuses from mothers with untreated GDM already at 30 weeks of gestation. The introduction of more stringent IADPSG criteria for the diagnosis of GDM contributed to a decrease in the incidence of cardiomyopathy, as well as the rate of perinatal morbidity and mortality. According to Billionet et al.,[9] GDM is associated with a moderately increased risk of adverse perinatal outcomes, which is higher in insulin-treated GDM than in non-insulin-treated GDM for most outcomes.

Table 4.

Diabetic fetopathy and cardiomyopathy in newborns from GDM women

Groups	Diabetic fetopathy	Diabetic cardiomyopathy
Group 1 (n=188)	79(42.0%)	8(4.2%)
Group 2 (n=216)	56(25.9%)	0
P-value	0.0006	0.002

Conclusions

The state of health of newborns from mothers with GDM is characterized by a high incidence of macrosomia and hepatomegaly, as well as hypoglycemia, neonatal CNS depression, and morpho-functional immaturity.

Diabetic cardiomyopathy, as a marker of insufficient glycemic compensation and a more severe course of the disease, was found in newborns from mothers with insulin-treated GDM. However, the incidence of this complication was low.

Diabetic fetopathy in newborns from mothers with GDM is manifested by morpho-functional immaturity of organs and systems developing in unfavorable hyperglycemic conditions.

Competing Interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

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Dyslipidemia as Predictor of Missed Miscarriage

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Abstract

***Background*:** This study aimed at finding the diagnostic and prognostic possibilities of determining apoC-II, as a serological marker for MM in early gestation.

***Methods and Results*:** The study included 182 pregnant women aged between 18 and 45 years at gestational age under 11 weeks. All women were divided into 3 groups. Group 1 included 90 women with MM; Group 2 included 52 women with spontaneous miscarriage; Group 3 included 40 women without pathology (control group). Lipid metabolism disorders were diagnosed according to the Russian national recommendations of the VII revision(the Russian Society of Cardiologists [RSC, 2020]), considering the European recommendations (2019). Proteomic analysis of the blood serum was performed using liquid chromatography-mass spectrometry. Abnormalities in the lipid profile, manifested as isolated hypercholesterolemia, and combined hypercholesterolemia with hypertriglyceridemia, were more common in patients with MM and spontaneous abortions: 62.2% and 59.7% of cases, respectively, which correlates with the identified marker apoC-II in Group 1 and Group 2.

Conclusion: ApoC-II can be considered as the most promising serologic marker for MM in the early gestation period for women with dyslipidemia.(**International Journal of Biomedicine. 2021;11(4):418-421.**)

Key Words: missed miscarriage • dyslipidemia • apolipoprotein C-II • serological markers

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Abbreviations

ApoC-II, apolipoprotein C-II; **ApoC-II**, apolipoprotein C-II; **HDL**, high-density lipoprotein; **LDL**, low-density lipoprotein; **LPL,** lipoprotein lipase; **LC-MS**, liquid chromatography-mass spectrometry; **MM**, missed miscarriage; **TC**, total cholesterol; **TG**, triglycerides; **VLDL**, very low-density lipoprotein.

Introduction

Missed miscarriage (MM) is one of the unsolved problems in the Russian Federation.[1,2] Among the risk factors for MM, the leading place belongs to dyslipidemia and endothelial dysfunction, both in the maternal body, in the fetoplacental complex and in the arteries of the umbilical cord.[1,3]

In a normal pregnancy, lipid parameters including total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL) and phospholipid gradually increase starting in the 12th week of gestation and continue to do so throughout pregnancy.[4-7]

The accumulation of maternal fat depots and hyperlipidemia are the two principal changes in lipid metabolism during pregnancy.[8] Pregnancy is typified by

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an increase in serum levels of TC and TG pushed by the rise in estrogen, progesterone and lactogen.[9] Both TG and TC are essential for the development of the fetus. However, high levels of maternal TC and/or TG are associated with preterm birth,[10-12] pregnancy-induced hypertension,[13] preeclampsia,[14-16] and large for gestational age.[17-19]

Recently, the ABCD study showed that atherogenic lipid profiles during the first trimester confer an increased risk of adverse pregnancy outcomes including maternal morbidity, mortality, and preterm delivery.[20,21]

The role of dyslipidemia, manifested by abnormally elevated TC, TG, and LDL against the background of low HDL in the pathogenesis of MM in patients with recurrent miscarriage, has been shown in a number of studies.[22,23] Low HDL level can lead to vascular thrombosis and the development of hypoxia in the placenta.[24,25]

Apolipoprotein C-II (apoC-II) is the potent physiological activator of LPL; therefore, it plays a central role in the metabolism of plasma TG. LPL is the main enzyme that hydrolyses plasma TG on triglyceride-rich lipoproteins, such as chylomicrons and VLDL, and on HDL, particularly during fasting.[3] ApoC-II has also been shown to act as an inhibitor of LPL at higher concentrations,[26] although the mechanisms by which this activation and inhibition take place remain poorly understood. Palva et al.[24] showed that the decrease of LPL activity in the heart, along with the inhibitory effects of excess apoC-II, may contribute to the hypertriglyceridemia observed in apoC-II transgenic mice. Fornengo et al.[27] reported a case of drug-resistant hypertriglyceridemia in a patient with increased levels of apoC-II. To date, the mechanisms by which physiological levels of apoC-II activate and excess apoC-II inhibits LPL are not fully understood.

In the modern literature, there are few data on serological markers of MM, a lack which creates the prerequisites for conducting this study aimed at finding the diagnostic and prognostic possibilities of determining apoC-II, as a serological marker for MM in early gestation.

Materials and Methods

In the period from April 2020 to February 2021, 182 pregnant women aged between 18 and 45 years at gestational age under 11 weeks were examined. All women were divided into 3 groups. Group 1 (Gr1) included 90 women with MM; Group 2 (Gr2) included 52 women with spontaneous miscarriage; Group 3 included 40 women without pathology (control group [CG]).

Inclusion criteria were MM, spontaneous miscarriage or physiological pregnancy at gestational age under 11 weeks. Exclusion criteria were autoimmune diseases, infectious diseases, and diseases of the thyroid gland, including thyroid dysfunction.

Examination of patients included clinical methods, questionnaire survey, analysis of case histories, laboratory tests (clinical blood test, blood levels of TG, HDL-C, and LDL-C, urine test, and determination of antibody titer to the TORCH complex), and pelvic ultrasound. All women underwent an assessment of vaginal microcenosis and the quantitative and qualitative composition of the biotope of the cervical discharge.

Lipid metabolism disorders were diagnosed according to the Russian national recommendations of the VII revision(the Russian Society of Cardiologists [RSC, 2020]), considering the European recommendations (2019): TC >5,0 mmol/l; TG >1.7 mmol/l; HDL-C <1.2 mmol/l in females; LDL-C >3.0 mmol/l.

Antibodies to thyroperoxidase (AT-TP) and thyroglobulin (AT-TG) in the blood serum were determined by ELISA using standard test systems from "CHEMA-MEDIKA" (Russia).

Proteomic analysis of the blood serum was performed using LC-MS.

The study was conducted in accordance with ethical principles of the WMA Declaration of Helsinki (1964, ed. 2013) and approved by the RUDN University Ethics Committee. Written informed consent was obtained from all participants.

Statistical analysis was performed using the *Statistica* 8.0 software package (*StatSoft Inc*, USA). Kruskal-Wallis test was used to compare means of 3 groups of variables not normally distributed. A value of $P<0.05$ was considered statistically significant.

Results

For the analysis of potentially significant markers, we studied serological markers found in all 3 groups; in particular, we compared the ratios of protein concentrations in the study groups. If the concentration ratio of the investigated marker in the Gr1/CG pair was close to 1 or equal to 1, then the marker was considered nonspecific; if the concentration ratio of the investigated marker in the Gr1/Gr2 pair was close to 1 or equal to 1, then the marker was also considered nonspecific; however, if the studied ratio was close to 1 or equal to 1 in the Gr1/Gr2 pair and at the same time >1 in the Gr1/CG pair, then the marker was considered potentially specific.

Table 1.

The potentially specific serorlogical marker of MM

Group pair	ApoC-II	ApoC-IV	Complement C5	Complement factor H
Gr1/CG	2.468	0.495	0.494	0.414
Gr1/Gr2	1.658	0.579	0.373	0.399
Gr2/CG	1.489	0.823	1.324	1.035
P-value	0.00016	0.01324	0.00428	0.00119

As can be seen from Table 1, the most specific serorlogical marker of MM may be apoC-II, since the ratio of its concentration was 2.468 in the Gr1/CG pair, 1.658 in the Gr1/Gr2 pair, and 1.489 in the Gr2/CG pair. For all other markers, differences between groups were less pronounced.

Moreover, the potential role of apoC-II was most pronounced in Gr1, which suggests that apoC-II may be the specific marker for MM. When the patients were divided into groups depending on the lipid profile, women with an impaired lipid profile, manifested as isolated hypercholesterolemia, and combined hypercholesterolemia with hypertriglyceridemia, significantly prevailed in gGr1 compared with the CG.

Abnormalities in the lipid profile were more common in patients with MM and spontaneous abortions: 62.2% and 59.7% of cases, respectively, which correlates with the identified marker apoC-II in Gr1 and Gr2 (Table 2, Fig.1). In the control group, abnormalities in the lipid profile were found only in 42.5%.

Table 2.

Abnormalities in the lipid profile in the study groups

Groups		No Dyslipidemia	Dyslipidemia
Group 1 (n = 90)	n	34	56
	(%)	37.7	62.2
Group 2 (n = 52)	n.	21	31
	(%)	40.3	59.7
Group 3 (n = 40)	n	23	17
	(%)	57.5	42.5
P-value P_{1-3} P_{2-3}		0.1012 0.0365 0.1033	0.1013 0.0365 0.1033

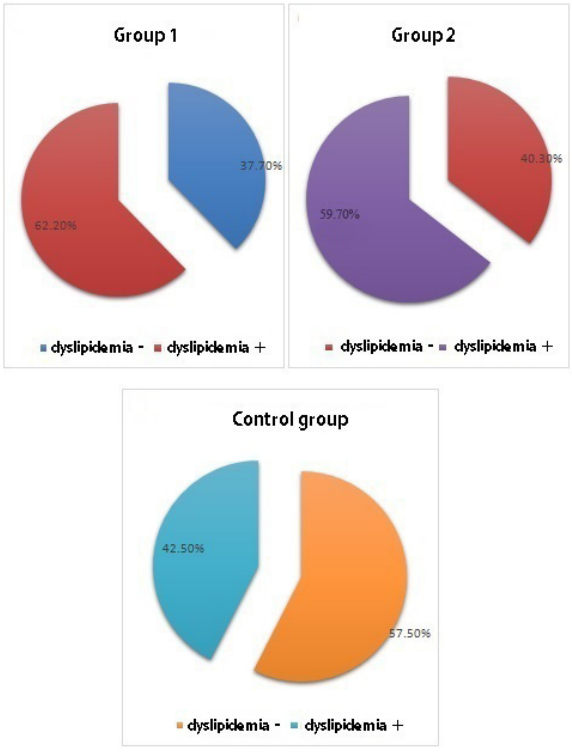

Fig.1 Abnormalities in the lipid profile in the study groups

Conclusion

The results of our study show that apoC-II can be considered as the most promising serologic marker for MM in the early gestation period for women with dyslipidemia. It seems appropriate before planning a pregnancy to conduct an additional examination of women with dyslipidemia who show a high apoC-II value in order to predict the outcome of pregnancy.

Competing Interest

The authors declare that they have no competing interests.

Sources of Funding

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ORIGINAL ARTICLE

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Prediction of Adverse Perinatal Outcomes and Preeclampsia in Pregnant Women with Chronic Arterial Hypertension

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Abstract

The objective of this study was to determine predictors and develop a prognostic model for preeclampsia (PE) and adverse perinatal outcomes in pregnant women with chronic arterial hypertension (CAH).

Methods and Results: The study cohort included pregnant women (n=376) with hypertensive disorders: Group 1 –pregnant women with CAH (n=134), Group 2 – with PE on the background of CAH (n=242). Healthy pregnant women made up the control group (n=34). The diagnosis of pregnant women with CAH was made on the basis of existing national and foreign recommendations that an increase in SBP ≥140 mmHg and/or DBP ≥ 90 mmHg indicates CAH. All patients underwent the following examinations: a survey on a special questionnaire; an anthropometric examination; physical examination; an assessment of the content of uric acid (UA) in the blood serum and microalbuminuria (MAU) in the urine at 6-8 and 16-17 weeks of pregnancy; 12-lead ECG, echocardiography, 24-hour ambulatory blood pressure monitoring, sonography of the uterus, fetus, and placenta at 6-8 and 16-18 weeks. It was found that an inappropriate left ventricular mass (LVM) in pregnant women with CAH, a certain "phenotype" of 24-hour ABPM and indicators of metabolic disorders demonstrate the systemic nature of organ damage and appear to be a predictor of adverse perinatal outcomes and the development of PE. The revealed changes in the LV structure, which are more significant in PE on the background of CAH, suggest the association of concentric left ventricular hypertrophy and disorders of uteroplacental blood flow. Detecting abnormal blood flow from early pregnancy will reduce not only perinatal morbidity and premature birth, but also the probability of organ (LV myocardium, kidney) damage in women with CAH. To predict the risks of adverse perinatal outcomes in pregnant women with CAH and PE, a number of factors were identified that have a statistically significant relationship with the studied complications. The developed model makes it possible to predict the probability of PE and unfavorable perinatal outcomes in pregnant women suffering from CAH with high efficiency (91.1%).(**International Journal of Biomedicine. 2021;11(4):422-427.**)

Key Words: chronic arterial hypertension • preeclampsia • left ventricular mass • microalbuminuria • uric acid

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Abbreviations

ABPM, ambulatory blood pressure monitoring; **AUC**, area under the ROC curve; **BMI**, body mass index; **BP**, blood pressure; **CAH**, chronic arterial hypertension; **DBP**, diastolic BP; **FGR**, fetal growth retardation; **HU**, hyperuricemia; **IVST**, interventricular septal thickness; **LVPWT**, left ventricular posterior wall thickness; **LVM**, left ventricular mass; **LVMI**, left ventricular mass index; **LVH**, left ventricular hypertrophy; **LVDD**, left ventricular diastolic dysfunction; **MAU**, microalbuminuria; **MBP**, mean blood pressure; **PE**, preeclampsia; **PI**, pulsation index; **RWT**, relative wall thickness; **SBP**, systolic BP; **TDI**, tissue Doppler imaging; **UA**, uric acid; **PP**, pulse pressure; **UAr**, uterine arteries; **UCA**, umbilical cord artery.

Introduction

Chronic arterial hypertension (CAH), defined by clinical practice guidelines as SBP (≥140 mmHg) and or DBP ((≥90 mmHg) before pregnancy or up to 20 weeks, complicates up to 5% of pregnancies.[1,2] Modern advances in the study of hypertensive disorders in pregnant women indicate the need to expand the possibilities of preclinical PE prediction on the background of CAH.[3,4] CAH is regarded as an independent clinical risk factor for PE.[5] The probability of developing PE on the background of CAH is 20%-30%,[2-4] with severe hypertension (>170/110 mmHg) – 46%.[6]

ABPM is considered to be a better predictor of PE and FGR than conventional office monitoring, but it is not sensitive or specific enough for routine screening.[7] In pregnant women with CAH, an unfavorable prognostic value is reported if there is no physiological decrease in BP at night or an increase in a*verage* nighttime BP. A chronic increase in BP without damage to target organs with stable indicators is associated with a smaller negative effect on the condition of the pregnant woman and the fetus than with PE.[8] Magee et al.[9] showed that "less strict" control of DBP does not lead to worsening of perinatal outcomes, compared to "strict" control, but increases the probability of severe hypertension.

Numerous studies indicate hypertension as a risk factor for pregnancy complications;[10] however, the prognostic value of the structure and function of the left ventricular (LV) myocardium in pregnant women with CAH for the purpose of individualizing treatment appears to be poorly studied. The data on LVDD in pregnant women with CAH, which is effectively diagnosed by TDI, are widely presented.[11]

During pregnancy, MAU is considered as a preclinical marker of kidney damage in CAH; after 20 weeks, it proves to be a predictor of early PE in the presence of CAH.[12] High sensitivity and low positive significance of MAU in predicting PE (88.9% and 22.2%, respectively) were shown by Wang et al.[13] Proteinuria threshold values for predicting adverse perinatal outcomes range from 300 mg/day to 500 mg/day.[14]

An increase in UA levels is associated with the development of PE;[15] a stronger connection is found in gestational hypertension.[16] Hyperuricemia (HU) is considered as a marker of inflammation of the trophoblast and placenta, development of fetal growth retardation (FGR),[17] and premature birth in pregnant women with PE.[18] It is reported that HU is associated with low gestational age and low weight of newborns, regardless of the presence or absence of proteinuria in pregnant women with CAH.[19]

The objective of this study was to determine predictors and develop a prognostic model for PE and adverse perinatal outcomes in pregnant women with CAH.

Materials and Methods

The study cohort included pregnant women (n=376) with hypertensive disorders: Group 1 – pregnant women with CAH (n=134), Group 2 – with PE on the background of CAH (n=242). Healthy pregnant women made up the control group (n=34). Written informed consent was obtained from all participants.

Inclusion criteria: single-child progressing pregnancy, the presence of CAH confirmed before pregnancy, the woman's informed consent for the use of biological material for scientific purposes.

The diagnosis of pregnant women with CAH was made on the basis of existing national and foreign recommendations that an increase in SBP ≥140 mmHg and/or DBP ≥90 mmHg indicates CAH. The program of a patient's examination included a survey on a special questionnaire and an anthropometric examination using a standard method with the calculation of the BMI (kg/m²). In all women, we assessed the content of UA in the blood serum and MAU in the urine at 6-8 and 16-17 weeks of pregnancy.

Office BP was measured using a mercury sphygmomanometer, according to Korotkov's method. BP was measured 3 times, and the means of these measurements were used in the analyses. The 24-hour ABPM was performed at 10-11, 21-22 and 32-33 weeks of pregnancy. The device was set to obtain BP readings at 15 min intervals during the day (7am–11pm) and at 30 min intervals during the night (11pm–7am).

The following BP indicators were taken into account: systolic (SBP), diastolic (DBP), pulse, mean arterial pressure (MAP). The degree of nighttime BP reduction, or daily index, was defined as the ratio of the difference between daytime and nighttime BP to daytime BP (in %). Nocturnal dipping was defined as a reduction in average SBP and DBP at night greater than 10% compared with average daytime values

Echocardiography was carried out according to the recommendations of the American Society of Echocardiography[20] in M- and B-modes. LVM was calculated using the formula R. Devereux.[21] The appropriate LVMM (in relation to height, SBP, gender, shock load) was determined [22]: predicted LVM = 55.37 + 6.64 × height ($m^{2.7}$) + 0.64 × SW (g-m/beat) – 18.07 × gender, where SW (stroke work) in gram-meters/beat [g-m/beat]) was computed as follows: cuff SBP × stroke volume × 0.0144; female gender coefficient = 2.

Observed LVM (oLVM) was divided by predicted LVM (pLVM) and was expressed as a percentage (oLVM/pLVM). Inappropriate LVM was defined as an excess of >28% from the predicted value (ie, oLVM/pLVM >128%).

RWT was calculated as IVST+LVPWT)/LV EDD. Increased RWT was defined as ≥0.45.[23]

LVMI was determined by dividing the LVMs by the body surface area in m^2. A cut-off of 110 g/m^2 was used to define an increased LVMI.[24]

Four types of different LV geometry patterns were defined based on LVMI and RWT, as recommended by the American Society of Echocardiography[25]:

-normal geometry (normal LV mass index and normal RWT),

-concentric remodelling (normal LV mass index but increased RWT),

-concentric hypertrophy (increased LV mass index and increased RWT),

-eccentric hypertrophy (increased LV mass index but normal RWT).

LV diastolic function was analyzed by tissue Doppler imaging.

Sonography of the uterus, fetus, and placenta was performed in real time according to the generally accepted method. Blood flow Dopplerometry at 6-8 and 16-18 weeks included the measurement of indices in the UA, after 24 weeks – in the UA, UCA and middle cerebral arteries of the fetus. The pulsation index (PI), resistance index (RI), and systolic-diastolic ratio (SDR) were assessed.

Statistical analysis was performed using statistical software package SPSS version 19.0 (Armonk, NY: IBM Corp.). The normality of distribution of continuous variables was tested by the Shapiro-Wilk test. Baseline characteristics were summarized as frequencies and percentages for categorical variables and as mean (M) and standard error of the mean (SEM) for continuous variables. Student's unpaired t-test was used to compare average values for data with normal distribution. Group comparisons with respect to categorical variables are performed using chi-square tests with Yates correction or, alternatively, Fisher's exact test when expected cell counts were less than 5. A probability value of $P \leq 0.05$ was considered statistically significant.

Results and Discussion

The average age of women in Group 1 was slightly higher than in Group 2 (Table 1). Excess body weight (BMI ≥25 kg/m^2) was detected in both trimesters to a greater extent in pregnant women in Group 2.

The study of the physiological functions of newborns revealed lower 1-minute and 5-minute Apgar scores in newborns from mothers of Group 2 and low birth weight, compared to the control group.

In general, 36.4% of newborns from mothers of Groups 1 and 2 had hypotrophy versus 8.8% of newborns from mothers of the control group (P=0.007). Cerebral ischemia was detected in 41.7% of newborns from mothers of Groups 1 and 2 versus 8.8% of newborns from mothers of the control group (P=0.003). Morphofunctional dismaturity was detected in 41.7% of newborns from mothers of Groups 1 and 2 and only in 2.9% newborns from mothers of the control group (P<0.05).

To predict the risks of adverse perinatal outcomes in pregnant women with CAH and PE, a number of factors were identified that have a statistically significant relationship with the studied complications (Table 2).

The identification of the presented risk factors made it possible to build a predictive model of the probability of the phenomena under study in each individual case.

The calculation was carried out according to the formula: $P = 1 / (1 + e ^ (- z))$, $z = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + ... + a_nx_n$, where p is the probability of the outcome, $x_1 ... x_n$ are the values of the predictors measured in a nominal, ordinal or quantitative scale, $a_1 ... a_n$ - regression coefficients.

The ROC (Receiver Operating Characteristic) analysis confirmed the high diagnostic value of the obtained model (Figure 1). The effectiveness of the prognostic model for identifying the contingent with the risk of developing PE and unfavorable perinatal outcomes was confirmed by AUC–0.92. The Nagelkerke index (0.75) indicates the possibility of explaining 75.0% of the variance of the variables. The high prognostic value of the model with a selection of

Table 1.

Characteristics of pregnant women and newborns in groups with hypertensive disorders

Variable	Group 1	Group 2	*P*-value
Age, yrs	34.6±3.7	28.4±2.6	0.171
BMI in the second trimester, kg/m^2	25.8±2.9	26.5±3.8	0.884
BMI in the third trimester, kg/m^2	26.7±3.2	28.2±1.7	0.679
BMI>25 kg/m^2 in the second trimester	49(28.5%)	91(44.6%)	0.841
BMI>25 kg/m^2 in the third trimester	59(34.3)	109(53.4%)	0.849
1-minute Apgar score	7.4±0.6	6.8±0.3	0.372
5-minute Apgar score	8.2±0.4	7.5±0.3	0.162
Birth weight, g	3152±320	2950.0±360	0.675
Hypotrophy of newborns	59(34.3%)	78(38.2%)	0.023
Morphofunctional dismaturity of newborns	39(22.7%)	64(31.4%)	0.580
Cerebral ischemia of newborns	66(38.4%)	92 (45.1%)	0.034

Table 2.

Risk factors for adverse perinatal outcomes in the presence of CAH and the development of PE

Factors	Regression coefficient B	Wald Statistics χ^2	P-value	Exp B
UA >370 mmol/l	2.0	13.9	0.000	7.8
MAU	0.96	4,2	0,04	2.6
LVDD	1.5	9.1	0,003	4.5
oLVM/pLVM >128%	1.9	14.4	0.000	6.5
Daily DBP >60 mmHg in the first half of pregnancy	1.1	4.6	0.03	2.8
Concentric LVH	1.2	4.4	0.035	3.4
Lack of early antihypertensive therapy	1.8	9.6	0.002	6.4
Lack of placental insufficiency prevention	1.6	9.5	0.002	4.9
PI (>1.55) in the uterine artery at 6-8 weeks	1.3	4.9	0.027	3.8
Night-pickers	1.9	14.8	0,000	7.3
BMI > 25 kg/m^2	1.5	9.0	0.003	4.4
Daily DBP>68 mmHg in the second half of pregnancy	1.1	5.1	0.024	2.9
Daily index (DBP) <10	-1.7	12.9	0.000	0.180
PI (>1.35) in UAr at 16-18 weeks	2.3	17.0	0.000	10.1
Constant	-8.2	44.7	0.000	0.000

predictors is proved by the following criteria: sensitivity – 84.3%, specificity – 93.8%, diagnostic efficiency – 91.1%, PPV (positive predictive value) – 96.5%, and NPV (negative predictive value) – 82.0%.The cut-off criterion (0.5) is a sign of high predictive efficiency of the model, which is realized when the indicator is more than 0.5.

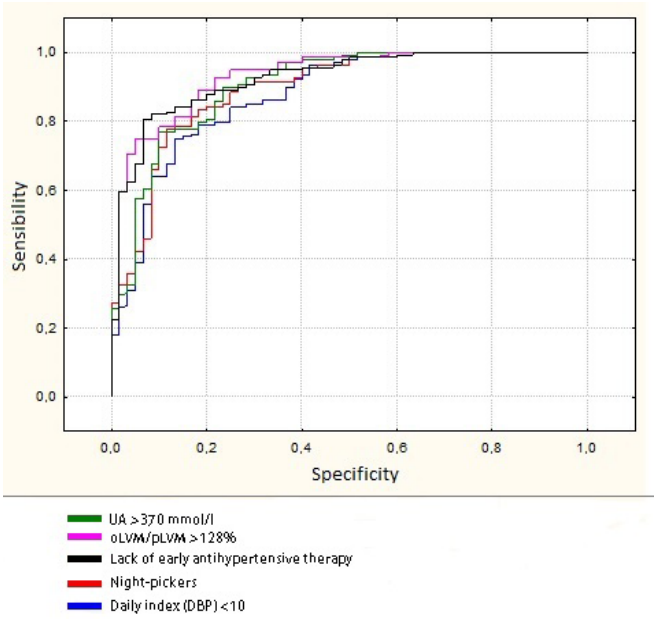

Fig. 1. *The prognostic model for predicting the risk of developing PE and unfavorable perinatal outcomes*

We have selected factors whose influence on the development of PE and adverse perinatal outcomes has been confirmed by many studies.

Evaluation of the uterine blood flow parameters as predictors of the consequences of limiting trophoblast invasion by the level of the decidual vascular segment allows us to identify violations of the cellular-molecular interactions between the mother and the fetus from the early stages of pregnancy.[26,27]

Our data confirm the informative value of the assessment of the PI in the UAr at 7-8 weeks (>0.55) and at 16-18 weeks (>1.35) for detecting placental abnormalities and predicting PE in pregnant women with hypertensive disorders from early pregnancy.[28] The diagnostic significance of measuring PI in UAr in each trimester of pregnancy (11-13.6 weeks, 20-22.6 and 32-33.6 weeks) has been shown earlier, along with other factors (age of the pregnant woman, presence of CAH, PE in the anamnesis).[29] The expert assessment recognized the greatest effectiveness of the prognostic model of the third trimester (sensitivity – 79%, specificity – 82%).

The role of excess body weight as a factor predisposing to the development of CAH and PE has also been noted earlier.

The obvious advantages of ABPM in predicting the risks of PE are determined by the detection of night hypertension,[7] belonging to night-pickers on the background of an average daily DBP >60 mmHg in the first half of pregnancy and >68mmHg in the second half – as predictors of unfavorable perinatal outcomes. Similar data on an increase in the risk of PE with mean DBP of ≥75 mmHg and MBP of ≥90 mmHg at 13–20 weeks of pregnancy are given by other authors.[30]

LV geometry analysis turned out to be informative from the standpoint of clarifying the presence of organ damage – the myocardium, kidneys and blood vessels, identified more often with prolonged hypertension. The process of cardiac remodeling, caused by a change in the thickness-radius ratio of the LV myocardium, with a greater LVM on the PE background, was realized in a change in the geometry of the concentric type, more often hypertrophy than remodeling.

The frequency of LV remodeling in pregnant women with CAH in the second trimester (76.3%) and the third trimester (85.4%) was higher than in foreign sources.[31] This fact is probably due to the high frequency of delayed hypotensive therapy in the sample.

Conclusions about the adverse effect of abnormal LV geometry on the prognosis of the disease prevail in concentric hypertrophy.[32] An increase of concentric LVH (from 28.2% to 38.6%) by the third trimester indicates the expediency of identifying women who are threatened by the progression of CAH.

The introduction of the variable oLVM/pLVM, which allows us to differentiate the risk group for severe hypertension and PE with an indicator ">128%," showing the presence of inappropriate LVM, regardless of the type of LVH, seems optimal when examining women with CAH.

The significance of LVDD, which is more often diagnosed in severe hypertension and the development of PE, has been confirmed by studies. In particular, Castleman et al. noted that in pregnancies complicated by CAH and PE, differentiating features from normal pregnancy were LV wall thickness of ≥1.0 cm, exaggerated reduction in E/A, and lateral e' of <14 cm/s.[33]

Thus, monitoring of the LV myocardium makes it possible to stratify pregnant women in a timely manner by risk groups for the development of severe hypertension, the most significant in the absence of early hypotensive therapy. We believe that the assessment of LV function using echocardiography for women with long-term hypertension (more than two years) before planning pregnancy is reasonable.

Our data allow us to disagree with the opinion that routine testing for HU is inexpedient.[34] The UA level >370 μmol/l is defined as a predictor of PE, preceding changes in the structure and function of the LV myocardium in pregnant women with CAH. The association of hyperuricemia with proteinuria and increased DBP, and with unfavorable perinatal outcomes, has been confirmed, in contrast to the findings of other authors.[35]

The presence of HU and MAU in pregnant women with CAH indicates a decrease in renal excretion on the background of parenchymal damage.[36,37] The absence of proteinuria in CAH should be considered as an organ-protective factor.

Data on the possibility of predicting premature birth, early PE and FGR in pregnant women with impaired uterine blood flow in UA and abnormal biochemical tests explain the prognostic role of the absence of placental insufficiency prevention in CAH.

Conclusion

Thus, an inappropriate LVM in pregnant women with CAH, a certain "phenotype" of 24-hour ABPM and indicators of metabolic disorders demonstrate the systemic nature of organ damage and appear to be a predictor of adverse perinatal outcomes and the development of PE. The revealed changes in the LV structure, which are more significant in PE on the background of CAH, suggest the association of concentric LVH and disorders of uteroplacental blood flow. Detecting abnormal blood flow from early pregnancy will reduce not only perinatal morbidity and premature birth, but also the probability of organ (LV myocardium, kidney) damage in women with CAH. The developed model makes it possible to predict the probability of PE and unfavorable perinatal outcomes in pregnant women suffering from CAH with high efficiency (91.1%).

Competing Interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

General Surgery

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Modern Clinical and Epidemiological Features and New Technological Possibilities in the Treatment of Bleeding Gastroduodenal Ulcers

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Abstract

The aim of this research was to study the current clinical and epidemiological features of ulcerative gastroduodenal bleeding (GDB) and to evaluate the outcomes of using new technological approaches in patients with bleeding from acute (symptomatic) and chronic (peptic ulcer disease) gastroduodenal ulcers.

***Methods and Results*:** The present study involved 221 patients with bleeding gastroduodenal ulcers. Depending on the source of bleeding, all patients were divided into 2 groups. Group 1 included 143 patients with acute symptomatic gastroduodenal ulcers; Group 2 consisted of 78 patients with peptic ulcer disease (PUD) complicated by bleeding. In the complex treatment of patients of all the studied groups, an individual approach was used that included the innovative technologies of endoscopic cytoprotective treatment of GDB, based on the combined use of traditional means of endoscopic hemostasis (EH) in combination with the use of endoscopic pneumoinsufflation of biologically active drainage sorbent of a new generation Aseptisorb-D and powdered hemostatic Zhelplastan. Our analysis showed that acute symptomatic gastroduodenal ulcers prevailed in the structure of GDB – 143(64.7%) patients; PUD complicated by bleeding was diagnosed in 78(35.3%) cases. To assess the type of ulcerative bleeding, the classification of J. Forrest (1974) was used. The use of new technological approaches, including cytoprotective treatment of bleeding defects with biologically active draining sorbents of a new generation in combination with local hemostatics in the complex therapy of patients with ulcerative GDB, has significantly improved the results of treatment, which is confirmed by high rates of final hemostasis (95.0%), indicators of the effectiveness of primary EH in type FIA-IB (93.1%) and prevention of recurrence of FIIA-IIB bleeding (92.5%), with a low frequency of repeated bleeding (4.07%), emergency surgeries (2.7%), and mortality (3.2%).

Conclusion: Currently, in the structure of ulcerative GDB, acute symptomatic gastroduodenal ulcers are the most common – 64.7%, and the share of PUD complicated by bleeding accounts for 35.3% of cases only. PUD and gastroduodenal ulcers complicated by bleeding have certain clinical and epidemiological features, which must be taken into account when treating these patients. Symptomatic gastroduodenal ulcers are more difficult to treat, which shows the need to develop new complex technologies for their treatment.(**International Journal of Biomedicine. 2021;11(4):428-434.**)

Key Words: bleeding gastroduodenal ulcers • endoscopic hemostasis • biologically active drainage sorbent

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Abbreviations

APC, argon-plasma coagulation; **EH**, endoscopic hemostasis; **GDB**, gastroduodenal bleeding; **GDUs**, gastroduodenal ulcers; **PUD**, peptic ulcer disease.

Introduction

The problem of treating patients with bleeding gastroduodenal ulcers (GDUs) is among the most difficult in modern urgent surgery. High mortality rates indicate an urgent need to improve modern technologies for the treatment of this pathology.[1-6]

The etiological structure of GDUs, according to many studies, has undergone significant changes in recent decades. If in the 1990s, in the structure of GDB, the share of complicated peptic ulcer disease (PUD) of the stomach and duodenum accounted for up to 70.2% of cases, currently, their number does not exceed 30.6%. At the same time, however, acute symptomatic GDUs are becoming increasingly important in clinical practice.[7-10]

The treatment of patients with acute bleeding GDUs is a difficult task of modern gastric surgery. These patients often have severe co-morbid pathology, multiple lesions of the mucous-submucosal layer of the digestive tract, disorders of platelet and coagulation links of hemostasis against the background of taking antiplatelet drugs, anticoagulants, and other drugs.[11-15]

Minimally invasive hemostasis technologies are becoming increasingly important in the treatment of patients with acute symptomatic GDUs. Various methods of endoscopic hemostasis (EH) have become widespread: argon-plasma coagulation (APC), laser coagulation, diathermocoagulation, injection hemostasis, clipping and combined techniques. But even when using combined methods of traditional endohemostasis, the frequency of recurrent bleeding reaches 10%-46%, which shows the need to use new technological capabilities in treating patients with ulcerative GDB.[16-20]

The aim of this research was to study the current clinical and epidemiological features of ulcerative GDB and to evaluate the outcomes of using new technological approaches in patients with bleeding from acute (symptomatic) and chronic (PUD) GDUs.

Materials and Methods

A clinical study was conducted in the Voronezh City Specialized Center for the treatment of patients with gastrointestinal bleeding (Voronezh City Clinical Emergency Hospital №1) in 202-2021. The present study involved 221 patients with bleeding GDUs. Depending on the source of bleeding, all patients were divided into 2 groups. Group 1 included 143 patients with acute symptomatic GDUs; Group 2 consisted of 78 patients with PUD complicated by bleeding.

The clinical and epidemiological characteristics of the study groups are presented in Table 1.

Our analysis showed that acute symptomatic GDUs prevailed in the structure of GDB –143(64.7%) patients; PUD complicated by bleeding was diagnosed in 78(35.3%) cases. The structure of the general incidence of bleeding GDUs was dominated by persons of retirement age – 53.0% of patients. At the same time, the retirement age was typical for Group 1 (66.6%). However, there was an opposite picture among patients of Group 2: 71.7% of patients were of working age, and only 28.3% of retirement age.

Table 1.

Clinical and epidemiological characteristics of the study groups

Indicator	Group 1 (n=143)	Group 2 (n=78)	*P*-value	General group (n=221)
Social status of patients				
Employee	20(13.9%)	24(30.7%)	P=0.0028	44(19.9%)
Non-employee	28(19.5%)	32(41.0%)	P=0.0006	60(27.1%)
Pensioner	95(66.6%)	22(28.3%)	P=0.0000	117(53.0%)
Age and gender of patients				
Age (years)	69.0 (54.0;79.0)	51.0 (37.0;61.0)	P<0.001	62.0 (47.0;72.0)
Men	84(58.7%)	61(78.2%)	P=0.0036	145(65.6%)
Women	59(41.3%)	17(21.8%)	P=0.0036	76(34,4%)
Localization of the source of bleeding				
Duodenal ulcers	72(50.3%)	55(70.5%)	P=0.0038	127(57.5%)
Stomach ulcers	71(49.7%)	12(15.3%)	P=0.0000	83(37.5%)
Gastroentero-anastomosis-ulcers	-	11(14.2%)	-	11(5.0%)
Number of gastroduodenal ulcers				
One	119(83.2%)	71(91.0%)	P=0.1102	190(85.9%)
Two	12(8.4%)	7(9.0%)	P=0.8821	19(8.6%)
Multiple	12(8.4%)	-	-	12(5.5%)
Localization of stomach ulcers				
Body of the stomach	43(60.5%)	6(50.0%)	P=0.0001	49(59.0%)
Antrum	22(30.9%)	5(41.6%)	P=0.0516	27(32.5%)
Cardiac and fundus of the stomach	4(8.6%)	1(8.4%)	P=0.6587	5(8.5%)
Localization of duodenal ulcers				
Anterior wall	29(40.2%)	24(43.6%)	P=0.0809	53(41.7%)
Posterior wall	12(16.6%)	5(9.0%)	P=0.5974	17(13.4%)
Upper wall	24(33.3%)	20(36.3%)	P=0.1151	44(34.6%)
Lower wall	8(9.9%)	6(11.1%)	P=0.7471	14(10.3%)
Bleeding type [21]				
Forrest IA	15(10.4%)	2(2.5%)	P=0.0346	17(7.7%)
Forrest IB	9(6.2%)	3(3.8%)	P=0.4428	12(5.4%)
Forrest IIA	11(7.6%)	8(10.2%)	P=0.5159	19(8.6%)
Forrest IIB	34(23.7%)	14(17.9%)	P=0.3154	48(21.7%)
Forrest IIC	64(44.7%)	40(51.2%)	P=0.3529	104(47.1%)
Forrest III	10(7.4%)	11(14.4%)	P=0.0850	22(9.5%)

Table 1.

Clinical and epidemiological characteristics of the study groups (continued)

	Group 1 (n=143)	Group 2 (n=78)	*P*-value	General group (n=221)
The degree of severity of blood loss [22]				
Mild	68(47.5%)	44(56.4%)	P=0.4444	112(50.7%)
Moderate	25(17.5%)	12(15.4%)		37(16.7%)
Severe	50(35.0%)	22(28.2%)		72(32.6%)
Helicobacter pylori test results				
Positive	16(11.1%)	32(41.0%)	P=0.0000	48(21.7%)
Negative	127(88.9%)	46(59.0%)		173(78.3%)

The average age of patients of Group 2 was 51.0(37.0;61.0) years, while in Group 1, the average age was significantly higher - 69.0(54.0;79.0) years (P<0.001)..

The gender composition of patients was as follows: 78.2% men and 21.8% women in Group 2, 58.7% men and 41.3% women in Group 1.

Depending on the location of the source of bleeding, patients with bleeding GDUs were distributed as follows: bleeding ulcers of the duodenum – 57.5%, stomach ulcers and gastroenteroanastomosis – 37.5% and 5.0%, respectively. In Group 1, duodenal ulcers accounted for 50.3% of cases, and stomach ulcers for 49.7%. In Group 2, duodenal ulcers accounted for 70.5% of cases, stomach ulcers for 15.3%, and gastroenteroanastomosis ulcers for 14.2%. It should be noted that GDUs in 85.9% of cases were single; paired and multiple GDUs were found in 8.6% and 5.5% of cases, respectively.

When analyzing the localization of ulcerative defects, we found that for stomach ulcers, the most typical localization was the stomach body – 59.0% of cases; the antrum of the stomach –32.5%; ulcers of the cardiac region and the fundus of the stomach were less common – 8.5% of cases. For duodenal ulcers, damage to the anterior and upper walls of the duodenum was the most frequent (41.7% and 34.6%, respectively), damage to the posterior and lower walls was less frequent (13.4% and 10.3%, respectively).

To assess the type of ulcerative bleeding, the classification of J. Forrest was used.[21] The most frequent type of ulcerative bleeding was FIIC – 47.1% of cases. FIIB type was detected in 21.7% of patients, FIII in 9.5%, FIIA in 8.6%, FIA in 7.7%, and FIB was observed in only 5.4% of cases. It should be noted that in patients of Group 1, the type of bleeding FIA was statistically significantly more common than in patients of Group 2: 10.4% and 2.5%, respectively (P=0.0346).

According to the severity of blood loss,[22] the patients were distributed as follows: the most common were mild and severe degrees of blood loss – 50.7% and 32.6% of cases, respectively. A moderate degree of blood loss was observed in 16.7% of patients. At the same time, there were no statistically significant differences in the volume of blood loss between the study groups.

All patients were examined for the presence of *Helicobacter pylori*. Positive results of the examination were revealed in 21.7% of patients; the negative urease test was found in 78.3% of patients. At the same time, positive test results for *Helicobacter pylori* were detected in 41.0% of cases in Group 2 and only in 11.1% of cases in Group 1 (P<0.0001).

The structure of concomitant pathology in patients with ulcerative GDB is presented in Table 2. In the structure of concomitant pathology in patients with ulcerative hemorrhages, diseases of the cardiovascular system prevailed (66.0%). It should be noted that for patients of Group 1, compared with Group 2, diseases of the cardiovascular system were much more common. Also, in patients of Group 1 diabetes mellitus was more common than in Group 2 – 13.9% and 3.8%, respectively (P=0.018).

Table 2.

Structure of concomitant pathology

	Group 1 (n=143)	Group 2 (n=78)	*P*-value	General group (n=221)
Diseases of the cardiovascular system	108(75.5%)	38(48.7%)	P=0.0001	146(66.0%)
Respiratory system diseases	13(9.1%)	7(8.9%)	P=0.9748	20(9.04%)
Digestive system diseases	33(20.1%)	18(23.1%)	P=0.8671	51(23.1%)
Diseases of the musculoskeletal system	5(3.5%)	1(1.28%)	P=0.4278	6(2.7%)
Diseases of the urinary system	21(14.7%)	7(8.9%)	P=0.2225	28(12.7%)
Nervous system diseases	14(9.8%)	5(6.4%)	P=0.3916	19(8.6%)
Oncopathology	9(6.3%)	5(6.4%)	P=0.7989	14(6.3%)
Diabetes mellitus	20(13.9%)	3(3.8%)	P=0.0203	23(10.4%)
Ulcerative medications				
Anticoagulants	22(15.4%)	1(1.28%)	P=0.0013	23(10.4%)
Disaggregants	30(21.0%)	11(14.1%)	P=0.2089	41(18.6%)
NSAIDs	14(9.8%)	4(5.12%)	P=0.3061	18(8.1%)

Analyzing the effect of ulcerative medications on the development of ulcerative bleeding, it was found that 18.6% of patients, just before the development of the bleeding episode, took disaggregants, 10.4% of patients – anticoagulants and 8.1% – NSAIDs. It is important to note that anticoagulants were much more often taken by patients in Group 1 – in 15.4% of cases compared to 1.28% of cases in Group 2 (P=0.001).

In the complex treatment of patients of all the studied groups, an individual approach was used that included the innovative technologies of endoscopic cytoprotective

treatment of GDB, based on the combined use of traditional means of EH in combination with the use of endoscopic pneumoinsufflation of biologically active drainage sorbent of a new generation Aseptisorb-D and powdered hemostatic Zhelplastan (Patent RF № 2633588).[23]

Thus, in patients with ongoing bleeding (FIA-FIB), bleeding was initially stopped by injection of a 5% solution of ε-aminocaproic acid at the source of bleeding, followed by APC. EH in the group always ended with endoscopic insufflations of powdered Zhelplastan in combination with Aseptisorb-D.

In patients with unstably stopped bleeding FIIA-FIIB, the method of endoscopic prevention of the resumption of hemorrhage was used by conducting APC of the thrombosed vessel with the type of bleeding FIIA; and with the type FIIB, the clot was washed from the ulcerative defect with subsequent coagulation of the source of hemorrhage. In order to increase the reliability of hemostasis and prevent recurrence of bleeding and endoscopic cytoprotective treatment, each case of therapeutic endoscopy always ended with pneumoinsufflation of Zhelplastan in combination with Aspetisorb-D.

In patients with bleeding type FIIC and FIII, endoscopic treatment was performed through combined insufflations of Zhelplastan and Aspetisorb-D in order to accelerate the processes of reparative regeneration and prevent possible resumption of bleeding.

In complex treatment, patients received modern drug therapy according to the standards of treatment of patients with ulcerative bleeding: antisecretory drugs – proton pump inhibitors (esomeprazole), general hemostatics with the inclusion of modern hemostatics (tranexamic acid, aminomethylbenzoic acid), infusion-transfusion and symptomatic therapy. The indication for surgical treatment in patients with ulcerative bleeding was continued bleeding with the impossibility or ineffectiveness of EH and recurrence of hemorrhage in hospital PUD inpatients, according to National Clinical Guidelines for the treatment of patients with ulcerative GDB.[24]

The following indicators assessed the effectiveness of the treatment: reliability of primary hemostasis in type FIA-IB, effectiveness of endoscopic prevention of recurrent bleeding in types FIIA-IIB and FIIC, frequency of bleeding recurrence, prevention of emergency operations, mortality rates.

Statistical analysis was performed using Microsoft Excel software package. For descriptive analysis, results are presented as median (Me) and interquartile range (*IQR*). Wilcoxon rank sum test was used to test for difference in medians. Group comparisons with respect to categorical variables are performed using chi-square tests with Yates correction or, alternatively, Fisher's exact test when expected cell counts were less than 5. A probability value of $P<0.05$ was considered statistically significant.

The study was carried out in compliance with Ethical Principles for Medical Research Involving Human Subjects, Adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, and amended by the 64th WMA General Assembly, Fortaleza, Brazil, October 2013. The study was approved by the Ethics Committee of Voronezh State Medical University named after N. N. Burdenko. Written informed consent was obtained from all participants.

Results and Discussion

All patients in the studied groups received personalized preventive technologies of cytoprotective treatment of GDUs complicated by bleeding with biologically active granular sorbent of a new generation Aseptisorb-D in combination with the hemostatic agent Zhelplastan, according to the developed technique. Studies have shown that immediately after pneumoinsuflation, Aseptisorb-D in combination with Zhelplastan formed a powerful layer of biologically active hydrogel on ulcerative defects, which protected the ulcerative defect, thrombosed vessel, and coagulation necrosis from the aggressive action of gastric and duodenal contents, showing local hemostatic and cytoprotective effects, preventing the resumption of bleeding and contributing to the favorable course of the reparative process in ulcerative defects.

At the same time, it was noted that in 24 patients of Group 1 with the FIA-FIB type of bleeding, primary EH was achieved in 23(95.8%) patients.

Clinical example. In a 47-year-old woman, against the background of uncontrolled Warfarin intake, continued bleeding from an acute ulcer of the duodenum was not stopped endoscopically. This patient was urgently operated on – a bleeding vessel in the ulcer was stitched. The postoperative period proceeded without complications, the patient was discharged for outpatient treatment on Day 13 after inpatient treatment.

There were no episodes of resumption of bleeding after successful primary EH in Group 1. In this group of patients, a 66-year-old man with the FIA type of bleeding died: His ongoing bleeding was stopped successfully and no repeated bleeding was noted during inpatient treatment, but the patient died from decompensation of concomitant pathology against the background of acute blood loss.

In Group 2, out of 5 patients with ongoing bleeding (FIA-FIB), primary EH was effective in 4 patients (80%). One patient was operated on "at the height of bleeding." There were no relapses of bleeding or fatal outcomes in this group.

When analyzing the results of treatment of patients in Group 1 with unstable stopped bleeding (FIA-FIB), 3(6.67%) of 45 patients had repeated bleeding. All of these 3 patients had disorders of the blood coagulation system due to taking anticoagulants. In 2 patients, repeated bleeding was stopped endoscopically using the developed techniques in combination with complex hemostatic therapy, which made it possible to avoid surgical treatment. One patient was operated on due to the failure of repeated hemostasis and died of pneumonia in the postoperative period. Also in this group of patients, 2 more, aged 81 and 84, died from decompensation of concomitant diseases on the background of anemia.

In Group 2, hemorrhagic relapses were observed in 2(9.1%) of 22 patients with unstably stopped bleeding (FIA-FIB). Both patients were operated on; they underwent the

operation "stitching a bleeding vessel" in an ulcer. One patient died in the postoperative period.

In 64 patients of Group 1 with the FIIC type of bleeding, repeated bleeding was observed in 2(3.1%) patients. These patients also took disaggregants and anticoagulants. All of them underwent repeated EH, according to the developed technique, which was effective in both patients. There were no surgical interventions in this group, but one, a 40-year-old patient, died from decompensation of cardiovascular pathology on the background of anemia.

Of the 40 patients in Group 2 with the FIIC type of bleeding, repeated bleeding was observed in 2(5.0%) patients. One recurrent bleeding was stopped endoscopically, the second underwent surgical treatment – resection of the stomach. There were no fatal outcomes in this group of patients.

With type FIII bleeding in both groups of patients, there was no recurrence of bleeding, emergency operations, or deaths.

Considering the results of treatment of patients in Group 1, it should be noted that the resumption of bleeding from acute GDUs in all 5 patients occurred on 1.5(1.0;2.0) days, which was caused primarily by pronounced hypocoagulation on the basis of taking medications. All these patients underwent repeated EH. In 4(80.0%) out of 5 patients with repeated GDB, the use of the developed innovative technologies of EH made it possible to carry out final hemostasis and avoid surgery; in 1 patient with an acute ulcer of the posterior wall of the duodenum, repeated bleeding could not be stopped endoscopically, and the patient was operated on.

Summarizing the treatment of Group 1 patients with acute GDUs, we noted that in order to achieve final hemostasis in these patients, repeated therapeutic endoscopies, a large number of therapeutic manipulations are necessary, which reduces the effectiveness of the treatment and worsens the quality of life of patients. It is important to note that the cause of acute erosive and ulcerative GDB in most of these patients was taking antiplatelet drugs, which caused them to have disorders of the blood clotting system. The difficulty for the doctor was that most of the patients in this group had a high and very high risk of thromboembolic complications and needed to resume anticoagulant therapy after bleeding was stopped. The decision on the timing of the resumption of therapy with antiplatelet drugs was decided individually, taking into account the risk-benefit ratio in each case.

In addition, of the patients of Group 1 with acute symptomatic GDUs, 66.6% were of retirement age with severe concomitant pathology. The developing GDB in these patients led to decompensation of somatic pathology, which, along with the bleeding itself, posed a direct threat to the life and health of patients. In treating such patients, it is necessary to maximize all the possibilities of conservative therapy aimed at preventing the resumption of GDB and correcting decompensated somatic pathology.

In treating patients with acute GDB and decompensated concomitant pathology, we also used a multidisciplinary approach. All these patients were hospitalized in the intensive care unit, where a dynamic resuscitator constantly monitored them. In addition, a team of doctors of various specialties was immediately involved in their treatment: therapists, cardiologists, neurologists, pulmonologists, clinical pharmacologists, etc. This approach allowed us to stabilize the condition of patients. However, despite the efforts made, 6(4.19%) patients of Group 1 died from decompensation of the underlying disease against the background of anemia.

In patients of Group 2 with gastric ulcer and duodenal ulcer, recurrence of ulcerative bleeding was observed on Day 4.0(2.5;6.0) of inpatient treatment. The cause of bleeding recurrence in 1 patient was an ulcer of the posterior wall of the duodenum penetrating into the head of the pancreas, and in 3 patients, the development of bleeding recurrence was due to the presence of erosive vessels in the ulcerative defect. All these patients were operated on "at the height of bleeding"; 1 patient died in the postoperative period.

The final results of the treatment of patients are presented in Table 3.

Table 3.

Comparative effectiveness of patient treatment

	Group 1 (n=143)	Group 2 (n=78)	*P*-value	General group (n=221)
The effectiveness of primary EH in FIA-IB	23(95.8% with respect to n=24)	4(80% with respect to n=5)	P=0.3202	27(93.1% with respect to n=29)
The effectiveness of endoscopic prevention of recurrent bleeding in FIIA-IIB	42(93.3% with respect to n=45)	20(90.9% with respect to n=22)	P=0.9999	62(92.5% with respect to n=67)
Overall re-bleeding rate	5(3.49%)	4(5.1%)	P=0.7235	9(4.07%)
Day of appearance of repeated bleeding	1.5(1.0;2.0)	4.0(2.5;6.0)	P=0.048	2,.(1.5;5.5)
Final hemostasis	137(95.8%)	73(93.5%)	P=0.5242	210(95.0%)
Emergency surgeries	2(1.4%)	4(5.12%)	P=0.1882	6(2.7%)
Suturing a bleeding vessel in an ulcer	2(1.4%)	3(3.8%)	P=0.3482	5(2.3%)
Stomach resection	-	1(25.0%)	-	1(16.7%)
Mortality	6(4.19%)	1(1.3%)	P=0.4258	7(3.2%)

The use of new technological approaches, including cytoprotective treatment of bleeding defects with biologically active draining sorbents of a new generation in combination with local hemostatics in the complex therapy of patients with ulcerative GDB, has significantly improved the results of treatment, which is confirmed by high rates of final hemostasis (95.0%), indicators of the effectiveness of primary EH in type FIA-IB (93.1%) and prevention of recurrence of FIIA-IIB bleeding (92.5%), with a low frequency of repeated bleeding (4.07%), emergency surgeries (2.7%), and mortality (3.2%).

Conclusion

Currently, in the structure of ulcerative gastroduodenal bleeding, acute symptomatic gastroduodenal ulcers are the most common – 64.7%, and the share of peptic ulcer disease complicated by bleeding accounts for 35.3% of cases only. Peptic ulcer disease and gastroduodenal ulcers complicated by bleeding have certain clinical and epidemiological features, which must be taken into account when treating these patients. The use of modern, new technological approaches based on the introduction of innovative endoscopic technologies with the use of biologically active draining sorbents and local hemostatic agents allowed us to improve the results of treatment, to ensure the possibility of final hemostasis in 95.0% of cases, to reduce the number of recurrent hemorrhages to 4.07% of cases and total mortality to 3.2%. Symptomatic gastroduodenal ulcers are more difficult to treat, which shows the need to develop new complex technologies for their treatment.

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Competing Interests

The authors declare that they have no competing interests.

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Use of the Hemostatic Agent Zhelplastan in Combination with a Granulated Sorbent in the Treatment of Patients with Mallory Weiss Syndrome. International Journal of Biomedicine. 2021;11(2):160-163. doi:10.21103/Article11(2)_OA7
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ORIGINAL ARTICLE

Radiology

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Comparison of a Portable and Non-Portable Ultrasound Machine in the Evaluation of Children with Sickle Cell Disease: A Pilot Study

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Abstract

Background: Transcranial color Doppler (TCCD) ultrasound is used to identify children with sickle cell disease (SCD) at high risk of developing stroke. There is anecdotal evidence to suggest that different ultrasound equipment can give different blood flow velocities. The purpose of this study was to compare two different TCCD ultrasound machines.

Methods and Results: A flow phantom was used to compare PSV measurements from a Philips IU-22 and Zonare Z-One ultrasound machine. Twenty-five children with SCD (aged between 2 and 16 years) attending the outpatient clinic at St. Mary's Hospital, Imperial College Healthcare NHS Trust, as part of the NHS Sickle Cell & Thalassaemia (SC&T) screening program were studied. The two ultrasound machines compared the TAMM velocities in the middle cerebral artery and stroke risk categorization. PSV measurements using a flow phantom were underestimated by Philips IU-22 (31%) and Zonare Z-One (53%). TAMM velocities varied considerably between machines, with a poor agreement in stroke risk categorization. As a result, three children identified at increased risk of stroke by Philips IU-22 were not identified by Zonare Z-One.

Conclusion: Two ultrasound machines were found to underestimate PSV using a flow phantom. The two ultrasound machines were shown to positively correlate, and this was statistically significant. However, there was variation in the TAMM velocities recorded by the machines which resulted in the different categorization of the stroke risk of a small number of the subjects. This pilot study confirms the feasibility and clinical significance of this investigation.(**International Journal of Biomedicine. 2021;11(4):435-440.**)

Key Words: sickle cell disease • stroke • ultrasound • transcranial Doppler

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Abbreviations

MCA, middle cerebral artery; **SCD**, sickle cell disease; **PSV**, peak systolic velocity; **TCD**, transcranial Doppler; **TCCD**, transcranial color Doppler; **TAMM**, time-averaged maximum mean.

Introduction

Sickle cell disease (SCD) is a genetic disorder arising from a point mutation in the gene encoding the hemoglobin protein beta-globin. When inherited in homozygosity or compound heterozygosity with other relevant beta-globin mutations, the resultant mutant hemoglobin causes the development of the clinical syndrome. These genetic abnormalities result in anemia, intermittent severe pain, susceptibility to infection, chronic damage to lungs, joints, kidneys and other bodily

organs, and reduction in life expectancy.[1] Additionally, up to 11% of patients with SCD develop strokes by age 20, with the highest risk of ischemic strokes in HbSS genotype.[2]

Screening for stroke risk can be undertaken using transcranial Doppler vascular ultrasound, either by non-imaging (TCD) or transcranial color Doppler (TCCD) ultrasound.[3] These are non-invasive scans that allow real-time evaluation of the intracranial cerebral circulation by insonation of the cerebral circulation via transcranial windows that include studying the blood flow velocities and the presence of turbulence or stenosis within arteries. A TCD ultrasonography in SCD utilizes the temporal window to examine the middle, anterior and posterior cerebral arteries (MCA, ACA, and PCA, respectively) and distal internal carotid arteries. TAMM velocities greater than 200 cm/s in all arteries are considered abnormal (except in ACA, where a lower threshold of >170 cm/sec is considered abnormal). Readings below 170 cm/sec are normal and those between 170-199 cm/sec are termed conditional.[4]

A large, randomized, controlled study, The Stroke Prevention Trial in Sickle Cell Anaemia, demonstrated the clinical utility of chronic transfusion therapy in children with abnormal TCD readings in primary stroke prevention.[4] This led to the uptake of routine TCD screening in many clinical SCD programs. Several reports indicate that stroke prevention programs using TCD surveillance and transfusion therapy in at-risk children have reduced the incidence of childhood stroke in SCD from 11% [2] to 1.9% by 18 years.[5] Clinical and cost-effective data in the UK context provide further support for screening programs for stroke risk assessment.[6]

The UK Forum on Haemoglobin Disorders (UKFHD) has recommended that UK SCD centers offer annual TCD scans to children aged 2-16 with severe SCD.[7] Another critical document published by UKFHD, the Quality Standards-Health Services for People with Haemoglobin Disorders, also stipulate the need for adequate training, maintenance of competencies, and quality assurance of the TCD screening program.[8] Both imaging and non-imaging Doppler techniques are used in this context, based mainly on the local availability of suitable scanners. Several studies have compared TCD with TCCD,[9] and the current consensus is to use the same arterial velocity cut-offs in either scanning modality.[7] To ensure universal uptake of TCD screening, many Primary Treatment Centers for SCD in the UK have developed outreach TCD surveillance programs in collaboration with local clinical teams to provide TCD screening closer to the patients' homes. [10] These are usually undertaken by using portable vascular ultrasound machines.

Whilst several studies have compared imaging versus non-imaging vascular ultrasound scans in SCD, no studies exist that indirectly compare a portable TCD machine with a non-portable one. Both are used within our SCD program, and it was essential to ensure that comparable readings were achieved using these machines. This study aimed to directly compare TCD readings using the available scanners within our service—the non-portable Philips IU-22 scanner with an S5-1 phased array transducer (5.0-1.0MHz) and portable Zonare Z-One scanner with a P4-1C phased array transducer (4.0-1.8 MHz). We hypothesized that velocity measurements from the portable ultrasound machine would not be significantly different from those obtained by the non-portable laboratory-based machine.

Methods

The study design was a prospective study. Patients with SCD, aged between 2 and 16 years, attending our tertiary pediatric hematology clinic for stroke surveillance, were invited and eligible for the study. Based on patient numbers within the service, we aimed to study 25 consecutive patients referred to the vascular sciences department for routine TCD scanning. This study was approved by the National Research Ethical service, UK Ref number (Ref 13/LO/1503). This study was also registered at the CT.gov, ref number (NCT02090881); written informed consent was obtained from legal guardians. National Research Ethical service permission was obtained for the study.

Study participants were scanned by a single operator who has more than 5 years of experience with imaging TCCD. Initially, a full TCD assessment was performed using the non-portable Philips IU-22 with an S5-1 phased array transducer (5.0-1.0MHz). The measurement was repeated in every patient in only the MCA on each side of the head using the portable Zonare Z-One scanner with a P4-1C phased array transducer (4.0-1.8MHz). For both examinations, a US standardized screening protocol was used, recording velocities from the transtemporal windows, a 5 mm sample volume size, with no angle correction. The gain was adjusted to see the spectral Doppler velocity waveform without noise in the background. For this study, the TAMM velocities were compared because stroke risk categorization, according to the STOP trial, was based on TAMM velocity, not peak systolic or end-diastolic velocities. Each study was categorized as normal if the TAMM velocity <155 cm/sec, conditional if the TAMM velocity was 155-179 cm/sec and abnormal or high risk if TAMM velocity ≥180 cm/sec, or inadequate according to criteria previously developed by the STOP protocol. The participants were not permitted to sleep during the scan to avoid an increase in blood carbon dioxide levels in sleep, which can increase in TCD velocities.

A pulsatile flow-simulating phantom (Fig.1), which generates pulsatile flow from a computer-controlled pump, was used to eliminate patient variability. The manufacturer had calibrated the speed to 120 cm/sec. The flow simulator was used to pump blood-mimicking fluid through a vessel surrounded by an agar-based, tissue-mimicking material. A glycerol/water mix was used to give a liquid with a density and viscosity close to human blood to mimic the speed of sound of 1540 m/sec;[11] 850 ml of water was mixed with 150 ml glycerol. PSV was measured using the Philips IU-22 non-portable scanner with an S5-1 phased array transducer (5.0-1.0MHz) and a Zonare Z-One portable scanner with a P4-1C phased array transducer (4.0-1.8MHz). The measurement of PSV was repeated 25 times with each transducer and the mean PSV was calculated. No angle correction was used with the phased array transducers with a sample volume of 5mm, as per the SCD screening protocol.

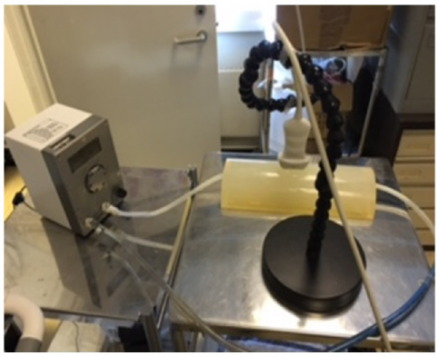

Fig 1. *Pulsatile flow-simulating phantom design for the measurement's accuracy.*

Statistical analyses were performed using SPSS Statistics version 21 (IBM). Spearman's correlation coefficients were calculated to assess the relationships between different parameters in order to assess the strength of the association between the two measures. Intraclass coefficient correlation (ICC) and 95% confidence intervals (CI) were used to measure the strength of the association between the measurements from both pieces of equipment and therefore the validity of the measurement. It has been stated that ICC>0.75 implies acceptable validity.[12] Bland-Altman plots were used to estimates the agreement between TAMM velocity measurements by determining how much the velocity from one machine differs from another. The level of statistical significance was defined as $P<0.05$.

Results

Twenty-five children were included in the study; all had HbSS genotype. The children underwent routine steady-state laboratory tests on the day of their scan or within two weeks. On the day of the scan, all children were reviewed by a clinician as part of their routine clinical assessment. This ensured that they were well at the time of the scan. Table 1 presents demographic details of study participants.

Table 1.

Demographic details of study participants

		N
Genotype	HbSS	25
Female		14
Median age in years (IQR)	9 (6-13)	25
Median Haemoglobin in g/l (IQR)	83 (76.5-90.5)	25
Median Height in cm (IQR)	134.5 (116.8-149.1)	22
Median weight in kg (IQR)	27.4 (19.8-38.8)	23

IQR: Interquartile range

Table 2 outlines the TAMM velocities recorded in the right and left MCA using both scanners. The data demonstrated a significant negative correlation between age and TAMM velocities in the left MCA using the Philips IU-22. There was no statistically significant correlation between the hemoglobin concentration and recorded TAMM velocity. Adequate tracings were obtained from 23 of 25 examinations of the right MCA and 25 of 25 examinations of the left MCA. This was because two of the subjects had poor right temporal windows, and so velocities were not recorded from the MCA. It was noted that the MCA was more difficult to visualize and obtain an optimal signal with the Zonare Z-One because of the limitations of the size of the screen and the resolution.

Table 2.

TAMM velocities recorded in the right and left MCA using Philips IU-22 and Zonare Z-One

			Spearman's correlation with age		Spearman's correlation with Hb levels	
	N	Median (IQR)	rho	*P*	rho	*p*
Philips Right MCA (TAMM)	23	119 (110-128)	-0.425	0.043	-0.111	0.651
Zonare Right MCA (TAMM)	23	105 (95-116)	-0.462	0.027	0.258	0.235
Philips Left MCA (TAMM)	25	120 (102-130)	-0.266	0.199	-0.15	0.473
Zonare Left MCA (TAMM)	25	115 (100-125)	-0.494	0.012	0.292	0.157

IQR: Interquartile range

The velocities from the right and left MCA were analyzed separately, and interhemispheric differences were assessed. A statistically significant correlation between the right and left MCA was demonstrated using both pieces of equipment. Additionally, ICC statistics demonstrated a good level of agreement between the two pieces of equipment; see Table 3 for details.

Table 3.

Correlation of readings from two instruments

Spearman's Correlation Coefficient between right and left TAMM velocity			
	rho	*P*-value	
Philips IU-22	0.675	<0.001	
Zonare Z-One	0.687	<0.001	
ICC between both instruments measuring TAMM velocity in the right and left MCA			
	ICC	95% CI	*P*-value
Right MCA	0.366	(-0.066-0.683)	0.005
Left MCA	0.558	(0.213-0.778)	0.001

The PSV measurements from the two ultrasound machines and the flow-simulating phantom are presented in Table 4, which shows the percentage error of these measurements.

Table 4.

Descriptive statistics of PSV (cm/sec) recorded using the Philips IU-22 ultrasound machine with a linear and phased array probe and the Zonare Z-One using a phased array probe

	Philips IU-22 Phased Array	Zonare Z-One Phased Array
n	25	25
Mean±SD	83.44 ± 5.55	56.64 ± 2.08
95% CI	81.15 - 85.73	58.78 - 60.50
Median (IQR)	84 (78 - 89)	59 (59 - 61)
% Error	31	53

Bland-Altman analysis (Figures 2 and 3) shows that the velocities recorded in the right and left MCA are within the limits of agreement, suggesting agreement between Philips IU-22 and Zonare Z-One. However, the 95% confidence intervals are wide, reflecting the small sample size and the significant variation of the differences. There is a divergence as the velocities increase.

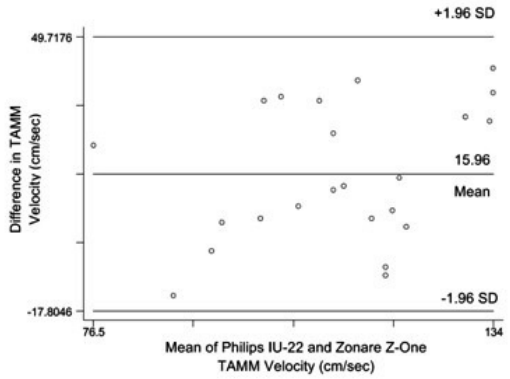

Fig. 2. *Right MCA: Bland-Altman analysis: Agreement between TAMM velocity (cm/sec) measured by Philips IU-22 and Zonare Z-One showing the 95% limits of agreement -17.8 to 49.8 (1.96 Standard Deviation)*

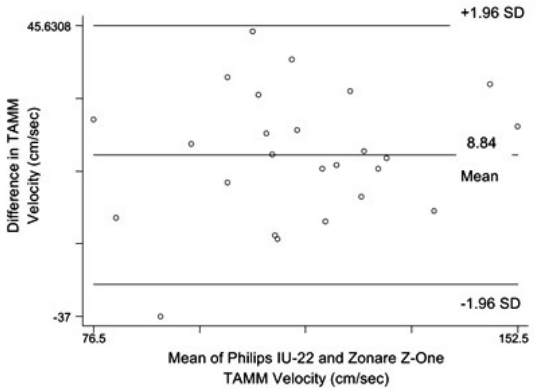

Fig. 3. *Left MCA: Bland-Altman analysis: Agreement between TAMM velocity (cm/sec) measured by Philips IU-22 and Zonare Z-One showing the 95% limits of agreement -17.8 to 49.8 (1.96 Standard Deviation)*

Stroke risk categorization according to TAMM velocities in both MCAs measured in both instruments were in full agreement, and all readings were normal in the 25 study participants.

TCCD with sample volume in the MCA using the Zonare Z-One machine and the Philips IU-22 machine are presented in Images 1 and 2.

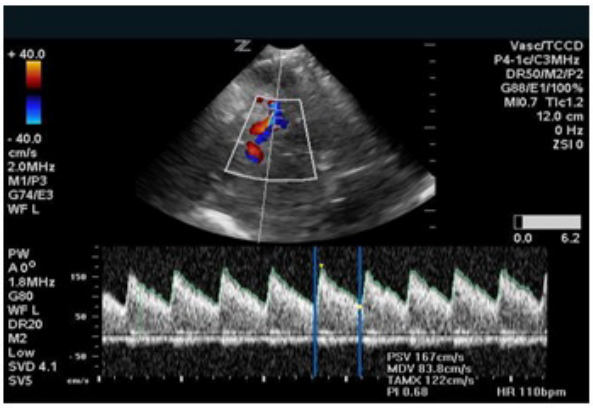

Image 1. *TCCD with sample volume in the MCA using the Zonare Z-One machine. Spectral waveform is enclosed in an envelope with measurement over one cardiac cycle giving PSV, Minimum Diastolic Velocity (MDV) and Time-Averaged Max (TAMX) velocity.*

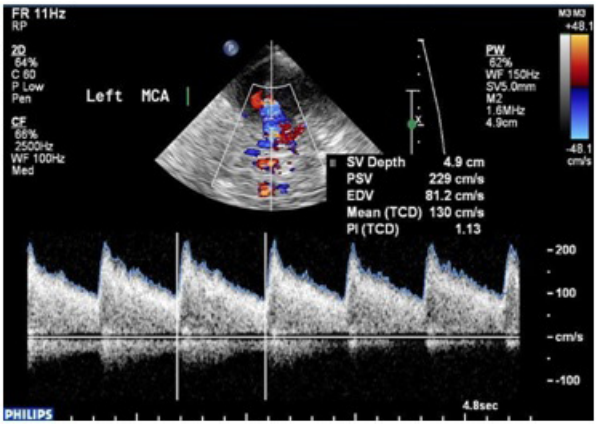

Image 2. *TCCD with sample volume in the MCA using the Philips IU-22 machine. Spectral waveform is enclosed in an envelope with measurement over one cardiac cycle giving PSV, End Diastolic Velocity (EDV) and Mean (TCD) which is the TAMM velocity. The sample volume (SV) depth was also recorded.*

Discussion

Moderate values of correlation between TAMM velocities in the right and left MCA of children with SCD were found, indicating little interhemispheric difference. This finding is supported by a previous study.[13] There was a moderate negative correlation between age and TAMM velocity, reflecting the known inverse relationship between blood flow TCD velocities and age.[4] However, unlike previous studies,[14] no significant correlation was found between TAMM and hemoglobin levels. This may be explained by the fact that recently transfused children were not excluded from the study, and recent transfusions can reduce flow velocity.[15]

This study has demonstrated a moderate correlation in the TAMM velocity readings in the right and left MCA between the two ultrasound machines in children with SCD.

This indicates an association between the TAMM velocities but does not necessarily imply agreement.[16]

Several factors may explain minor differences in readings between the two instruments. Firstly, it is recognized that TCCD ultrasonography does have limitations because it is operator-dependent. The difference between the two machines may be accounted for by minimal differences in probe position and sample volume placement, resulting in significant differences in velocity measurements.[11] This may account for some discrepancy in the readings; however, the effort was made to minimize this by measuring the velocity at the same sample volume depth in the vessel. Variability in velocity measurements can be introduced because velocity measurements depend on the site where a particular vessel segment is sampled, and the blood flow distribution may not be uniform across curved and tortuous arteries.[17] Discrepancies can be due to different sampling sites as the distribution of blood flow across the artery may not be uniform and there are variations in anatomy and tortuosity of the MCA.[11]

No angle correction was used during this study as it is assumed that the angle of insonation between the ultrasound beam and the direction of the blood flow in the MCA is close to 0° so that the velocity can be measured accurately.[18] However, differences in anatomy of the vessels may introduce variability and errors. TCCD allows for visualization of the vessels and placement of the sample volume to try to keep the site of sampling the same. However, human error could have been a factor due to the differences in the machines. On the Zonare Z-One portable machine, the operator rated the ease of obtaining the TAMM velocity measurement in the MCA in terms of the adequacy of view of the circle of Willis and visualization of the distal MCA. The study carried out by Lui et al. found that the waveform display size was important and could be a factor when using the Zonare Z-One as the display size and resolution are not comparable to the Philips IU-22. The transducers are of similar size so that the positioning in the temporal window was consistent.[19]

The MCA was chosen for the comparison because previous studies have suggested that there is less variation in velocities obtained in this vessel due to its anatomy and position relative to the ultrasound beam.[12,20] The measurement of the Doppler shift is dependent on the flow velocity and the angle of insonation. In this situation, the insonation angle is unknown but is assumed to be close to zero due to the anatomy of the MCA. Angle-corrected TCCD velocities are not widely used because it has been suggested that they can overestimate the risk of stroke.[21]

The examinations were performed by the same operator, which removed inter-operator variability. The gain was adjusted to ensure optimal signal-to-noise ratio with the precise automatic tracing of the waveforms, and no manual measurements of velocity were made during the study. We thought that this was less time-consuming as well as more precise and consistent. This adjustment did not vary between machines in an effort to reduce discrepancies in velocity measurements. However, it is not known if there is any discrepancy between the automatic tracing features of the ultrasound machines, so this may need further investigation. Velocity measurements may also be influenced by inherent differences in signal processing.

Although the machines were shown to significantly correlate, Bland-Altman analysis demonstrated wide lines of agreement and variation in differences. It also suggested that as TAMM velocities increase, the variation may also increase. Only one value was greater than two standard deviations, indicating that the repeatability is acceptable.

However, the direct comparisons must be treated with caution. This study focused on the agreement between the two ultrasound machines, rather than the accuracy of the portable ultrasound machine itself. Philips IU-22 was the assumed standard; however, the phantom showed that this standard may also underestimate velocities (data not shown). Further investigation of these differences may require a string phantom, which produces a well-defined velocity[21] and is recommended for annual quality control by the Institute of Physics and Engineering in Medicine.[22]

Physiological factors in the children, such as CO_2 and hemoglobin, were minimized by performing the measurements on the same day to reduce hemodynamic and hematological differences. However, it should be taken into account that the study was performed such that the examination on the Zonare Z-One was carried out after the examination using Philips IU-22. Some of the children were becoming restless during this second examination and may have reduced the opportunity to optimize the examination.

A further limitation is the relatively small sample size in the study, which was related to the difficulties of recruiting children with SCD. However, we believe that our sample was sufficient to provide a valid comparison of the machines. The examinations were performed by the same operator, which may be seen as a limitation because the individual may have been more proficient with a particular machine and was not blinded to the velocities recorded during the previous examination.

Conclusion

The purpose of this pilot study was to evaluate the use of a portable ultrasound machine by comparison with an established, laboratory-based ultrasound machine. Both machines were found to underestimate PSV using a flow phantom. This study demonstrated complete agreement in stroke risk categorization between the two instruments. As a pilot study, it confirms the feasibility and clinical significance of this investigation. The children identified to be at a higher risk by Philips IU-22 were not identified by Zonare Z-One. A larger sample size with a greater range in TAMM velocities is needed to investigate further the correlation between different ultrasound machines at extremes of measurement, such as very high or very low velocities. Further evidence is needed to establish the implications these differences may have on screening.

Acknowledgments

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Competing Interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

Ophthalmology

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The Effectiveness of Laser Treatment for Diabetic Retinopathy in Patients with Chronic Kidney Disease

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Abstract

Background: Panretinal photocoagulation (PRP) remains one of the effective methods of treatment in pre- and proliferative forms of retinopathy with high efficiency. The aim of this study was to investigate the efficacy of PRP depending on the somatic status, laboratory parameters, and the severity of chronic kidney disease (CKD) in patients with type 2 diabetes (T2D) and a history of diabetic retinopathy (DR).

***Methods and Results*:** The study included 76 patients (50 women and 26 men) with T2D who underwent PRP for DR (152 eyes) using a VISULAS® 532s solid-state laser (ZEISS). The patients were divided into two groups depending on the severity of CKD. Group 1 (n=32, 64 eyes) included patients with CKD Stage 1, Group 2 (n=44, 88 eyes) included patients with CKD Stage 2. All patients underwent standard ophthalmological examination: visometry, tonometry, perimetry, biomicroscopy of the anterior segment of the eye and vitreous body, and fundus ophthalmoscopy. Thickness map of the retina was obtained using the RTVue-100 OCT (Optovue, Fremont, CA) EMM5 scan protocol and the Stratus OCT (Carl Zeiss Meditec, USA) radial scan protocol. Laboratory methods included a general blood test, PPG, FG, HbA1c, general urine analysis, and the assessment of blood levels of creatinine, ALT, and AST.

PRP was carried out according to the standard method, gradually, in three stages; the interval between the stages of laser treatment was 1 month. After laser treatment, all patients, regardless of the treatment stage, were prescribed topical Broxinac® (Bromfenac ophthalmic solution 0.09%). The dynamics of corrected visual acuity (CVA) parameters and the retinal thickness of the macular region were assessed before PRP and 3 months after the complex treatment.

Multivariate analysis revealed a linear and nonlinear effect of lipid spectrum indicators on the formation of CL (crystalline lens) pathology. After treatment, a significant increase in CVA was noted in both study groups. The effectiveness of PRP coagulation depended on the severity of the CKD stage in T2D patients with DR.

Normalization of morphometric parameters of the macular region of the retina was noted in 93.8% of cases in Group 1 and in 86.4% of cases in Group 2. The decrease in the effectiveness of treatment was associated with the presence of macroangiopathy (coronary artery disease), concomitant diseases (chronic heart failure, hypertension and dyslipidemia), and CKD stage.

Conclusion: Prolonged administration of the non-steroidal, anti-inflammatory drug Bromfenacum® for a month after each stage of PRP is effective.(**International Journal of Biomedicine. 2021;11(4):441-445.**)

Key Words: diabetic retinopathy • chronic kidney disease • panretinal photocoagulation

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Abbreviations

AST, aspartate transaminase; **ALT**, alanine transaminase; **BMI**, body mass index; **CKD**, chronic kidney disease; **CAD**, coronary artery disease; **CL**, crystalline lens; **CHF**, chronic heart failure; **CVA**, corrected visual acuity; **DR**, diabetic retinopathy; **DME**, diabetic macular edema; **GFR**, glomerular filtration rate; **HDL**, high-density lipoprotein; **LDL**, low-density lipoprotein**; PRP**, panretinal photocoagulation; **T2D**, type 2 diabetes; **TC**, total cholesterol.

Introduction

Diabetic retinopathy (DR) and diabetic nephropathy are typical microvascular complications of T2D caused by chronic hyperglycemia.[1-4] Prevalence of CKD and DR increases proportionally to the disease duration in T2D.[5-7]

DR associated with visual impairment has a significant impact on health-related quality of life.[8] To treat early diabetic neovascular retinopathy, Beetham et al. in 1969 applied the first ruby laser photocoagulation.[9] To date, the methods of retinal laser photocoagulation continue to be improved; the indications and contraindications for this type of treatment are being formed.[10] PRP remains one of the effective methods of treatment in pre- and proliferative forms of retinopathy with high efficiency.[11,12] The effectiveness of PRP varies from 60% to 99% and depends on the degree of suppression of vascularization, stabilization, and improvement of visual functions.[13,14] Many researchers associate such a range of efficacy with the influence of somatic factors, significant fluctuations in laboratory parameters, and the presence of diabetic macular edema, which remains the main cause of vision loss in patients with DR.[10.15,16]

The aim of this study was to investigate the efficacy of PRP depending on the somatic status, laboratory parameters, and the severity of CKD in patients with T2D and a history of DR.

Materials and Methods

The study included 76 patients (50 women and 26 men) with T2D who underwent PRP for DR (152 eyes) using a VISULAS® 532s solid-state laser (ZEISS).

The patients were divided into two groups depending on the severity of CKD. Group 1 (n=32, 64 eyes) included patients with CKD Stage 1, Group 2 (n=44, 88 eyes) included patients with CKD Stage 2.

In this study, the DR classification proposed by Kohner and Porta (1992) was used. The median age of the patients was 62[30;77] years.

The exclusion criteria were T1D, the presence of the inflammatory, post-traumatic, and dystrophic diseases of the eyeball not associated with DM, as well as hereditary and congenital eye pathologies, CKD Stage 3.

All patients underwent comprehensive clinical examination. All patients were examined by a neurologist, therapist, endocrinologist, cardiologist, and podiatrist. CKD was diagnosed with determination of the blood creatinine level and further calculation of the GFR using the Cockcroft&Gault formula.

All patients underwent standard ophthalmological examination: visometry, tonometry using non-contact pneumotonometer (Reichert Technologies), perimetry using PNR-2-01, biomicroscopy of the anterior segment of the eye and vitreous body on an SL-140 slit lamp (Carl Zeiss Meditec AG, Germany), and fundus ophthalmoscopy using a non-contact Ocular MaxField High Mag 78D Lens.

Thickness map of the retina was obtained using the RTVue-100 OCT (Optovue, Fremont, CA) EMM5 scan protocol and the Stratus OCT (Carl Zeiss Meditec, USA) radial scan protocol.

Laboratory methods included a general blood test, PPG, FG, HbA1c, general urine analysis, and the assessment of blood levels of creatinine, ALT, and AST.

PRP was carried out according to the standard method, gradually, in three stages; the interval between the stages of laser treatment was 1 month. After laser treatment, all patients, regardless of the treatment stage, were prescribed topical Broxinac® (Bromfenac ophthalmic solution 0.09%). The dynamics of CVA parameters and the retinal thickness of the macular region were assessed before PRP and 3 months after the complex treatment.

The study was approved by local ethics committee, and written informed consent was obtained from all participants.

Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp. The normality of distribution of continuous variables was tested by one-sample Kolmogorov-Smirnov test. Continuous variables with normal distribution were presented as mean (standard error of the mean [SEM]); non-normal variables were reported as median (interquartile range (IQR; 25th to 75th percentiles). Student's unpaired and paired t-tests were used to compare two groups for data with normal distribution. Mann-Whitney U test was used to compare means of 2 groups of variables not normally distributed. The Wilcoxon criterion was used to compare the differences between the paired samples. The results are graphically presented in the form of a Box and Whisker Plot. A value of $P<0.05$ was considered significant.

Results

Comparative characteristics of clinical and laboratory parameters (Table 1) revealed significant differences in the study groups by age (P=0.025), duration of the disease (P=0.015), and GFR (P=0.015). The groups did not differ in laboratory parameters of the lipid profile, FG, PPG, HbA1c, and liver function tests (Table 1).

After the combined treatment, there was a significant increase in CVA in both groups: from 0.60(0.2;1.0) to 0.69(0.3;1.0) (P=0.000) in Group 1 and from 0.53(0.04;1.0) to 0.63(0.04;1.0) (P=0.000) in Group 2.

The quality of vision and visual prognosis in patients of both groups was influenced by the CL transparency and the state of the macular region of the retina (Table 2). In Group 1, every second patient had CL pathology, and 12.5% of patients had pseudophakia. In Group 2, the incidence of the CL pathology was 77.3%, including pseudophakia in 27.3% of patients, which had a positive effect on visual acuity indicators. Thus, in Group 1, patients with posterior chamber intraocular lenses accounted for 12.5% (6.25% women, 6.25% men); in Group 2, this indicator was 2 times higher - 27.26% (11.36% women, 15.9% men).

Multivariate analysis revealed a linear and nonlinear effect of lipid spectrum indicators (TC and LDL) on the formation of CL pathology, including in patients who received surgical treatment (Fig.1). BMI and HDL indices on their own did not have a reliably significant effect on CL pathology.

Table 1.

Characteristics of clinical and laboratory parameters in study groups

Variable	Group 1 (n=32)	Group 2 (n=44)	*P*-value
Age, yrs	59.81±9.88	64.14±6.60	0.025
Gender, M/F	20/12	30/14	0.606
Disease duration, yrs	12.06±5.99	15.55±6.14	0.015
BMI, kg/m²	31.63±5.92	30.36±5.30	0.33
TC, mmol/L	5.00±1.17	4.65±1.1	0.183
LDL, mmol/L	3.09±1.04	3.0±0.94	0.685
HDL, mmol/L	1.04±0.66	1.35±0.49	0.676
AST, U/L	22.07±10.54	24.16±18.49	0.566
ALT, U/L	25.54±10.54	24.52±16.85	0.772
FG, mmol/L	9.29±3.17	8.82±3.59	0.483
PPG, mmol/L	13.64±3.88	12.96±2.67	0.365
HbA1c, %	8.87±1.83	9.39±1.65	1.199
GFR, ml/min/1.73m²	98.34±3.19	73.21±0.00	0.000

Table 2.

The structure of the CL pathology in the study groups

Pathological condition	Group 1 (n=64)	Group 2 (n=88)	*P*-value
Without CL pathology	32(50.0%)	20(22.7%)	0.000
Male	16(25.0%)	8(9.1%)	
Female	16(25.0%)	12(13.6%)	
CL pathology	32 (50%)	68(77.3%)	0.000
Cataract,	24(37.5%)	44(50.0%)	0.126
Male	4(6.25%)	6(6.8%)	
Fmale	20(31.25%)	19(43.2%)	
Pseudophakia	8 (12.5%)	24(27.3%)	0.027
Male	4(6.25%)	14(15.9%)	
Female	4(6.25%)	10(11.4%)	

The study showed a more pronounced incidence of CL pathology in patients of Group 2 and revealed a significant effect of age (r=0.4, *P*=0.045), disease duration (r=0.5, *P*=0.017) and GFR (r=0.6, *P*=0.016).

Morphometric parameters of the perimacular and macular areas of the retina did not reveal significant differences between the groups before PRP. After the full course of treatment, a significant decrease in the retinal thickness was noted in the nasal (*P*=0.015) (Fig. 2), temporal (*P*=0.041), and inferior segments (*P*=0.044). There were no negative dynamics in CVA and morphometric parameters of the retina against the background of combined treatment (PRP and Bromfenac ophthalmic solution 0.09%) in patients with CKD Stages 1 and 2.

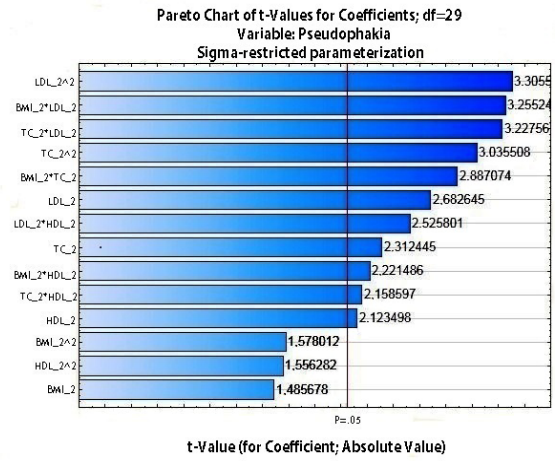

Fig. 1. *Multivariate analysis. Effects of lipid spectrum indicators on the formation of CL pathology, including in patients who received intraocular lens implantation surgery.*

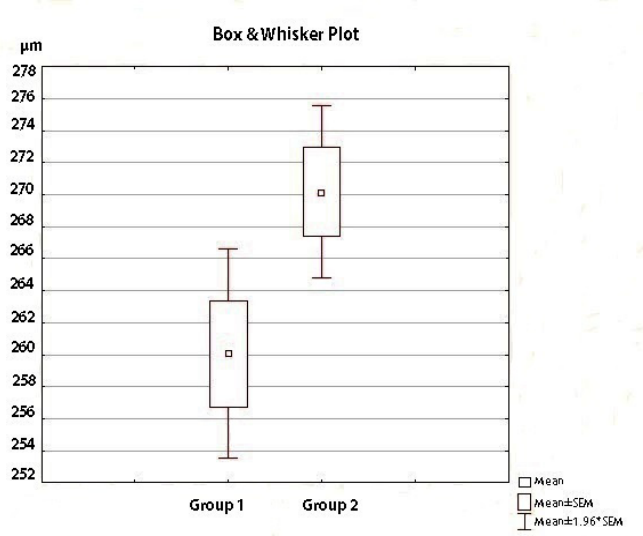

Fig. 2. *Retinal thickness (µm) in the nasal segment after the full course of treatment in the study groups.*

In Group 1, DME in both eyes was diagnosed in 25% of patients (12.5% of women, 87.5% of men) with disease duration of 12.75±4.9 years. In Group 2, DME was observed in 34.1% of patients (46.2% of women and 53.8% of men) with disease duration of 18.38±4.1 years.

Diabetic macroangiopathies in patients with DME were represented only by coronary artery disease. In Group 1, concomitant diseases were represented by dyslipidemia

(75%), obesity (75%), arterial hypertension (62.5%), and chronic heart failure (37.5%). In Group 2, obesity, arterial hypertension, and dyslipidemia were encountered with the same frequency (92.3% of cases), and chronic heart failure in 69.2% of cases

In Group 1, during treatment, complete normalization of the retinal thickness was achieved in 6(75.0%) patients; DME remained in the temporal and superior segments in 2(25.0%) patients. In Group 2, there was a tendency towards a decrease in retinal thickness in the temporal and superior segments, and in 9(60.0%) patients, retinal thickness in the nasal, inferior, and central segments was normalized. Analysis of the somatic status of patients with incomplete regression of DME revealed the presence of coronary artery disease, chronic heart failure and dyslipidemia, which can be considered as predictors for DME in DR patients against the background of CKD Stages 1 and 2.

Discussion

Domestic and foreign scientists have demonstrated the effect of the somatic status features on the development and severity of DR and visual prognosis.[2,4,10] In our study, CVA depended on the CL transparency and the morphometric parameters of the macular region. Our results show a significant effect of the CKD stage, somatic status (patient's age, disease duration, body mass index), laboratory parameters (total cholesterol, low-density lipoprotein) on CL opacity in T2D patients. However, in both study groups, there were patients who underwent surgery to replace the cloudy lens with an intraocular lens, which could affect the CVA values. In Group 2, CL pathology occurred by 27.3% more often than in Group 1. Thus, CKD is a trigger for CL opacity, which depends on the severity of kidney pathology. These data require further clinical research. Against the background of topical use of the non-steroidal, anti-inflammatory drug Bromfenacum® 0.09%(for a month after each stage of treatment), PRP was shown to be effective in 93.8% of patients in Group 1 and in 86.4% of patients in Group 2. In DME, the normalization of morphometric parameters of the macular region of the retina was influenced by the presence of macroangiopathies, concomitant diseases, and the CKD stage.

Thus, macroangiopathies, concomitant diseases, and the CKD stage can also be attributed to biological markers for the DME development. It is necessary to continue monitoring patients with incomplete regression of DME and further correction of the scheme for topical and systemic treatment with normalization of the somatic status in order to restore the morphometric integrity of the retina and reduce the risk for the progression of retinal proliferative processes.

Conclusion

Based on the monitoring of clinical and functional indicators, a significant increase in CVA was noted in both study groups. Analysis of the medical history, somatic status, and laboratory parameters helps to identify biological markers of CL opacity.

The effectiveness of PRP coagulation depends on the severity of the CKD stage in T2D patients with DR.

Normalization of morphometric parameters of the macular region of the retina was noted in 93.8% of cases in Group 1 and in 86.4% of cases in Group 2. The decrease in the effectiveness of treatment was associated with the presence of macroangiopathy (coronary artery disease), concomitant diseases (chronic heart failure, hypertension and dyslipidemia), and CKD stage.

Prolonged administration of the non-steroidal, anti-inflammatory drug Bromfenacum® for a month after each stage of PRP is effective.

Competing Interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

Experimental Medicine

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Effect of the p38 MAPK Inhibitor on the Expression of Metalloproteinases and Their Inhibitors during the Formation of Abdominal Adhesions

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Abstract

Background: The aim of this study was to assess the effect of blockade of the p38 mitogen-activated protein kinase (MAPK) on the expression of genes encoding metalloproteinases (MMPs) during the formation of adhesions in the abdominal cavity.

Methods and Results: The experiments were carried out on male Wistar rats (*n*=70). The studies were carried out in two groups: Group 1 (control, n=35) – modelling the adhesive process; Group 2 (experimental, n=35) – modelling the adhesive process with intraperitoneal administration of Seroguard®—a prolonged form of the p38 MAPK inhibitor. The expression of the *MMP1a*, *MMP2*, *MMP7*, *MMP9*, and *TIMP* genes was assessed using real-time PCR.

In the control group, overexpression of the *MMP1a* and *MMP7* genes began from 6 hours after modeling the adhesive process, *MMP9* – from Day 1, *MMP2* – from Day 7 and persisted until the end of observation. With local blockade of p38 MAPK, the level of overexpression of genes encoding MMPs in the early stages was higher than in the control group (*MMP1a* – by Day 1; *MMP7* – by 6 hours and Day 1, *MMP9* – by 12 hours). From Day 3 to Day 14, the *MMP1a* and *MMP7* expression in the experimental group was significantly lower than in the control group.

Conclusion: The performed study demonstrated the involvement of different types of MMPs—collagenases (MMP1a), gelatinases (MMP2 and 9), matrilysins (MMP7)—in the rearrangement of the extracellular matrix during the process of adhesion formation in the abdominal cavity.(**International Journal of Biomedicine. 2021;11(4):446-450.**)

Key Words: adhesive process • p38 MAPK • MMP • TIMP

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Introduction

The formation and remodelling of the extracellular matrix are involved in the development of a range of diseases. Matrix metalloproteinases (MMPs) and their inhibitors play an important role in this process. MMPs are classified based on various criteria such as preferred substrate, enzymatic reaction mechanism, soluble or transmembrane domains, and structural homology.[1] Collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and others are isolated.[2]

For humans, 23 MMPs are known.[3] Collagenases (MMPs 1, 8, 13, 18) are able to break down collagens of types I, II and III. The main substrates of gelatinases (MMPs 2 and 9) are type IV collagen and gelatin. Stromelysins (MMPs 3, 10 and 11) have a broad ability to break down extracellular matrix proteins but are unable to split triple-helical fibrillar collagens. Matrilysins (MMPs 7 and 26) break down some components of the extracellular matrix. Membrane-type MMPs (MMPs 14, 15, 16, 17, 24, and 25) are expressed on the cell surface and activate proMMP. Other MMPs not classified in the previous categories include MMPs 12, 19, 20, 21, 23, 27, and 28.[2] However, it is becoming more and more obvious that this division is somewhat artificial, since there is a whole range of MMPs that do not fit into any of the traditional groups.

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The catalytic activity of MMPs is regulated by tissue inhibitors of metalloproteinases (TIMPs).[4,5] Traditionally, TIMPs have been thought to control the extracellular matrix by directly inhibiting MMP-dependent proteolysis. This classical role of TIMP suggests that elevated TIMP levels lead to fibrosis, while loss of TIMP leads to increased proteolysis of the matrix. Currently, it is believed that the interaction of MMP and TIMP is much more complex and depends on the specific tissue.[6]

The balance between MMP and TIMP levels controls the degree of local degradation of the extracellular matrix at the periphery of cells and thereby influences cellular processes such as migration, proliferation, and survival.[7]

MMPs are widely studied in the context of their involvement in extracellular matrix remodelling in acute and chronic diseases of inflammatory genesis.[8] However, at present, their biological functions are being reassessed, which has revealed many unexpected targets for MMPs, including the processing of chemokines, cytokines, and cell surface receptors.[9,10]

Considering the role of MMPs in extracellular matrix remodelling, MMPs are the object of study in diseases characterized by excessive growth of connective tissue. MMPs play a complex dual role in the development of fibrosis. MMPs can reduce fibrosis by proteolytic cleavage of extracellular matrix components, but under special circumstances, upregulation of certain MMPs also has an adverse effect that leads to the progression of fibrosis in the liver, lungs, and kidneys. Thus, it has been shown that dysregulation and overexpression of MMPs lead to excessive growth of connective tissue and the formation of rough scars.[11]

MMP production is activated in idiopathic pulmonary fibrosis along with inflammatory agents such as TGF-β and INF-γ. The increased levels of expression of MMP9, TIMP1, MMP1 were noted.[12,13] It is believed that increased regulation of MMP1, 2, 10, 11, and 14 is responsible for the progression of fibrosis.[14, 15] At the same time, an increase in MMP19 reduces the severity of fibrosis.[16]

MMP2, 3, 8, 10, 12, 13, and 14 are significantly increased with liver damage, which, according to some authors, accelerates the process of fibrosis formation.[17,18] Interestingly, an increase in MMP9 expression is associated with both the ability to stimulate development and eliminate fibrosis in the liver.[19] An increase in MMP9 expression in myocardial infarction has been proven.[20, 21]

The role of MMPs has also been studied in adhesions in the abdominal cavity. It has been shown that the levels of MMP2 and 9 in the blood serum in an experimental model can serve as prognostic markers for the detection of postoperative adhesions.[22] In clinical observation, it was shown that the concentration of MMP9 in the peritoneal fluid was significantly lower in women with adhesions in the pelvis than in healthy women, and the MMP9/TIMP1 ratio was significantly higher in women with significant adhesions during repeated laparoscopy compared with women with minimal adhesions or without adhesions.[23]

The aim of this study was to assess the effect of blockade of the p38 mitogen-activated protein kinase (MAPK) on the expression of genes encoding MMPs during the formation of adhesions in the abdominal cavity.

Materials and Methods

In this study, we used 70 nine-month-old male Wistar rats weighing 220–250 g. The rats were sedated using Ketamine 50 mg/kg, Droperidol 2.5 mg/kg and Atropine 0.4 mg/kg. An aseptic inflammatory process in the abdominal cavity was simulated by opening the serous-muscular layer of the cecum with a 1 cm incision, followed by closing the wound using screw sutures and the scarification of the right lateral canal.[24, 25]

The animals were kept in accordance with good laboratory practice. The experiments were performed in accordance with the norms for the humane treatment of animals, which are regulated by the International Guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care in accordance with the protocol approved by the Institutional Animal Care and Use Committee of the Irkutsk Scientific Center of Surgery and Traumatology (Protocol No. 6 of 04.18.2017).

The studies were carried out in two groups: Group 1 (control, n=35) – modelling the adhesive process; Group 2 (experimental, n=35) – modelling the adhesive process with administration of Seroguard® (conjugate the 4-[4-(4-fluorophenyl)-2-(4-methylsulfylphenyl)-1H-imidazole-5-pyridine with polyvinyl-imidazole, JSC "Pharmasyntez") in a volume of 3 ml during the completion of the operation.[26]

The animals were sacrificed, and tissues were collected for examination at 7 time points, ranging from 6 hours to 30 days.

The study of gene expression in the serous-muscular layer of the cecum of intact animals (n=5) was used to determine the basic expression of genes.

The samples of the caecum lesion zone were collected from the experimental animals, placed in RNAlater solution (Ambion, Canada, Cat. N 7020), and then crushed in liquid nitrogen. RNA extraction was performed using the RNeasy Mini Kit (Qiagen GmbH, Germany, Cat. N 74104). RNA purification was performed with the Rnase-Free DNase Set (Qiagen GmbH, Germany, Cat. N 79254). cDNA synthesis was performed using the cDNA – RT2 First Strand Kit (Qiagen GmbH, Germany, Cat. N 330401).

The gene expression analysis was performed using real-time polymerase chain reaction (PCR) on a CFX96 Bio Rad device (USA). Gene expression was determined using the RT2-Profiler ™ Array Rat Wound Healing Kit (Qiagen GmbH, Germany). The genes are listed in Table 1. We used the RT2 SYBR Green qPCR Mastermix oligonucleotide kit (Qiagen GmbH, Germany, Cat. N 330503). The relative fold difference in gene expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Table 1.
Genes tested using real-time PCR

Notation	Gene name	GenBank	Unigene
MMP1a	Matrix metallopeptidase 1a (interstitial collagenase)	NM_001134530	Rn.79007
MMP2	Matrix metallopeptidase 2	NM_031054	Rn.6422
MMP7	Matrix metallopeptidase 7	NM_012864	Rn.10282
MMP9	Matrix metallopeptidase 9	NM_031055	Rn.10209
TIMP1	TIMP metallopeptidase inhibitor 1	NM_053819	Rn.25754

Results

We have evaluated the effect of local application of the p38 MAPK inhibitor on the expression of genes encoding metalloproteinases MMP1a, MMP2, MMP7, MMP9, and the TIMP1 inhibitor in the adhesion formation zone. For this purpose, Seroguard® was used. It is known that Seroguard® is a prolonged form of the p38 MAPK inhibitor intended for intraperitoneal administration.[26, 27]

It was found that in the control group, overexpression of the *MMP1*a and *MMP7* genes began as early as 6 hours after modeling the adhesive process, *MMP9* – from Day 1, *MMP2* – from Day 7 and persisted until the end of observation, indicating the ongoing restructuring of tissues in the damaged zone. The differences were significant with the indices of intact animals for the *MMP1a* and *MMP7* genes at all observation periods, for the *MMP9* gene – from Day 1 to Day 30, for the *MMP2* gene – from Day 7 to Day 30.

The overexpression of the *MMP1a*, *MMP7* and *MMP9* genes has two peaks, on Days 3 and 14, and the overexpression of *MMP2* has one peak – on Day 14 (Figure 1).

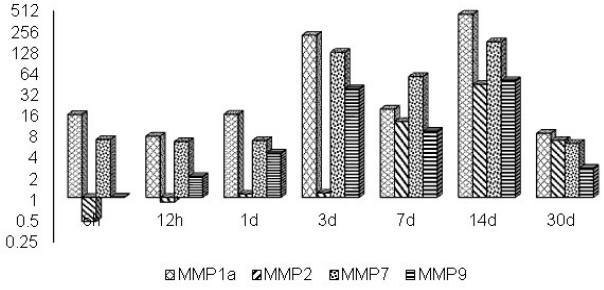

Fig. 1. *Expression of genes encoding MMPs in animals of the control group.*

In the experimental group, all genes encoding MMPs, except for *MMP2* (increased from Day 3), were overexpressed as early as 6 hours after modeling the adhesive process (Figure 2), and gene activity was increased until the end of observation. The differences were significant with the indices of intact animals for all genes encoding MMPs, except for *MMP2*, at all periods of observation. The first peak of activity for *MMP1a* falls on Day 1, *MMP9* – on Day 3, and *MMP7* – on Day 7. The second peak of activity for all genes encoding MMPs is observed on Day 14.

In the experimental group, from 6 hours to a day after injury, the level of overexpression of genes encoding MMPs in the injury zone was higher than in the control group. The differences were significant for *MMP1a* in a period of Day 1, for *MMP7* – in a period of 6 hours and Day 1, for *MMP9* – in a period of 12 hours. From Day 3 to Day 14, the activity of genes encoding MMPs in the experimental group was significantly lower than in the control group. Differences were significant for *MMP1a* at Day 3 and Day 14, and for *MMP7* – at Day 3. However, by the end of the observation (30 days), the expression in the experimental group again exceeds that of the control group. The differences were significant for the *MMP7* and *MMP9* genes.

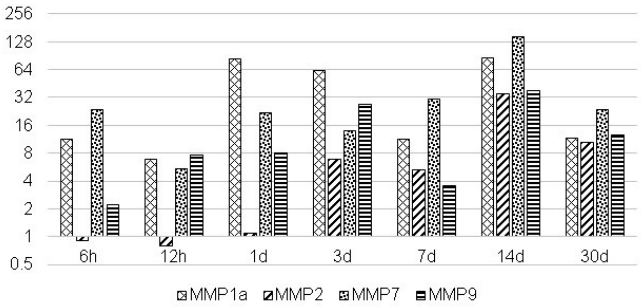

Fig. 2. *Expression of genes encoding MMPs in animals of the experimental group.*

At the same time, in the experimental group, the expression level of *TIMP1* (known as an inhibitor of MMP1) in the early stages (Day 3) was significantly higher than in the control group. In the rest of the periods, no significant differences were found between the groups (Figure 3).

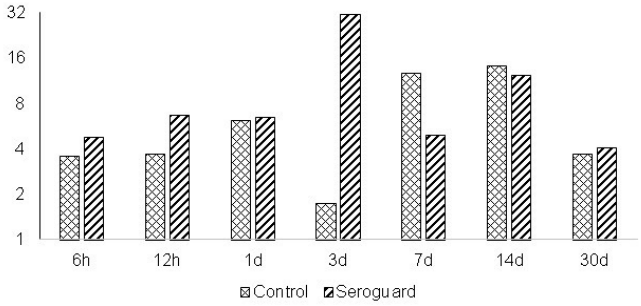

Fig. 3. *The TIMP1gene expression in animals of the control and experimental groups.*

Interestingly, in animals of the control group, the severity of overexpression of the *MMP1a* gene was significantly higher than the *TIMP1* gene, with the maximum severity of differences on Days 3 and 14 (Figure 4).

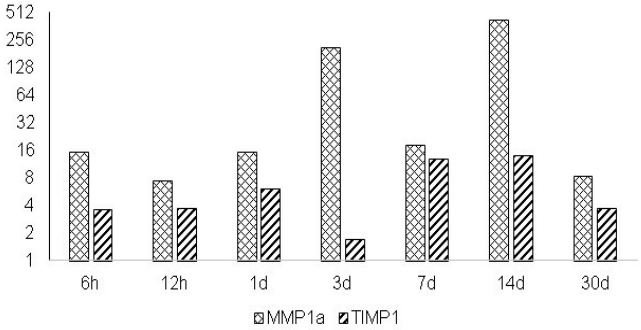

Fig. 4. *Expression of the TIMP1 and MMP1a genes in animals of the control group.*

In animals of the experimental group, the difference was less pronounced (Figure 5).

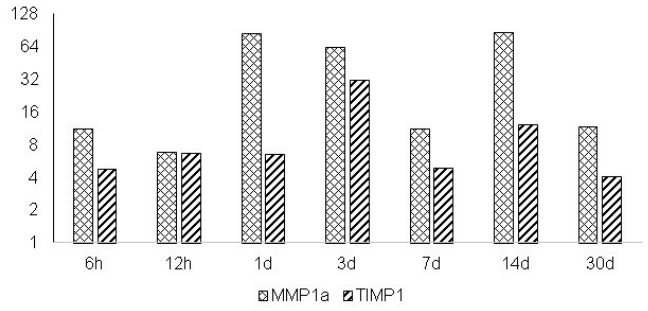

Fig. 5. *Expression of TIMP1 and MMP1a in animals of the experimental group.*

Conclusion

The performed study demonstrated the involvement of different types of MMPs—collagenases (MMP1a), gelatinases (MMP2 and MMP9), matrilysins (MMP7)—in the rearrangement of the extracellular matrix during the process of adhesion formation in the abdominal cavity. Moreover, the peaks of metalloproteinase activity coincide with the periods of active restructuring and formation of the extracellular matrix and fall on Days 3 and 14 (control group). The overexpression of the *MMP*2 and *MMP7* genes are most pronounced.

Local blockade of p38 MAPK affects the overexpression of MMPs in the peritoneal injury zone, leading to early pronounced overexpression (up to Day 1) of the *MMP1a*, *MMP7*, and *MM9* genes. However, in the control group, the expression of the *MMP1*a and *MMP7* genes on Days 3 and 14 is higher than in the experimental group with local blockade of p38 MAPK.

Competing Interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

Dentistry

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Effect of Air-Abrasion on Shear Bond Strength of Resin Composite to Dentin: A Study in Vitro

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Abstract

The purpose of this study was to evaluate in vitro the efficacy of alumina, sodium bicarbonate and erythritol-based tooth air-abrasion on shear bond strength (SBS) of resin composite to dentin.

Methods and Results: In order to assess the strength of the adhesive bond of the resin composite to tooth dentin, 50 tooth samples were prepared in accordance with the Ultradent Shear Bond Test method. All samples were divided into 5 groups. In Group 1 (n=10) and Group 2 (n=10), for air-abrasion of dentin surface 2 powders based on aluminum oxide with a particle size of 50 μm and 27 μm, respectively, were used (RONDOflex plus 360, KaVo, Biberach, Germany). In Group 3 (n=10) and Group 4 (n=10), other abrasive powders based on sodium bicarbonate (40 μm) and erythritol (14 μm), respectively, were used for a similar purpose (Air-Flow Classic comfort, Air-Flow Plus, EMS, Nyon, Switzerland). The control group (n=10) consisted of the remaining tooth samples in which the dentin surface, after preparation with a carbide bur, was not subjected to an air-abrasion.The one-day adhesive strength of bonded interfaces was evaluated on an UltraTester device (Ultradent Products Inc., USA) after resin bonding without aging simulation. The speed of movement of the test clamp with the installed sample was set to 1 mm/min. The maximal value of bonding failure was fixed in pounds (lb). The dentin surface ultrastructure was studied on 10 additional tooth samples, which were prepared for SEM analysis.

It was found that the treatment of dentin surface with air-abrasive powders based on alumina (50 μm and 27 μm) and sodium bicarbonate (40 μm) did not improve the strength of the adhesive bond of resin composite to dentin. The strength of adhesion of the resin composite to dentin decreased significantly after air-abrasion of the tooth surface with erythritol-based powder.(**International Journal of Biomedicine. 2021;11(4):451-455.**)

Key Words: air abrasion • dentin • shear bond strength

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Introduction

The longevity of cosmetic dental restorations is mainly limited by the quality of the adhesive bonding of composite materials with dentin and enamel because under conditions in the oral cavity, adhesive and hybrid layers are exposed to progressive degradation.[1-3]

For many years of practical experience and scientific research in the field of adhesive dentistry, it has been emphasized that a poor integration of resin material with tooth hard tissues is among the most frequently mentioned reasons for the loss of adhesive restorations and contributes to a violation of marginal seal and development of carious demineralization.[4-7]

Reliable adhesion of resin composite to dentin substrate may be determined by the formation of a hybrid layer resulting from the penetration of adhesive into the dentinal tubules, forming adhesive tags. Concurrently, there is a great deal of

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evidence indicating a noticeable improvement in the strength of adhesion of resin materials to dentin, especially in surface segments with a high density of dentinal tubules. Therefore, a perfect infiltration of an etched dentin surface with adhesive resin would yield a greater number of adhesive tags and better integration.[8-10] However, in accordance with the results of other studies, there is no correlation between adhesive bond strength, depth of resin penetration, and the number of resin tags.[11,12]

Nevertheless, achieving a strong adhesion of resin composites to the tooth dentin is not easy because of relatively high moisture and organic content in the tissue. Also, there is a smear layer on a tooth surface, which occurs after the preparation of the carious cavity and negatively influences dentin bonding.[13,14] Removal of the smear layer is a necessary step when a total-etch approach is chosen, and for many decades a phosphoric acid in the form of gels has been used to make it.[15,16]

However, due to the extension of indications for the application of air-abrasion in professional dental care, some studies have demonstrated the effectiveness of this method in adhesive dentistry in order to improve the surface micro-roughness and to increase the area of bonding surface.[17] In addition, as a result of another study carried out in this field, it was found that the use of air-abrasion of the dentin surface after traditional preparation using conventional rotary instruments would contribute to a significant decrease in the thickness of the smear layer.[18]

It should be noted that for a long time the main instruments used for mechanical preparation of hard tooth tissues still remain diamond and carbide burs. However, because new devices and materials are used in adhesive dentistry for making air-abrasive preparations, there is an urgent need for preliminary in vitro studies in order to assess their influence on the quality and strength of adhesion to hard tooth tissues.[19,20]

In dentistry, alumina and sodium bicarbonate abrasive powders are among the most commonly used for tooth surface abrasion with air-born particles. However, taking into account the results of a large number of studies that indicate the probable loss of healthy hard tissue during pseudo-mechanical tooth preparation using compressed air and inorganic powders, low-abrasive organic particles, such as erythritol, began to find their place in daily practice.[21-23]

Available databases contain a large amount of inconsistent data indicating both the positive and negative efficiency of using air-abrasive powders based on alumina and sodium bicarbonate to improve the adhesion of composite materials to tooth hard tissues.[22,24,25] These databases also lack information highlighting the consequences of the air-abrasion with low-abrasive particles. For these reasons, the purpose of this study was to evaluate in vitro the efficacy of alumina, sodium bicarbonate and erythritol-based tooth air-abrasion on shear bond strength (SBS) of resin composite to dentin.

Material and Methods

In order to assess the strength of the adhesive bond of the resin composite to tooth dentin, 50 tooth samples were prepared in accordance with the Ultradent Shear Bond Test method.

On all samples, in order to get a uniform smear layer, the surface of dentin was treated with an application of tungsten carbide bur under constant water cooling. The prepared surfaces were rinsed with a water spray for 30 seconds and dried using an air-water syringe of the dental unit. After that, all samples were divided into 5 groups. In Group 1 (n=10) and Group 2 (n=10), for air-abrasion of dentin surface 2 powders based on aluminum oxide with a particle size of 50 μm and 27 μm, respectively, were used (RONDOflex plus 360, KaVo, Biberach, Germany). In Group 3 (n=10) and Group 4 (n=10), other abrasive powders based on sodium bicarbonate (40 μm) and erythritol (14 μm), respectively, were used for a similar purpose (Air-Flow Classic comfort, Air-Flow Plus, EMS, Nyon, Switzerland). The control group (Group 5, n=10) consisted of the remaining tooth samples in which the dentin surface, after preparation with a carbide bur, was not subjected to an air-abrasion.

In each case of air-abrasion, the nozzle of the instrument was angulated at 45° to the dentin surface. The treatment was carried out with a constant stream of particles under 0.25 MPa pressure in a handpiece for 30 seconds, slowly moving a nozzle with sweeping motions above the surface of tooth samples at a distance of 5mm, after which the tooth surface was thoroughly washed with an air-water spray for 30 seconds.

In all samples, the adhesive protocol was carried out by means of the wet bonding method using the adhesive resin OptiBond Solo Plus (Kerr, Italia).

Dentin etching was carried out for 15 seconds using a 37% phosphoric acid gel (FineEtch®37, Spident Co., Ltd, Korea), after which the etched surface was rinsed with distilled water for 15 seconds and excess moisture was carefully blotted out. Application and polymerization of adhesive resin were done in accordance with the manufacturer's instructions. The light-curing composite GRADIA DIRECT (Japan) served as a resin composite of choice. The composite was cured for 20 seconds using a VALO lamp (Ultradent Products Inc., USA) in a standard mode.

The one-day adhesive strength of bonded interfaces was evaluated on an UltraTester device (Ultradent Products Inc., USA) after resin bonding without aging simulation. The speed of movement of the test clamp with the installed sample was set to 1 mm/min. The maximal value of bonding failure was fixed in pounds (lb).

The dentin surface ultrastructure was studied on 10 additional tooth samples, which were prepared for SEM analysis. The dentin surface was treated in accordance with the methods used in the study. Therefore, there were 2 tooth samples for each of the 5 methods of dentin surface treatment.

After an appropriate mechanical treatment of the dentin surface in each group all prepared samples were thoroughly washed for 30 seconds and dried using a water-air syringe. Before the SEM analysis, the dried samples were coated with a 10 nm thick layer of gold on a magnetron sputtering machine (Quorum Q150R ES, UK). Morphological studies of the dentin surface were carried out using a scanning electron microscope SEM - EVO MA 10 (Zeiss, Germany). Images were taken using SmartSEM software.

Statistical analysis was performed using StatSoft Statistica v6.0. The mean (M) and standard deviation (SD) were calculated. Multiple comparisons were made with one-way ANOVA and post-hoc Tukey HSD test. A probability value of $P<0.05$ was considered statistically significant.

Results

Results obtained after evaluation of the SBS of resin composite adhesion to tooth dentin are presented in Table 1. According to the presented data, the lowest SBS among the groups was demonstrated in Group 4. The SBS values in Groups 1, 2, 3, and the control group did not differ significantly. The SBS value in Group 3 was slightly higher than in Group 4. The SBS value in Group 4 was significantly lower than in the control group (Group 5) and Group 1.

Table 1.
SBS of resin composite adhesion to tooth dentin in the study groups

Variable	Group 1	Group 2	Group 3	Group 4	Group 5
SBS, lb	28.53±4.13	27.61±3.44	25.92±6.0	23.05±3.33	28.35±3.3
Statistics	P=0.0282; P_{1-2}=0.9876, P_{1-3}=0.6307; P_{1-4}=0.0393, P_{1-5}=1.0000 P_{2-3}=0.8929, P_{2-4}=0.1218, P_{2-5}=0.9946, P_{3-4}=0.5429, P_{3-5}=0.6904, P_{4-5}=0.0498				

During the analysis of micrographs taken from the surface of tooth samples of the control group, it was found that preparation of dentin using a rotary instrument had led to the formation of a pronounced smear layer with a small number of visible tubule orifices (Fig.1). However, the use of air-abrasive powders had facilitated an effective removal of the smear layer from the dentin surface of tooth samples, exposing a large number of dentinal tubules in all experimental groups (Fig.2-5).

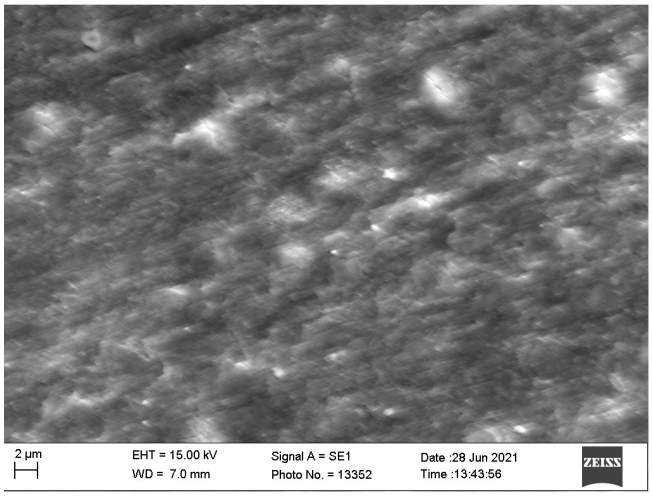

Fig. 1. *Dentin surface after preparation with a tungsten carbide bur (×2000)*

The comparative analysis of configurations of 2D profiles of different dentin surface segments in samples of Groups 1 and 2 (Fig.2,3) did not reveal significant visual differences. It was also found that on the dentin surface of samples in Groups 3 and 4, there were abrasive powder residues, especially in samples abraded with erythritol (Fig.4).

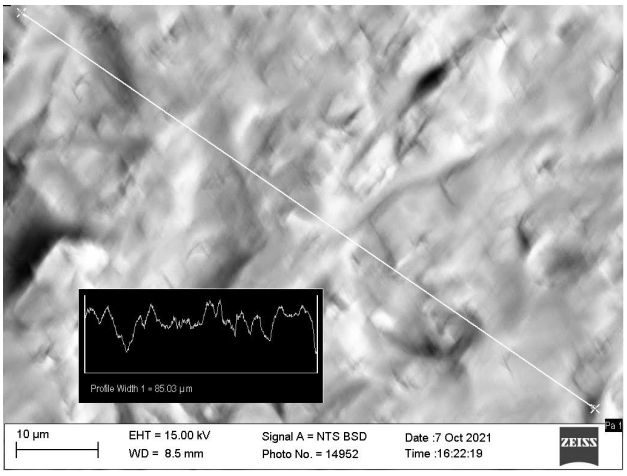

Fig. 2. *Dentin surface after preparation with alumina powder with a particle size of 50 µm (×1500)*

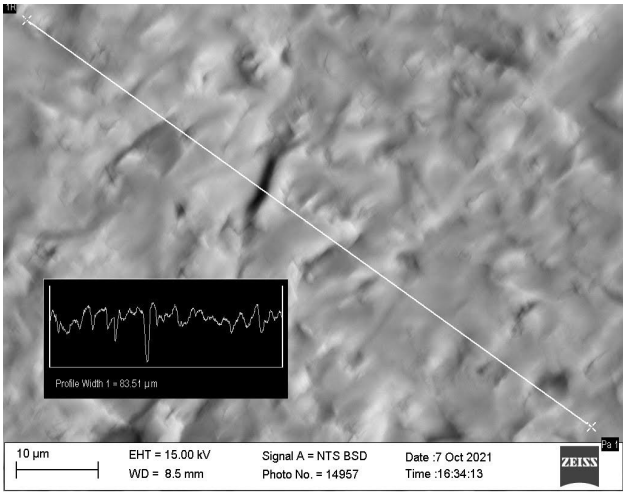

Fig. 3. *Dentin surface after preparation with alumina powder with a particle size of 27 µm (×1500)*

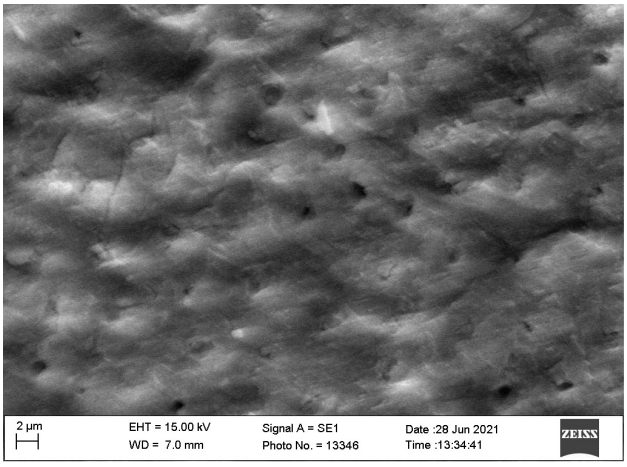

Fig. 4. *Dentin surface after preparation with sodium bicarbonate (40 µm) (×2000)*

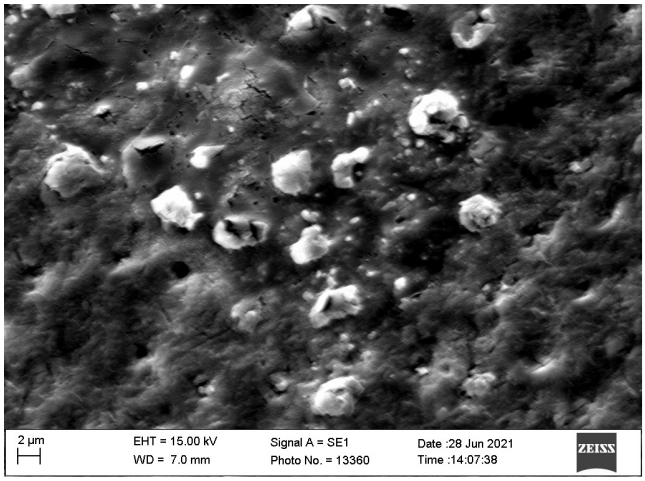

Fig. 5. *Dentin surface after preparation with erythritol (14 µm) (×2000)*

Discussion

The possibility of effective use of air-abrasive powders for the preparation of dental tissues was proposed for the first time by R.B. Black.[26] However, due to the high cost of the method and absence of high-velocity suction units, time consumption, and lack of technologies and materials at that time to perform highly aesthetic tooth restorations, this approach to tooth preparation was not as much demanded as the traditional method of cutting affected hard tissues with rotary instruments.

Revival of air-abrasion technology for tooth cleaning and preparation was not just facilitated by the understanding of the entire complexity of tooth enamel and dentin surface ultrastructure, but also by the emergence of nanomaterials for tooth cosmetic restoration and instructions for performing a proper adhesion in order to get long-term aesthetic and functional treatment results.[27]

In fact, an air-abrasion of natural pits and fissures facilitates detection of the extent of enamel demineralization in the presence of a diagnosed decay and also helps to perform high-quality cleaning of tooth surfaces from organic deposits and microbial biofilm.[28]

Nevertheless, despite the extensive knowledge in the field of tooth hard tissue air-abrasive preparation, the number of studies in the discipline does not decrease, which is directly related to the development and implementation of new abrasive powders.

The present study was aimed at investigating the effect of different air-abrasive treatment strategies on the adhesion strength of resin composite to dentin without aging. We used 3 traditional abrasive powders based on Al2O3 (50 µm and 27 µm) and sodium bicarbonate, and a relatively new powder based on erythritol, which is a food polyol. It is soluble in water and has a lower relative surface hardness, according to the Mohs scale.

Examination of micrographs of the dentin surface treated with an abrasive powder based on erythritol revealed particle residues in some areas, which was apparently a result of the destruction of larger particles upon collision with the dentin surface. Most of the residues were located in the orifices of dentinal tubules and might represent a mechanical obstacle for penetration of the adhesive resin into dentin.

A similar analysis of micrographs obtained from the dentin surface of samples of Groups 1, 2, and 3 revealed that only in the case of sodium bicarbonate abrasion were there sporadic fragments of abrasive particles, which could be seen on the dentin surface. However, it did not result in a significant decrease in adhesive strength when compared with other groups.

In the present study, the adhesion strength of resin composite to tooth dentin in samples of the control group, in which traditional adhesive protocol was used according to the Etch & Rinse principle, did not have a statistical difference from the mean value when compared with other groups where the main part of the smear layer was removed by air-abrasion before an etching step. The same results were confirmed by other researchers who used abrasives based on alumina (50 µm) and different adhesive resins in an attempt to improve adhesion to dentin. They concluded that air-abrasion allows decreasing the thickness of the smear layer and increases the thickness of the hybrid layer. However, the bond strength was slightly improved due to the large number of defects observed in the hybrid layer on the dentin surface.[29-31] Besides, air-abrasive treatment of tooth surfaces using alumina-based powders caused a loss of hard tissues.[22,32]

In the present study, during the preparation of several tooth samples, excessive dentin tissue removal was also noted with the formation of pits on the surface of samples after air-abrasion with 50 µm alumina particles.

Thus, within the limits of the study, it was found that the treatment of dentin surface with air-abrasive powders based on alumina (50 µm and 27 µm) and sodium bicarbonate (40 µm) did not improve the strength of the adhesive bond of resin composite to dentin. The strength of adhesion of the resin composite to dentin decreased significantly after air-abrasion of the tooth surface with erythritol-based powder.

Conflict of Interests

The authors declare that they have no conflicts of interest.

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Production, Properties and Swelling of Composite Agar-Pectic Gel Particles in an Artificial Gastroenteric Environment

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Abstract

The purpose of the present work was to obtain and study the properties of composite calcium-agar-pectic gel particles (CaAPGPs) obtained from aqueous solutions of agar (AA) and apple pectin (AP), from aqueous solutions of agar (AA) and pectin heracleuman (HS) in the presence of Ca^{2+} ions (0.34 M). The swelling of the obtained composite CaAPGPs in an artificial gastroenteric environment was also investigated.

***Methods and Results*:** We used commercial AP AU701 (AP, Herbstreith & Fox KG, Germany), HS isolated from the aerial part of the Sosnovskyi hogweed *Heracleum sosnowskyi* Manden, and food agar (AA). Spherical composite CaAPGPs were obtained from low-methyl esterified AP with a molecular weight of 406 kDa, pectin HS with a molecular weight >300 kDa, and food agar (AA) in the presence of Ca^{2+} ions (0.34 M) as a cross-linking agent by the method of ionotropic gelation.

It was found that dry CaAPGPs based on AP (Ca-AA-AP) have a diameter of 1.16±0.14–1.23±0.05 mm, which was greater than the diameter of dry CaAPGPs based on HS (Ca-AA-HS) (0.95±0.12–1.16±0.05 mm). The density of dry CaAPGPs based on AP (Ca-AA-AP) with an increase in the concentration of AP in their composition from 1% to 2% increased by 1.7 times – from 0.37±0.07 mg/mm^3 to 0.63±0.05 mg/mm^3. Dry composite CaAPGPs based on HS (Ca-AA-HS) were denser. With an increase in the HS concentration in their composition from 1% to 2%, the degree of particle density increases by 2.2 – from 0.45±0.03 mg/mm^3 to 0.97±0.19 mg/mm^3.

The swelling and degradation of the obtained dry composite CaAPGPs in an artificial gastroenteric environment were studied. It was found that CaAPGPs formed from 1% AP and 2% AA degraded almost immediately in simulated intestinal fluid. Whereas, CaAPGPs formed from 2% AP and 1% or 2% AA completely degraded in simulated colonic fluid after 1 hour of incubation in it.

CaAPGPs formed from 1% HS and 2% AA, and particles obtained from 2% HS and 1% AA, remained stable in simulated intestinal fluid, and then completely degraded immediately upon entering in simulated colonic fluid. CaAPGPs, consisting of 2% HS and 2% AA, dissolve in simulated colonic fluid after 1 hour of incubation in it.(**International Journal of Biomedicine. 2021;11(4):456-459.**)

Key Words: apple pectin • heracleuman • calcium ions • composite agar-pectic gel particles • artificial gastroenteric environment

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Abbreviations

AP, apple pectin; **HS**, heracleuman; **CaAPGPs**, calcium-agar-pectic gel particles; **GIT**, gastroentestinal tract; **SGF**, simulated gastric fluid; **SIF**, simulated intestinal fluid; **SCF**, simulated colonic fluid.

Introduction

In recent years, a surge of interest has been devoted to the research of natural polysaccharides (pectin in particular) for their wide range of biological properties and different applications in pharmacology. Pectin is a polysaccharide with a core consisting of α-1,4-linked D-galacturonic acid and α-1,2-L-rhamnose units in turn, as well as a large number of neutral sugars, including

arabinose, galactose, and lesser amounts of other sugars. Pectins have a linear anionic backbone with regions having no side chains known as "smooth regions," and regions with non-ionic side chains known as "hairy regions." The structural classification of pectin includes: homogalacturonan (HG), rhamnogalacturonan I (RG-I), and substituted galacturonans such as rhamnogalacturonan II (RG-II).[1-3]

In the human diet, pectin is one of the most important sources of dietary fiber. Like other types of dietary fiber, pectin is practically not depolymerized by endogenous enzymes of the gastrointestinal tract when passing through the stomach and small intestine. However, partial degradation of pectin is possible under the physicochemical conditions of the stomach and small intestine. More or less completely pectin is fermented in the human large intestine using pectinolytic enzymes produced by symbiotic microflora.[2]

Pectins, like other non-starch polysaccharides (NSPs), are used in the treatment of inflammatory bowel disease (IBD). NSPs exhibit the potential to be promising agents for adjuvant therapy and for the prevention of IBD.[4]

The use of an artificial gastroenteric environment as a model digestive system will make it possible to study the processes of biotransformation of plant food, and in particular pectins, in a healthy and/or inflamed gastrointestinal tract. The properties of pectic-gel particles largely depend on the chemical composition and macromolecular structure of pectin polysaccharides. Swelling and degradation of pectic-gel particles in the gastrointestinal tract depend on the structural and mechanical characteristics of pectin, the concentration of pectin and the type of metal ion as a cross-linking agent in the composition of pectic-gel particles, concentration of pectinases in the large intestine, pH, and temperature.[2, 5]

Composite gels based on natural polymers allow the development of new biomaterials with new physicochemical properties that will improve their functionality. Different physicochemical characteristics of composite gel particles based on different pectins can influence their stability and swelling properties in the simulated gastric environment, the environment of the small and large intestines. Composite gel particles based on the different pectins exhibit potential applications as carrier materials in controlled release systems and particularly serve as promising systems for colon-targeted drug delivery.[5]

Previously, the properties of composite gel particles based on pectin and chitosan,[6] pectin and alginate,[7] pectin and k-carrageenan[8] were obtained and studied. There is information on the preparation and properties of composite gel particles formed from two different pectins.[5] However, there is practically no data on the preparation and properties of composite gel particles formed from pectin and polysaccharide agar.

The purpose of the present work was to obtain and study the properties of composite calcium-agar-pectic gel particles (CaAPGPs) obtained from aqueous solutions of agar (AA) and apple pectin (AP), from aqueous solutions of agar (AA) and pectin heracleuman (HS) in the presence of Ca^{2+} ions (0.34 M). The swelling of the obtained composite CaAPGPs in an artificial gastroenteric environment was also investigated.

Materials and Methods

We used commercial AP AU701 (AP, Herbstreith & Fox KG, Germany), pectin heracleuman (HS) isolated from the aerial part of the Sosnovskyi hogweed *Heracleum sosnowskyi* Manden,[9] and food agar (AA).

Spherical composite CaAPGPs were obtained from low-methyl esterified AP with a molecular weight of 406 kDa, pectin HS with a molecular weight >300 kDa, and food agar (AA) in the presence of calcium ions (0.34 M) as a cross-linking agent by the method of ionotropic gelation.[10]

Composite CaAPGPs based on AP (1% AP+2% AA, 2% AP+2% AA, 2% AP+1% AA) were obtained by slowly stirring a mixture of the appropriate amount of AP (10 mg or 20 mg) and the corresponding amount of AA (10 mg or 20 mg) in distilled water (1 ml) with a magnetic stirrer MM-5 (Russia) at heating (45 °C) for 4 hours until complete dissolution. Composite CaAPGPs based on HS were prepared in a similar way.

Gel particles of spherical form were prepared by drop-by-drop injection of corresponding solutions of mixtures of pectins with agar from a syringe through a needle with a hole diameter of 0.6 mm at a distance of 4-5 cm in the slowly stirrred calcium chloride solution (0.34 M) and further stirring for 30 min at room temperature. The resulting gel particles were then washed three times in distilled water with stirring for 5 minutes and dried for 10-14 h at 37°C.

The diameter and density of CaAPGPs were determined using an optical microscope (Altami, Russia) with a camera and an image analysis program (ImageJ 1.46r program, National Institutes of Health, USA). For calibration, a linear scale was used; one pixel corresponded to 0.024 mm.

To study the swelling of dry composite CaAPGPs under conditions simulating a gastroenteric medium, artificial gastric medium (SGF), small intestine medium (SIF), and large intestine medium (SCF) were prepared as previously described.[2,6]

To determine swelling, the corresponding dry composite CaAPGPs (1–2 mg) were placed in Petri dishes (diameter 3.5 cm) and subsequently incubated in 3 ml of SGF (2 h), SIF (4 h), and SCF solutions with shaking in a shaker (Titramax 1000, "Heidolph", Germany) at 100 rpm and at 37°C. At regular intervals, the diameter and density of the gel particles were determined as described above. The experiments were performed in triplicate. The degree of gel swelling (SD,%) was determined by the formula: $SD = (D_t\text{-}D_0)/D_0 \times 100\%$, where D_1 – diameter of the particles (mm) after a certain time of incubation in the medium, D_0 – initial particle diameter of the particles (mm).[11]

The statistical analysis was performed using the statistical software BioStat (version 4.03) and Microsoft Office Excel 2007.

Results and Discussion

The morphological (size, shape) and structural-mechanical (density) characteristics of the obtained wet and dry composite CaAPGPs were studied.

Table 1 shows the morphological and structural-mechanical characteristics of wet composite CaAPGPs. Wet spherical composite CaAPGPs formed on the basis of AP (Ca-AA-AP) have a diameter in the range from 3.80±0.25 mm to 4.28±0.25 mm, which is larger than the diameter of wet CaAPGPs formed on the basis of pectin heracleuman (Ca-AA-HS), with a diameter from 3.27±0.15 mm to 3.50±0.15 mm.

The density of wet composite CaAPGPs formed on the basis of AP (Ca-AA-AP) varies from 0.35±0.05 mg/mm^3 to 0.59±0.10 mg/mm^3, which is lower than the density of wet agar-pectin gel particles formed on based on pectin heracleuman (Ca – AA – HS), which ranges from 0.61±0.05 mg/mm3 to 1.02±0.14 mg/mm^3.

Table 1.

Morphological and structural-mechanical characteristics of wet composite CaAPGPs

Gel particles	Diameter, mm	Density, mg/mm^3
1% AP + 2% AA	4.28 ± 0.25	0.35 ± 0.05
2% AP + 2% AA	3.80 ± 0.25	0.59 ± 0.10
2% AP + 1% AA	4.03 ± 0.20	0.48 ± 0.09
1% HS + 2% AA	3.47 ± 0.08	0.61 ± 0.05
2% HS + 2% AA	3.27 ± 0.15	1.02 ± 0.14
2% HS + 1% AA	3.50 ± 0.15	0.74 ± 0.09

Table 2 shows the morphological and structural-mechanical (density) characteristics of dry CaAPGPs. It was found that dry CaAPGPs based on AP (Ca-AA-AP) have a diameter of 1.16±0.14-1.23±0.05 mm, which was greater than the diameter of dry CaAPGPs based on HS (Ca-AA-HS) (0.95±0.12-1.16±0.05 mm). The density of dry CaAPGPs based on AP (Ca-AA-AP) with an increase in the concentration of AP in their composition from 1% to 2% increased by 1.7 times – from 0.37±0.07 mg/mm^3 to 0.63±0.05 mg/mm^3. Dry composite CaAPGPs based on HS (Ca-AA-HS) were denser. With an increase in the HS concentration in their composition from 1% to 2%, the degree of particle density increases by 2.2 – from 0.45±0.03 mg/mm^3 to 0.97±0.19 mg/mm^3.

Table 2.

Morphological and structural-mechanical characteristics of dry composite CaAPGPs

Gel particles	Diameter, mm	Density, mg/mm^3
1% AP + 2% AA	1.23 ± 0.05	0.37 ± 0.07
2% AP + 2% AA	1.16 ± 0.14	0.63 ± 0.05
2% AP + 1% AA	1.19 ± 0.05	0.46 ± 0.08
1% HS + 2% AA	1.16 ± 0.05	0.45 ± 0.03
2% HS + 2% AA	0.95 ± 0.12	0.97 ± 0.19
2% HS + 1% AA	1.14 ± 0.07	0.69 ± 0.12

The swelling and degradation of the obtained dry composite CaAPGPs in an artificial gastroenteric environment were studied.

It was found that CaAPGPs formed from 1% AP and 2% AA degraded almost immediately in SIF. Whereas, CaAPGPs formed from 2% AP and 1% or 2% AA completely degraded in SCF after 1 hour of incubation in it (Fig. 1).

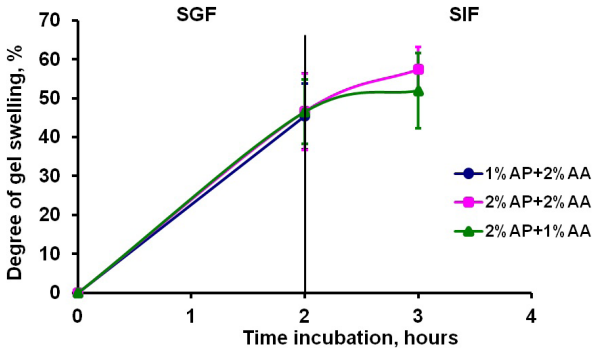

Fig. 1. *Swelling and degradation of composite CaAPGPs formed on the basis of AP in an artificial GIT.*

CaAPGPs formed from 1% HS and 2% AA, and particles obtained from 2% HS and 1% AA, remained stable in SIF, and then completely degraded immediately upon entering in SCF. CaAPGPs, consisting of 2% HS and 2% AA, dissolve in SCF after 1 hour of incubation in it. (Fig. 2).

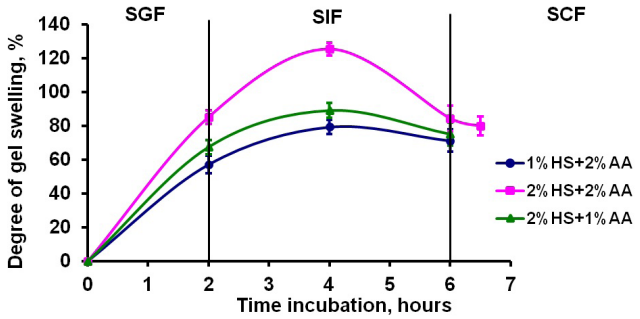

Fig. 2. *Swelling and degradation of composite CaAPGPs formed on the basis of pectin HS in an artificial GIT.*

Thus, our results indicate the promise of studying the obtained CaAPGPs in conditions of the artificial GIT as adsorbents of heavy metals that can adsorb heavy metals and remove them from the body.

Competing Interest

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

Biotechnology

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Interaction of Long-Chain Alcohol with Dry Yeast, Cholesterol, and Sea Firefly Luciferase

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Abstract

Background: A hand sanitizer containing alcohol, usually ethanol or isopropanol, is typically used for disinfection, but given that cholesterol is one of the main components of virus envelopes, long-chain alcohol may be more effective. To better understand the potential disinfection activity of long-chain alcohols, we studied their interactions with dry yeast, cholesterol, and sea firefly luciferase.

Methods and Results: We measured, at 30 ºC and 39 ºC, the minimum inhibition concentration (MIC) of dry yeast fermentation and the stability of cholesterol and sea firefly luciferase with alcohols, diols, cetyltrimethylammonium chloride, and stearyltrimethylammonium chloride. The MIC decreased with the chain length at C≤12 for dry yeast and cholesterol with alcohol at 30 ºC. At C_{13} and higher, the cut-off region was observed. At 39 ºC, the cut-off region shifted to C_{15} and higher. The reduction of MIC was measured with the diol or sea firefly luciferase at C≤14.

Conclusion: The presence of the cut-off region is suggested to be related to whether the alcohol is in the liquid state. For the liquid alcohol, the longer the chain length, the lower the MIC. This suggests a potential disinfection activity of long-chain alcohol. **(International Journal of Biomedicine. 2021;11(4):460-466.)**

Key Words: cut-off region • liquid alcohol • minimum inhibition concentration

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Introduction

During the COVID-19 pandemic, people are recommended to wash their hands with soap or an alcohol-based hand sanitizer.[1] The viral envelope of the COVID-19 virus is lipophilic/hydrophobic due to its composition of lipids and cholesterol. However, a question arises as to why we use ethanol or isopropanol but not long-chain alcohols.

The effects of ethanol and isopropanol on disinfection have been widely studied, reviewed, and summarized.[2-8] However, only a few studies have investigated the effect of long-chain alcohols. Kubo et al.[9] investigated the antimicrobial activity of long-chain alcohols and reported only the trend and qualitative results from the standpoint of the alcohol structure. Using alcohols, Mukherjee et al.[10] investigated the antibacterial properties of long-chain fatty alcohols against mycobacteria. Mukherjee et al.[10] found that the best activity was shown by alcohol with a C_{10} chain length, but this was not consistent with the report by Kubo et al..[9] Fletcher et al.[11] studied the inhibitory activity of long-chain alcohols on the growth of *Mycoplasma gallisepticum* and *Mycoplasma pneumoniae* and found that the primary saturated alcohols were more effective than both the unsaturated and secondary alcohols. However, the reason for this difference was not clear. Ingram and Vreeland[12] compared the effects of ethanol with that of hexanol only on the inhibition of the growth of *Escherichia coli*. Gill and Ratledge,[13] Teh,[14] and Kato and Shibasaki[15] studied the inhibition of growth of various microorganisms by *n*-alcohols and found that the inhibition potency increased with the chain length. In sum, few studies have been conducted on

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the activity of long-chain alcohols from the perspective of disinfection, and the effect is not fully understood.

Many previous studies delved into the impact of long-chain alcohol on the anesthetic effects (a local or general loss of sensation, including pain, achieved by action on the brain or peripheral nervous system to suppress responses to sensory stimulation)[16-18] and the growth of microorganisms[9,19] and reported the presence of a cut-off region (i.e., where alcohols or other compounds suddenly lose their activity and function). However, the reason for this cut-off phenomenon in anesthetic effects and microorganism growth is unknown although some explanations have been proposed.[18,20-22] Furthermore, to the best of our knowledge, no previous study has investigated whether the cut-off is observed in the case of disinfection with long-chain alcohol.

According to Veenstra et al.,[23] the long-chain alcohols showed low acute and repeat-dose toxicity with high-dose effects related to minimal liver toxicity. There was no evidence of toxicity to the reproductive system or the developing organism with these chemicals. The former was performed on male and female rats during premating, mating, and gestation. For up to a year, the latter was studied with rats and dogs. Alcohols with C_6–C_{11} chain lengths are generally considered irritants, whereas those with intermediate chain lengths (C_{12}–C_{16}) are considered mild irritants. These chemicals are broadly and safely used across the consumer products industry with the highest per person consumer exposures resulting from use in personal care products.

Therefore, to understand the activity of long-chain alcohol for disinfection, the dependence of the minimum inhibitory concentration (MIC) on the chain length of alcohol should be studied. Further, we should examine whether the cut-off region is present, and if it is, explain the reason for its presence.

In this work, we studied the MIC of alcohol for dry yeast fermentation and cholesterol stability, examined whether a cut-off region existed, and if yes, investigated the reason for the existence of the cut-off region. This study provides no direct evidence of the efficacy of long-chain alcohols as disinfectants. We believe, however, that it can present new and basic data for discussing a potential activity and possibility of these alcohols for disinfection.

In many studies on disinfection, real microorganisms, bacteria, and viruses were used. Some studies[24,25] have shown that dry yeast can be used to study disinfection, antimicrobial action, and infectious diseases. Moriyama[26] developed biological control materials and used mycovirus and dry yeast to prevent virus-induced rice diseases. The inhibitory effect of alcohols on mycoplasmas was investigated using cholesterol.[11] Furthermore, cholesterol is one of the main components of the envelope of viruses such as SARS-CoV-2. Therefore, we used dry yeast and cholesterol instead of real viruses. In this study, we also used dried sea firefly luciferase.

Materials and Methods

Chemicals

In this study, straight-chain alcohols and diols, cetyltrimethylammonium chloride, and stearyltrimethylammonium chloride were used. All chemicals listed in Table 1 (except 1,4-butanediol, 1,6-hexanediol, and 1,16-hexadecanediol), *N*, *N*-dimethylformamide (DFM), pH buffer solution (pH 6), cholesterol, and cholesterol fluorescence kit were purchased from Fisher Scientific Canada. MilliporeSigma supplied three diols for this experiment. Dried sea firefly was purchased in Monotaro, Japan. The Metro Supermarket supplied dry yeast (Instaferm by Lallemand Inc.) and sugar (granulated sugar by Lantic Sugar Ltd.) (Oakville, Ontario, Canada). Milli-Q Direct 8 equipment was used to create deionized water.

Solutions

The concentrations of ethanol, 1-butanol, 1,2-ethanediol, and 1,4-butanediol were adjusted by diluting the original solutions with deionized water. The long-chain alcohols, diols ($C_6 \leq$ chain length $\leq C_{16}$), cetyltrimethylammonium chloride, and stearyltrimethylammonium chloride were first dissolved in DFM and then their concentrations were adjusted. Since the fermentation of dry yeast is the most active at a pH of ~6, the pH buffer solution (pH=6) was added to all solutions. The final concentration of DFM was 1%. It was previously confirmed that the pH buffer solution and 1% DFM do not affect the fermentation of dry yeast or the stability of cholesterol and sea firefly luciferase.

Based on the preliminary tests, the concentrations of chemicals were decided as listed in Table 1 at first. Using the chemicals listed in Table 1, the lower limit of MIC was determined, below which dry yeast fermentation and the stability of cholesterol and sea firefly luciferase were observed, and the upper limit of MIC was determined, above which these characteristics were not observed. The chemicals were then prepared with concentrations that were slightly finely adjusted between the upper and lower limits of the MIC, and the experiment was repeated. The upper limit of the MIC decided with these slightly, finely adjusted concentrations was adopted as the final MIC value.

Minimum inhibitory concentration

Ishijima and Abe[25] demonstrated the use of the culture media, measurement of the growth inhibition circle, and assessment of the MIC for antibacterial, disinfection, and sterilization purposes. We first measured the MIC for dry yeast fermentation with ethanol using the Ishijima and Abe's method[25] and experimental procedure described below, and the MICs measured using both methods were identical. Therefore, in this study, we used the experimental procedure described below. To confirm the reproducibility of MIC, all measurements were performed 10 times in 10 independent and separate experiments.

Dry yeast fermentation

Five grams sugar and 1g dry yeast were added to a 50 mL centrifuge tube. After 10 mL alcohol solution was added to the tube, the tube was put in the incubator for 30 min. The temperature was controlled at 30 °C or 39 °C.

After 30 min of fermentation, the MIC was determined to be the lowest concentration that completely inhibited the fermentation of dry yeast.

The same procedure was applied with diols, cetyltrimethylammoniumchloride,orstearyltrimethylammonium chloride at 30 °C. The solubilities of cetyltrimethylammonium

chloride and stearyltrimethylammonium chloride are higher than those of alcohols with C_{16} and C_{18}.

Table 1.
Concentrations of chemicals

Chemicals	Target	Concentrations (M)
ethanol	dry yeast cholesterol	1×10^{-2}, 1×10^{-1}, 1, 1.5, 2, 7
1-butanol		1×10^{-2}, 5×10^{-2}, 1×10^{-1}, 5×10^{-1}, 8×10^{-1}, 1, 2
1-hexanol		1×10^{-3}, 1×10^{-2}, 3×10^{-2}, 5×10^{-2}, 1×10^{-1}
1-octanol		1×10^{-4}, 1×10^{-3}, 3×10^{-3}, 5×10^{-3}, 1×10^{-2}, 1×10^{-1}
1-decanol		1×10^{-5}, 5×10^{-5}, 8×10^{-5}, 1×10^{-4}, 5×10^{-4}, 1×10^{-3}
1-dodecanol		1×10^{-6}, 5×10^{-6}, 1×10^{-5}, 2×10^{-5}, 5×10^{-5}, 1×10^{-4}
1-tridecanol		1×10^{-7}, 1×10^{-6}, 3×10^{-6}, 5×10^{-6}, 1×10^{-5}
1-tetradecanol		1×10^{-7}, 5×10^{-7}, 1×10^{-6}, 2×10^{-6}, 3×10^{-6}, 5×10^{-6}, 1×10^{-5}
1-pentadecanol		1×10^{-7}, 1×10^{-6}, 1×10^{-5}, 1×10^{-4}
1-hexadecanol		1×10^{-7}, 1×10^{-6}, 1×10^{-5}, 1×10^{-4}
1,2-ethanediol		1×10^{-2}, 1×10^{-1}, 1, 1.5, 2, 7
1,4-butanediol		1×10^{-2}, 5×10^{-2}, 1×10^{-1}, 5×10^{-1}, 8×10^{-1}, 1, 2
1,6-hexanediol		1×10^{-3}, 1×10^{-2}, 3×10^{-2}, 5×10^{-2}, 1×10^{-1}
1,8-octaediol		1×10^{-4}, 1×10^{-3}, 3×10^{-3}, 5×10^{-3}, 1×10^{-2}, 1×10^{-1}
1,10-decanediol		1×10^{-5}, 5×10^{-5}, 8×10^{-5}, 1×10^{-4}, 5×10^{-4}, 1×10^{-3}
1,12-dodecanediol		1×10^{-6}, 5×10^{-6}, 1×10^{-5}, 2×10^{-5}, 5×10^{-5}, 1×10^{-4}
1,13-tridecanediol		1×10^{-7}, 1×10^{-6}, 3×10^{-6}, 5×10^{-6}, 1×10^{-5}
1,14-tetradecanediol		1×10^{-7}, 5×10^{-7}, 1×10^{-6}, 2×10^{-6}, 3×10^{-6}, 5×10^{-6}, 1×10^{-5}
1,15-pentadecanediol		1×10^{-7}, 1×10^{-6}, 1×10^{-5}, 1×10^{-4}
1,16-hexadecanediol		1×10^{-7}, 1×10^{-6}, 1×10^{-5}, 1×10^{-4}
cetyltrimethyl-ammonium chloride		1×10^{-8}, 5×10^{-8}, 1×10^{-7}, 1×10^{-6}
stearyltrimethyl ammonium chloride		1×10^{-9}, 1×10^{-8}, 5×10^{-8}, 1×10^{-7}
ethanol	dry sea firefly luciferase	1×10^{-2}, 1×10^{-1}, 5×10^{-1}, 8×10^{-1}, 1, 2
1-butanol		1×10^{-3}, 5×10^{-3}, 1×10-2, 5×10^{-2}, 1×10^{-1}
1-hexanol		1×10^{-4}, 5×10^{-4}, 1×10^{-3}, 5×10^{-3}, 1×10^{-2}
1-octanol		1×10^{-5}, 1×10^{-4}, 5×10^{-4}, 1×10^{-3}, 5×10^{-3}
1-decanol		1×10^{-6}, 5×10^{-6}, 1×10^{-5}, 5×10^{-5}, 1×10^{-4}
1-dodecanol		1×10^{-7}, 1×10^{-6}, 1×10^{-5}, 5×10^{-5}, 1×10^{-4}
1-tridecanol		1×10^{-7}, 1×10^{-6}, 2×10^{-6}, 5×10^{-6}, 1×10^{-5}
1-tetradecanol		5×10^{-8}, 1×10^{-7}, 5×10^{-7}, 1×10^{-6}, 5×10^{-6}
1-pentadecanol		1×10^{-8}, 1×10^{-7}, 1×10^{-6}, 1×10^{-5}
1-hexadecanol		1×10^{-8}, 1×10^{-7}, 1×10^{-6}, 1×10^{-5}

Stability of cholesterol

One gram of cholesterol was dissolved in 1% DFM. After diluting, 1 mM cholesterol solution was prepared. Then, 100μL cholesterol solution was mixed with 10 mL alcohol or diol in the 15mL centrifuge tube. After 30 min at 30°C or 39°C (incubator), 100 μL of the solution was taken from the tube and fed to the cholesterol fluorescence kit. At McMaster University (Ontario, Canada), the MIC was calculated using a fluorescence spectrometer (excitation: 560 nm, fluorescence: 590 nm). When cholesterol reacts with alcohol, it undergoes esterification and no fluorescence occurs. The MIC represents the concentrations of alcohol and other chemicals below which fluorescence is not detected.

Dried sea firefly luciferase

The bioluminescence of sea firefly (460 nm) is induced by a reaction in which luciferin is oxidized by the O_2 dissolved in the water under the catalytic action of the enzyme luciferase. The bioluminescence emission experiment was conducted according to the experimental procedure by Toya and Ito.[27]

First, 0.1 g of dried sea firefly was pulverized thoroughly in a mortar. Immediately after mixing the pulverized, dried sea firefly with a 1mL alcohol at 30°C, the bioluminescence was measured with the light detector.[28] The light detector converts light intensity to sound intensity. The light detector has a high sensitivity to blue light (460 nm) and was calibrated with a fluorescence spectrometer. It detected bioluminescence from 0.1g of ground dried sea firefly mixed with 1L of distilled water. The MIC was set to be the alcohol concentration at which the sound intensity became the background noise level.

Considering the good reproducibility of MIC measured in 10 experiments, the variation in the concentrations of luciferin and enzyme luciferase between the experiments was considered negligible.

Results

No difference was observed in the MIC values measured in the 10 experiments in this study. This suggests that the measurement data were highly reproducible. Therefore, error bars are not plotted in Figures 1–4 shown below.

Minimum inhibition concentration (MIC) by alcohol for dry yeast fermentation and cholesterol stability at 30°C and 39°C

The extent to which the MIC for the dry yeast fermentation and the stability of cholesterol depends on the chain length of alcohol at 30°C and 39°C are shown in Figures 1 and 2, respectively.

The MIC for dry yeast fermentation decreased with chain length up to C_{12} at 30°C. This is consistent with the lipophilicity/hydrophobicity. Furthermore, the cut-off region was observed at C_{13} and C_{14}. The anesthetic cut-off is a well-known phenomenon at C≥13.[16] This mechanism has yet to be solved. Figure 1 depicts a similar cut-off phenomenon in the inhibition of dry yeast fermentation.

Moreover, the MIC was measured at C_{13} and C_{14} at 39 °C, but not at C_{15} and C_{16}. That is, the cut-off region was shifted to a longer chain length range of alcohol at 39°C.

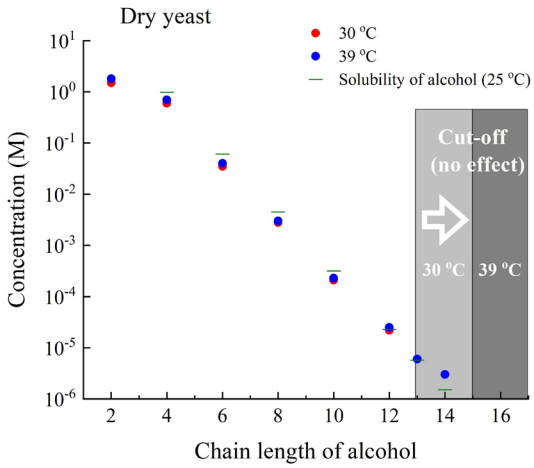

Fig. 1. *Dependence of the MIC for dry yeast fermentation on the chain length of alcohol and solubility of alcohol in water*[32]

The MIC for the stability of cholesterol was slightly smaller than that for dry yeast fermentation, but the trend of the former was the same as that of the latter. That is, the MIC decreased with the chain length of alcohol. There was a cut-off region at a long-chain range, and the threshold of the cut-off region shifted to a longer chain length at 39 °C.

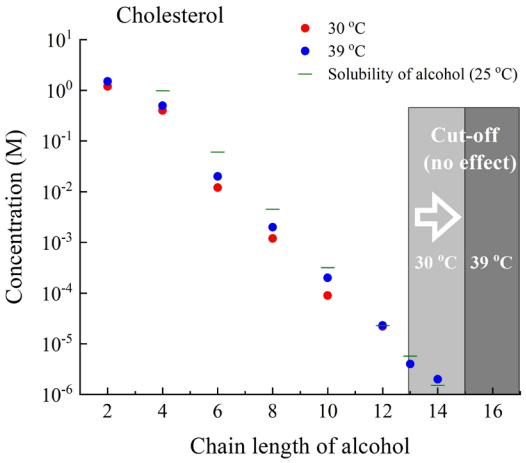

Fig. 2. *Dependence of the MIC for cholesterol stability on the chain length of alcohol and solubility of alcohol in water.*[32]

In the experiment with cholesterol, alcohol directly reacted with cholesterol in the solution. Further, to inhibit the dry yeast fermentation, alcohol had to interact with mannoprotein, β-glucan, chitin, and envelope.[29] This may be why the MIC for cholesterol was slightly smaller than that for dry yeast.

Minimum inhibition concentration (MIC) by diols and cetyltrimethylammonium chloride and stearyltrimethylammonium chloride for dry yeast fermentation and cholesterol stability at 30 °C

Figure 3 depicts the MIC by diol at 30 °C. The MIC decreased as the diol chain length increased. For both dry yeast fermentation and cholesterol stability, the MIC of diol was nearly equal to that of alcohol. The cut-off region was observed at C_{15} and higher at 30 °C.

Furthermore, it was also found that cetyltrimethylammonium chloride (C_{16}) and stearyltrimethylammonium chloride (C_{18}) inhibited the dry yeast fermentation and cholesterol stability at 30 °C.

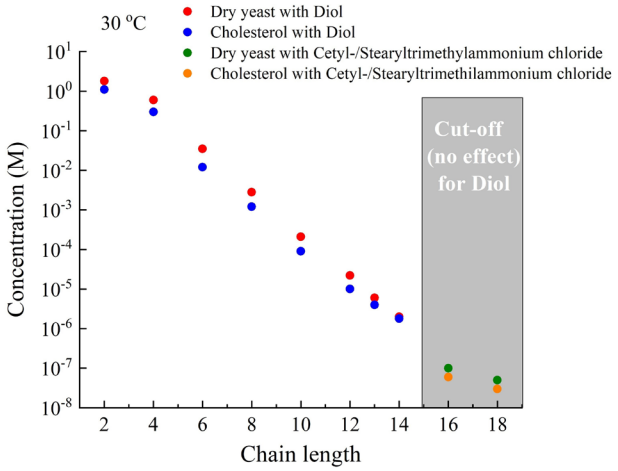

Fig. 3. *Dependence of the MIC for dry yeast fermentation and cholesterol stability on the chain length of the diol. The MIC values for cetyltrimethylammonium chloride and stearyltrimethylammonium chloride are included.*

Minimum inhibition concentration (MIC) by alcohol for dried sea firefly at 30 °C

The dependence of MIC for the bioluminescence of dried sea fireflies on the chain length of alcohol at 30 °C is shown in Figure 4. The MIC of alcohol decreased with chain length up to C_{14}, with the cut-off region observed at C_{15} and C_{16}. The MIC for bioluminescence was lower than the MIC for dry yeast fermentation and alcohol-induced cholesterol stability.

As Oba et al.[30] and Suzuki et al.[31] used firefly luciferase, the sea firefly luciferase is considered to have high sensitivity for alcohol. Hence, the MIC value for bioluminescence was smaller than that for dry yeast fermentation and cholesterol stability.

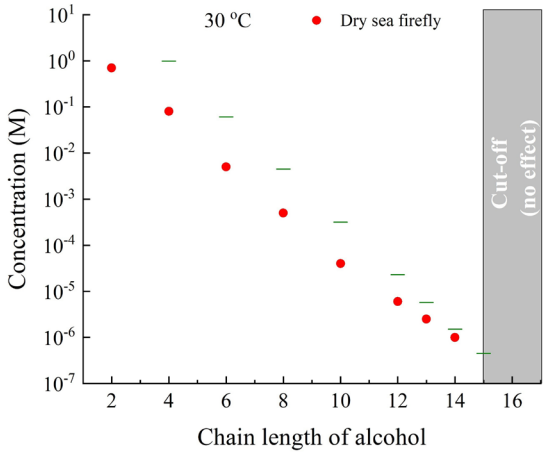

Fig. 4. *Dependence of the MIC for the bioluminescence of dried sea firefly on the chain length of alcohol at 30 °C and solubility of alcohol in water.*[32]

Discussion

Possible reasons for the origin of the cut-off region

Our study found (a) that the MIC decreased with the chain length of alcohols and diols in all our experiments and (b) that the cut-off region was clearly observed for longer chain alcohols and diols under our experimental conditions.

Figures 1 and 2 also show the plots of the solubility of alcohol in water at 25 °C.[32] The solubility of alcohol at 30 °C and 39 °C is unlikely to be lower than the solubility at 25 °C. The fermentation activity of dry yeast is hindered at high temperatures. In daily life, we do not use disinfectants at high temperatures such as 50 °C. The melting points of 1-tridecanol and 1-tetradecanol are 32.5 °C and 37.7 °C, respectively, whereas those of 1-pentadecanol and 1-hexadecanole are 45.5 °C and 49.3 °C, respectively. The solubility of solid alcohol increases upon melting. The melting point of alcohols with C≤12 is lower than 25 °C. Therefore, a temperature of 39 °C was selected in this study.

From Figures 1 and 2, the MIC values at C≤10 at 30°C and 39 °C were found to be smaller than the solubility of alcohol at 25°C; the MIC values at C_{12} at 30 °C and 39 °C were almost equal to the solubility at 25 °C. According to Bell,[32] the logarithm of the solubility of *n*-alcohol in water at 25 °C changes almost linearly with the chain length of alcohol up to C_{16}. When the temperature is raised to 30 °C and 39 °C, this linearity is unlikely to change. Assuming that both the logarithm of solubility at 30°C and the logarithm of MIC at 30 °C change linearly with the length of the alcohol chain, two linear plots intersect between C_{12} and C_{13}. Because solubility increases at 39 °C, the linear plot of solubility at 39 °C between C_{14} and C_{15} may intersect the MIC at 39 °C.

In Figure 3, we used chemicals with higher solubility than alcohol. The MIC values for diol at 30 °C were almost identical to those for alcohol at 30 °C. We could not find the quantitative value of solubility of diol up to C_{16} in water at 25 °C or 30 °C in any research papers. A straight-chain diol, however, has a higher solubility than straight-chain alcohol. Therefore, if the logarithm of diol solubility changes linearly with chain length, we can assume that the diol solubility linear plot intersects with the MIC for dry yeast fermentation and the stability of cholesterol between C_{14} and C_{15}. For cetyltrimethylammonium chloride and stearyltrimethylammonium chloride, which have much higher water solubility than alcohols and diols, the MIC was discovered even at C_{16} and C_{18}.

We also used the sea firefly luciferase, which is highly sensitive to alcohol (Figure 4). Then, we found that the MIC was less than that for dry yeast or cholesterol. The MIC was smaller than the solubility of alcohol up to C_{14}. We considered that the linear plot of solubility of alcohol at 30 °C intersects the linear plot of the MIC between C_{14} and C_{15}.

To summarize, for alcohol to inhibit the fermentation of dry yeast and the stability of cholesterol and sea firefly luciferase, it must be in a liquid state. In a liquid state, the longer the chain, the lower the MIC. When the solubility is smaller than the MIC, the cut-off phenomenon occurs.

Potential activity of long-chain alcohols

In this study, we investigated the interaction of alcohol with the dry yeast, cholesterol, and sea firefly luciferase, not with the real viruses. However, dry yeast was used to study disinfection, antimicrobial action, and infectious diseases. Further, cholesterol is one of the main components of the viral envelope. As a result, the findings of this study suggest that long-chain alcohols may be suitable for disinfection and that the longer the chain of the alcohol, the lower the concentration required for disinfection. Of course, ethanol and isopropanol are useful for disinfection. However, if we need to disinfect wide surfaces such as walls, floors, and ceilings of rooms, long-chain alcohols are convenient, effective, and useful because their costs are lower, and roughening of hands by alcohol can be prevented because of the very low concentration required.

The mechanisms of disinfection with alcohol against viruses and bacterias are not completely elucidated. However, possible mechanisms are as follows:[33-35] (a) alcohol passes through the viral envelope/bacteria membrane and penetrates the cell, increasing the pressure inside the cell. As a result, the cells are destroyed, and the virus is eliminated. (b) The cholesterol reacts with alcohol, converting to ester. Therefore, the envelope is punctured, allowing the contents of the cell to leak out, killing the virus. Alcohol must be in liquid form to work in this manner. Solid alcohol cannot enter the cell or interact with cholesterol. In terms of phenomenology, this is consistent with the possible reasons for the origin of cut-off regions discussed above.

The real viruses were not used to prove the activity of long-chain alcohols and the presence of a cut-off region. However, we consider that the possibility of the potential activity of long-chain alcohols as disinfectants were qualitatively demonstrated. Therefore, further study with real viruses is desired.

Conclusion

The MIC for the dry yeast fermentation and for the stability of cholesterol and sea firefly luciferase decreased with the chain length of the alcohol used. However, the cut-off phenomenon was observed at longer chain lengths. One possible explanation for the cut-off phenomenon is that alcohol is either liquid or solid. Only liquid alcohol can inhibit dry yeast fermentation and maintain the stability of cholesterol and sea firefly luciferase. The results obtained in this study suggest that long-chain alcohols may have a potential activity as a disinfectant.

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Disclaimers

The views expressed in the submitted article are our own and not an official position of the institution.

Conflict of Interests

The authors declare that they have no conflicts of interest.

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POINT OF VIEW

Family Planning Programs – An Opportunity to Integrate Preconception Health Services into Primary Health Care

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Abstract

A life-course approach to enhancing maternal and child health by improving parental health prior to conception is gaining international interest. Healthcare providers and researchers are seeking effective ways to increase women's and men's access to preconception care, especially through primary care. At the same time, preconception health includes conversations about pregnancy planning (or avoidance), which is a part of family planning (FP) services. Unfortunately, "family planning" has the same meaning as "contraception" for many people at the moment, which is too simplistic because there are multiple aspects of preconception health and well-being. Expanding the understanding of preconception health as inclusive of FP and primary care has the potential to increase access to this important care while respecting sexual and reproductive rights, according to a couple's reproductive plans.

Conclusion: Respecting the sexual and reproductive rights of the population requires that FP programs be focused not only on contraception but, equally, on preconception care.(**International Journal of Biomedicine. 2021;11(4):467-472.**)

Key Words: preconception health • preconception care • family planning • contraception • primary care • reproductive health

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Abbreviations

FP, family planning; **GPs**, general practitioners; **PCHC**, preconception health and care; **PC**, preconception care.

Improving the health of the mother and child through programs focused on reducing risks prior to pregnancy in the context of a life-course approach is gaining international interest. Such an evolution in maternal and child health is logical and important. While a focus on treating diseases and/or health complications has long been considered a priority in health care, the promotion of simple and cost-effective preventive measures with a positive impact on health are also of importance in medical practice. As such, the recommendation that couples planning a pregnancy should receive care may seem obvious to many medical workers and a considerable part of the population. Thus, discussions about the role of PCHC may seem unnecessary, and the general population may find them simple and unnecessary to address

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in the face of other more important and complicated issues. Preparing couples for a future pregnancy is a since-the-world-began topic that is still relevant. Globally, only about half of pregnancies are planned, and the number of couples who have received preconception preparation is much smaller.

A major impediment to progress is the lack of resources required to implement PCHC programs. At the European Congress on Preconception Health (2019), the participants, including those from economically developed countries, appreciated this issue as a very current one. In this context, one strategy recommended by many researchers is the integration of preconception care (PC) services into primary health care. It seems, however, that this process also faces some obstacles: frequently, the motivation of the family doctors/GPs is not enough to achieve the integration in daily practice.

At the same time, preconception preparation requires an interest in and participation of a couple or woman in thinking about pregnancy. Some experts have suggested that interventions to improve reproductive and preconception health could be more relevant and efficient by integrating PCHC services into FP programs, embedded into primary health care. The biggest challenge is that the concept of family planning, with some exceptions, is considered to be synonymous with contraception, i.e., FP = contraception. We propose a different approach: that "FP = contraception + preconception care."[1,2] There was a surprising amount of resistance by some well-known people in the field of medicine, as well as among researchers and some international organizations, who promote the first approach. It is difficult to explain why preconception healthcare services have been ignored by the same international organizations responsible for developing global strategies and policies to advance family health, which determine health policies and programs at the regional and national levels.

Contraception and PC are two components of FP that interact with each other. Before making the decision to discontinue contraception, the woman or couple should evaluate their preconception risks and have support to reduce them. Access to all contraceptive methods is equally important for couples who do not wish to conceive and those who need to wait until they have been able to address risk factors or built protective factors.

Preconception care

PC is defined by the WHO as providing biomedical, behavioral, and social health interventions before pregnancy to improve health and modify behaviors and environmental influences. PC can contribute to reducing maternal and childhood mortality and morbidity, and to improving maternal and child health in both high- and low-income countries.[3] Clinicians and health workers should provide the information that a woman and her partner need, in order to make informed choices about planning a pregnancy, to ensure the best possible outcome for the couple and the baby.[4]

PC is an important intervention to reduce risks in early pregnancy and lead to healthy outcomes for women and babies. General PC is directed at all prospective parents and takes place in primary care (GPs or midwife level). Most preconception health messages are important for women and couples regardless of their desire to have a child and include key preventive health measures that should be part of quality, routine care. PC includes risk assessment, health promotion, counseling and, if indicated, referral to a specialist to assist with unique needs (e.g., a nutritionist, an endocrinologist). Although the primary goal is to promote better reproductive outcomes, the changes induced by PC are also usually beneficial to the woman's health. Furthermore, PC aims to improve couples' informed decision making by providing information on reproductive options.[5] One size cannot fit all, however. The content and mechanisms of delivery of preconception will need to be tailored to the realities of different countries. Even where strong public health programs are in place across the life-course, they do not guarantee that women enter pregnancy in good health.[6] The concept of preconception health care was described almost two centuries ago when it was suggested that the medical treatment of children should begin with the earliest formation of the embryo and should include the mother before marriage as well as during pregnancy. Many years later, PC has not become part of the routine care of most reproductive-aged women.[7]

The paradox of the situation is that while more researchers have shown the important role of PC for the health of generations at all stages of life, the practical implementation of the recommendations of improving the preconception health remains suboptimal, with considerable differences between countries. It seems that the role of PC of couples in the prevention of maternal-fetal complications, both at the antenatal and postnatal stage, has become, over time, a declarative statement. Even in the countries with well-developed health management systems, frequently, program managers give priority to strategies with more immediate impact compared to interventions that will deliver results in the medium or long term. This is largely due to the social policies of the governments of many countries, especially in the context of limited budgets, which give priority to projects that have short-term outcomes. Thus, it is "more convenient" to purchase some modern ultrasonography devices to detect congenital defects or to strengthen the intensive care units for infants with incubators, than invest in the health of young adults for several years before they intend to start a family. While investment in any and all interventions aimed at improving the health of the mother and child are welcome, policies and programs need to include preventive strategies that ultimately can help sustain ongoing good outcomes for families. For example, ignoring the PC stage will still maintain the high frequency of congenital malformations in fetuses and newborns, and the constant number of preterm births will require increased effort from intensive therapy units for premature children, sophisticated medical equipment, expensive drugs, etc., as well as the economic, social and psychological costs, both at the family and community level.

Providing quality PC is the responsibility of all primary care providers, not just those who provide maternity care or handle a high volume of women's preventive health visits. Innovative strategies that incorporate PC into routine primary care visits are needed.[8] Transforming the way PC is delivered is critical to success. In order to successfully deliver PC, family

physicians must understand the risk factors for- and the realities of- unintended pregnancy; recognize the value of reproductive planning in reducing these risks, and assess preconception health risks during chronic disease management visits and acute care visits that are not specifically focused on women's health or maternity issues. Preconception care is primary care and it should be a priority for primary care providers in all settings. The majority of preconception health topics are important whether a woman desires a future pregnancy or not, so providing quality PC is essentially providing quality women's health care.

At the same time, doctors across specialties should also consider the possibility of pregnancy development in reproductive age women and they should inform them about possible risks for their health, for fetal and newborn risks, generated by disease evolution and treatment.[9]

Integrating preconception planning services into primary health care is important to ensure that services reach larger numbers of people in the reproductive age population and that people receive quality counseling and services from providers who know them.[10] In 2006, the Center for Disease Control (CDC) released Recommendations to Improve Preconception Health and Health Care – United States: A Report of the CDC/ ATSDR Preconception Care Work Group and the Select Panel on Preconception Care. This report was published in an effort to improve reproductive health outcomes. However, in spite of these national and international efforts, there continue to be barriers to preconception counseling in routine primary care.

Steel et al.[11] conducted a systematic review of barriers in the process of providing preconception services and programs. The most consistently cited barrier for PC provision, as described by primary care practitioners and specialist (obstetrician-gynecologist) doctors are insufficient clinical time and a lack of reimbursement for practitioner time spent on preconception counseling. Primary care practitioners participating in a United Kingdom survey asserted that a potential barrier to the success of PC programs is the perceived low value placed on PC held by women in the community. These health professionals purported that PC programs do not reach those women who do not plan their pregnancy. To add complexity to this issue, women's lack of knowledge about PC has also been highlighted by both Dutch midwives and Israeli obstetrician-gynecologists as a potential barrier to effective care delivery as the initial clinical encounter for many women often occurs in the early antenatal period. Conversely, a concern has been expressed by primary care practitioners that providing information on the teratogenic effects of medication within preconception counseling will lead to women avoiding important pharmaceutical care. This was of particular concern if the practitioner was not confident in identifying a woman's pregnancy plans during the clinical encounter.

Primary care providers also face difficulties with prioritizing PC together with other preventive care issues. Consequently, potential interventions for improving the delivery of PC guidelines should also focus on providing tools and resources to assist providers in delivering the content and evidence base of the guidelines. Understanding the views of both women and providers as well as the theoretical basis for changing their behavior will be essential when designing effective implementation strategies for improving the delivery and uptake of PC.[12] These strategies may also need to consider the role practice nurses and other health professionals may have in facilitating better uptake of PC, especially among high-risk patients who should need services the most.

A review by Shannon et al.[13] identified primary care as the most common setting for preconception health service. However, the authors also concluded that there is no agreed consensus on the best method to deliver care within primary care. It is possible that many strategies acting synergistically are needed to improve service delivery. Integration into routine care could be one strategy, but this would not be sufficient to reach the target population for routine preventive care as seen in some other countries. In another aspect, Ukoha and Dube [14] reported that although preconception care is recognized as an important factor in improving pregnancy outcomes, most primary healthcare nurses lack the necessary resources to provide PC. Given that the majority of preconception health messages are also key preventive health messages for women, there may either be confusion in terms of what PC counseling entails or providers may be providing suboptimal guidance and support for women's health overall. For example, all people should be screened for tobacco use and given guidance if they screen positive for use. This should be done regardless of a person's gender or age, and tobacco use has negative consequences. For a couple expressing an interest in pregnancy or struggling with fertility, preconception health messages may be an extra incentive for seeking support in tobacco cessation.

Pre-pregnancy preparing as a component of FP services

The shift to integrate preconception health promotion into the continuum of women's health care requires a diverse multi-level and multi-strategic approach involving a range of sectors and health professionals to improve access to care and address the determinants of health.[15] Considering multiple problems in the provision of preconceptional or interconceptional health services, especially the lack of resources for providers, on the one hand, and the modest motivation of the population to benefit from these services, on the other, managers in the field tried to integrate PC into FP programs. There are not many published data on this subject.

Hussein[16] mentioned that since the general practitioners' primary concern is that it is difficult to convince women to have a preconception assessment, it would seem feasible to reach them through FP clinics, which have the potential to be a good setting to deliver preconception assessment of reproductive risks.

Following the Population and Development Conference in Cairo in 1994, in many countries national programs were developed and implemented to offer FP services by opening specialized clinics and providing equipment and training for service providers. FP services were included in the basic packages of primary health services. The state programs in the field of FP encouraged cross-sector collaboration across the public systems of health, education, social assistance, etc. National health systems have also ensured the interdisciplinary cooperation of the medical providers of different specialties in promoting and providing FP services.

Preconception health messages, recommendations and guidelines originated in the USA and the preconception movement has gained momentum internationally with a variety of strategies developed and tested for improving preconception health and related outcomes.[10,17] The report titled Providing Quality Family Planning Services: Recommendations of CDC and the US Office of Population Affairs (2014) provides strategies developed collaboratively by the CDC and the Office of Population Affairs of the US Department of Health and Human Services. These agencies advance recommendations for the provision of quality FP services, which include contraceptive services, pregnancy testing and counseling, helping clients achieve pregnancy, basic infertility services, preconception health services, and sexually transmitted disease services. Gavin et al.[18] conclude that providers of FP services should offer preconception health services to female and male clients in accordance with CDC recommendations to improve preconception health and health care. Including PC in FP services could ensure a higher quality of couple preparation for pregnancy, including more active involvement of men.

Preconception preparing could be used more fully in cases of infertility, which is one of the FP important areas. According to the Royal College of Obstetricians and Gynecologists (2011), the lack of preconception care for infertile couples may be a reflection of a lack of continuity in women's health reported elsewhere.[19] PC should be actively offered by all healthcare professionals. Nurses in infertility clinics and GPs in primary care are ideally placed to open up a discussion about PC with couples seeking fertility assistance, promoting the agency of these patients during investigation and treatment stages.

Another category of target beneficiaries of FP services are adolescents. It is crucial to create a responsible attitude for future decisions regarding the conception of a child, in adolescents and young adults. The concept of maintaining/ fortifying health in general, as well as risk-free behavior, should be presented to young people as a condition of paramount importance for achieving their reproductive plans.

However, there are some controversies about this approach. In 2018, the WHO published "Family Planning. A Global Handbook for Providers (3rd edition)," which is considered to be a key document that contributes to ensuring the quality and safety of FP services, encouraging all national health systems and other interested organizations to engage in this important topic.[20] This document includes a very modest section regarding PC, the other part of the document being devoted to contraception. The information provided on the subject is connected by the fact that a woman who wants to have a baby can use advice on preparing for pregnancy and giving birth safely, thus having a healthy child. There are some very general recommendations for women only, including supplementation with folic acid and iron. This manual, along with other multiple sources, confirms that in principle, FP is meant only to prevent pregnancy; in other words, "FP = Contraception." The term "family planning" is often considered by the general population and healthcare providers as synonymous with contraception.

Regarding the subject of FP and respect for human rights, the document "Human Rights: Family Planning Providers' Contribution" mentions the following: "All people deserve the right to determine, as best they can, the course of their own lives. Whether and when to have children, how many, and with whom are important parts of this right. FP providers have the privilege and responsibility to help people to make and carry out these decisions. Furthermore, programs that honor their clients' human rights contribute to positive sexual health outcomes."[21] And yet, the manual titled "Family Planning - A global handbook for providers," only provides information about contraception for healthcare professionals in low- and middle-income countries. This approach can raise several questions, one of which could be: What is the interpretation (meaning) of the concept of FP for health providers in high-income countries? We believe it is unfair that the promotion and respect of people's sexual-reproductive rights depend on the economic status of the countries.

The results of PerConcept survey (2019), conducted among family physicians and other specialists in three countries: Republic of Moldova (Chisinau), Mexico (Mexico City), and the Russian Federation (Irkutsk), confirmed that the concept of family planning should really include the component of preconception preparation, along with contraception, the approaches being applied equally to both. The results of the survey are interesting because doctors, respondents from three regions of the world: Europe, Asia, and North America, have similar perceptions about including PC in FP services.[22] The majority of respondents considered that strategies and programs in the field of FP should be revised to include preconception health services. In the practical aspect, the interviewees mentioned the importance of ensuring that the population has access to information and services in reproductive health, including PC, as "an effective tool to prevent some risks in maternal and infant well-being."

The interaction between two componentsof FP: contraception and PC

There is an important connection between pregnancy prevention and pregnancy preparing. In some cases, PC requires the use of a safe contraception method until the couple is able to address health and behavioral problems to minimize the risks to a healthy pregnancy. At the same time, a couple's decision to stop using contraception in order to become pregnant offers a "teachable moment" to provide preconception preparation information. Frayne[23] suggests that providers can reach women who are planning a pregnancy with preconception health messages at scheduled contraception visits or annual wellness examinations. van der Zee et al.[5] consider that an important requirement for preconception consultation is pregnancy planning, which is possible through the provision of contraceptives. Incorporating pregnancy intention screening into primary care to address unmet preconception and contraception needs may improve the delivery of FP services and improve reproductive health and wellness overall.[24]

In the position paper on Preconception Care of the American Academy of Family Physicians, one of the General Recommendations for Preconception Interventions for Women and Men is contraception.[10] This position paper emphasizes

the importance of incorporating routine identification of pregnancy intention for men and women of reproductive age to inform shared decision-making around reproductive planning and risk reduction. Because reproductive choices and risks can change over time, preconception health risks and pregnancy desires should be routinely assessed during chronic disease management visits.

Women recognize that pregnancy planning is an important benefit of contraception; however, they recognize it as a disadvantage also, as it is difficult to decide when to stop its use and to try to become pregnant.[5] One of the determining causes of the situation described could be the lack of information on fertility, menstrual cycle, and fertile days, which are important components of PC. In another context, it should be realized that the values and ideas of "naturalness" and "romanticism" that people consider important in the process of becoming pregnant. In other cases, women (or men) may feel coerced into pregnancy, and in others, people may feel overall that they have limited control of their lives and therefore limited capacity to plan for the future. Due to this complexity, confidentiality is essential for people who ask medical workers for PC.[5,25]

Primary care for women of childbearing age should include routine assessment of a woman's reproductive goals and pregnancy intentions ("reproductive planning").[26,27] Women who could potentially become pregnant should be assessed for preconception risks and educated about the importance of maternal health in ensuring healthy pregnancies. Women may be motivated to address modifiable health risks by learning about the way their health will affect a future pregnancy. For women not intending to get pregnant in the short term, PC should include counseling on effective contraception. Women with chronic medical conditions should be counseled about highly effective reversible methods, such as intrauterine devices and contraceptive implants, which have few medical contraindications. Including preconception health services in FP care could provide more comprehensive care to meet the unique needs of all beneficiaries and will be more appropriately accepted by women and men. There are a number of key reports and recommendations from the CDC(2006, 2010) and WHO(2013) that encourage providing comprehensive services to people of reproductive age.

The strategies and resources used to facilitate the clinical and public health integration and dissemination of PC, including the role of group care, the medical home, workplace and school-based health promotion programs, and home visitation, will also impact future progress.[28] We also recognize that there are many factors that are out of the individual's control that can influence their fertility, health and well-being including environmental hazards in their work place and community, inadequate access to health care and follow-up resources, inadequate access to healthy food and places to exercise, and stress due to poverty and racism or bias. Telling people to change something that is not in their control can be counterproductive. Therefore, it is important that providers and clinics partner with public health professionals, community leaders, and policymakers to create healthy conditions where all people can survive and thrive.

Conclusions

Modifying FP strategies and programs to include the preconception component, will contribute to:

- opening up optimistic prospects for integrating PC services into primary care, as FP programs have been implemented in many countries, involving financing operations and the infrastructure services system, in particular with the involvement of family doctors/GPs;
- respect for the sexual and reproductive rights of all persons and couples, regardless of their reproductive plans;
- increased opportunities for providing health education and information about PC. Training in pregnancy preparing could be carried out in FP networks (specialized clinics and health centers for young people, medical and private institutions in the field of reproductive health, etc.) for medical care providers in primary care and women and men at the same time;
- continuity of reproductive health care;
- better sustainability of maternal and child health programs and projects, taking into account the longer-term benefits of FP activities, including PC;
- last but not least, increase the value of FP services for the population, as they will increase their impact on maternal and child health indicators.

Approaching PC as a component and integral part of FP can generate discussions between providers or researchers, and future high-quality studies will be needed to adjust public health policies and improve clinical practices.

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Conflict of Interests

The authors declare that they have no conflicts of interest.

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REVIEW ARTICLE

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Physical Exercise and Falling among Older Patients with Diabetes: A Narrative Review

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Abstract

Both diabetes mellitus (DM) and aging have an effect on gait behavior, balance, muscle performance, and other medical complications related to the development of diabetic neuropathy, hypoglycemia, hypotension, cognitive impairment, pain, disturbed proprioceptions, and polypharmacy. The main goal of the present review study was to identify risk variables for hypoglycemia-influenced falling in DM older people, to suggest protective interventions to reduce the occurrence and to explore the effect of physical exercise on falling among elderly individuals with DM. In July 2021, these keywords were used to search Google Scholar, PubMed, Embase: falling in elderly, DM complications, insulin, hypoglycemia, and physical exercise. Because falls are so common during activities, it is critical to figure out what elements influence balance and walking activity. Multi-medications, cognitive dysfunction, dementia, urinary incontinence, depression status, and hypoglycemia are just some of the issues that can affect the elements of controlling balance directly during motion. Others, such as multi-medications, cognitive dysfunction, dementia, urinary incontinence, depression status, and hypoglycemia, can affect balance control indirectly by disrupting posture mobility. Exercise training has been shown to increase body performance and reduce joint discomfort, as well as improve psychological status and quality of life, muscular strength and balance, lower the chance of falling, and improve overall health in the aged and older adults.(**International Journal of Biomedicine. 2021;11(4):473-479.**)

Key Words: older adults • falling • diabetes mellitus • physical exercise

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Diabetes mellitus (DM) is continuing to be an increasing international health burden.[1] Furthermore, diabetes is becoming more prevalent among the elderly worldwide as a result of increased life expectancy and the disease's widespread prevalence.[2] Diabetic older persons have unique treatment needs. Hypoglycemia is a potential complication that might arise as a result of diabetes treatment.[3,4]

DM affects more than 10% of the older population, compared to 6% of the overall adult population, and it affects as many as 25% of nursing home residents.[5] The care of older individuals comes with its own set of difficulties. Hypoglycemia episodes are a common consequence of DM treatment with oral and insulin medicines.[3] Hypoglycemia's repercussions are perhaps even more severe in fragile elderly individuals than others.[4]

For example, falling in the elderly is perhaps the first sign of hypoglycemia, with terrible implications such as bone fractures, poor life quality, and death. Not as prevalent as the elevated falling rate in this population, osteoporosis is also common, owing to the presence of concomitant disorders such as DM, which is linked to impaired bone healthiness.[6] Moreover, a number of drugs have been linked to the development of osteoporosis in older DM individuals.[6]

Older individuals are prone to a variety of health issues, the most common of which is falling, which is the primary reason for mortality among adults over age 65. A fall can cause different health difficulties, ranging from the immediate (trauma, fractures, and lacerations) to the long-term (muscle fatigue, weakness, and fear of falls).[7] Falling has a harmful

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influence on older individuals' life quality since it impacts their activities and mobility, and increases their death rate. Falling is very common among aged and older DM persons, particularly type 2 DM (T2DM), which is a major risk factor. In people over age 65, the incidence rate is around 39% each year, and it is more common among those with uncontrolled glycemia.[8] Despite the fact that there are many disorders linked to falling in the elderly, a probable common risk factor is a loss of balance.[9] Furthermore, numerous DM drugs have been linked to falls in the older population.[6]

The main goal of the present review study was to identify risk variables for hypoglycemia-influenced falling in DM older people, to suggest protective interventions to reduce the occurrence and to explore the effect of physical exercise on falling among elderly individuals with DM. In July 2021, these keywords were used to search Google Scholar, PubMed, Embase: falling in elderly, DM complications, insulin, hypoglycemia, and physical exercise.

Causes of falling among older patients with DM

The causes of falls in older people are multifaceted, and DM has been identified as one of the major risk factors in several studies.[10,11] Diabetic neuropathy, neuromuscular problems, musculoskeletal dysfunctions, neuropathic pain, diabetic foot, cognitive dysfunction, vestibular disturbance, pain, and anti-DM medicines that produce hypoglycemia all contribute to a higher risk of falling in diabetic older individuals. Furthermore, DM increases the incidence of osteoporosis and fractures.[10-12]

Because of changes in the structure and architecture of bones, such as collagen quality and bone minerals, the strength and quality of bone in diabetic and non-diabetic individuals are lowered.[13,14] The severity, duration, and complications of DM, as well as a lack of food control, are all linked to a higher risk of fractures.[13] Furthermore, excessive use of DM medicines, particularly thiazolidinediones (TZD), may increase the risk of fracture due to a decrease in bone quality and mass. DM may increase the number of impairment episodes in older people, resulting in a higher risk of falling.[15]

Due to the rising prevalence of DM worldwide, the risk of fractures and falls in DM individuals may become a major public health concern in the future, particularly among diabetic older people, who are frailer and more prone to bone loss and osteoporosis.[16]

Recognizing the number of factors linked to falling in diabetic older people is a difficult and time-consuming task. Many products have been shown to be of minor benefit to older people who are at risk of falling due to the difficulties of including them in clinical assessments. Furthermore, the most predictable determinant of falling is past falling; the susceptibility of falling increases as a result of previous experiences.[17] This predictor, on the other hand, does not detect persons who have not fallen but does recognize the danger characteristics associated with falling. In addition, this predictor provides a reduced direction to the reasons for previous falls. As the primary goal is to intervene before an event occurs, the value of falling history is restricted because it does not reflect the detection of elements that contribute to falling. Finally, the most effective way is to identify persons who are exposed to dangers prior to falling and intervene in a timely manner to reduce these risks.

Factors associated with falling among older patients with DM

Diabetic older people are more vulnerable to falling than non-diabetic older people. When aging is combined with DM, the chance of falling is increased by 17 times.[18] Furthermore, age and diabetic consequences, such as impaired sensibility(peripheral neuropathy, retinopathy, and diabetic foot), are substantial factors in an individual's risk of falling.[19] Age, BMI, insulin medicines, neuropathy, heart illnesses, osteoarthritis, decreased balance, and weakness of the lower limbs have all been shown to raise the associated factors of falling in DM older individuals.[20]

Hypoglycemia

One of the commonest causes of unintentional lesions among older people is falls, which account for about 90% of wrist and hip fractures. Head injuries account for between 24% and 60% of all injuries.[21] Long-term DM problems, such as diabetic foot ulcers, retinopathy, and diabetic neuropathy, are recognized to induce falling in senior individuals.[21] Falls in DM people have been linked to a variety of risk factors. Aging, insulin medications, peripheral neuropathy, disturbed balance, obesity, osteoarthritis, cardiac diseases, and impaired lower limb function are all factors to consider.[20]

Diabetic individuals over age 65 have been reported to have a higher chance of falling and becoming disabled. One of the most feared acute consequences of DM therapy, hypoglycemia, has been proven to increase the chance of falling. Cognitive ability disturbance, palpitations, shakiness, and trembling are all signs that can contribute to hypoglycemia-related falls.[22,23]

Falling caused by hypoglycemia may result in fractures, head and soft tissue injuries, and disorientation, which can lead to recurrent falls.[24] Other comorbidities, including osteoporosis, in older persons may result in fractured bones. Accidents resulting in hospital visits were retrospectively analyzed in a large USA database among T2DM individuals on medicines (excluding insulin).[22]

Retrospective and case-control studies have also revealed an increase in hypoglycemia-induced falling and bone fracture, notably in individuals on insulin therapy who had strict glycemic control, but not in those on oral anti-DM drugs.[25] Hypoglycemia is related to a considerably greater accident risk leading to hospitalization in T2DM individuals using insulin, including incidents involving falling and driving.[24]

In older patients, hypoglycemia has a significant negative effect on life quality.[22] Hypoglycemia is feared by individuals more than any other diabetic condition, according to clinical experience.[26] Hypoglycemia is related to diminished results in the elderly, including an increase of falling and fracture risks, as well as hospital admission, frailty, in-hospital mortality, and durable cognitive dysfunction, including dementia disorder.[27] It is also linked to altered electrophysiology, including a longer QT interval, which is a recognized cause of cardiac arhythmia and can cause older people to fall.[28]

Anti-diabetic medications

One of the associated factors for falling is the use of anti-diabetic medications.[18] It has been shown that taking

several prescription medications raises the falling risk in older individuals. It was discovered that DM individuals who took more than four drugs had a higher risk of falling.[29,30] As the number of drugs used increased, so did the risk of falling. Insulin-dependent diabetics have a greater hazard of falling and hypoglycemia than those who take oral DM medications.[24]

The risk of hypoglycemia is higher in those taking sulphonylureas than in people taking other oral DM medicines.[31] The use of oral insulin sensitizers, on the other hand, was not linked to falls. TZD and metformin, for example, have not been demonstrated to raise the risk of falling.[32] TZD, on the other hand, has been linked to increased fracture risks in DM individuals due to decreased bone tissue growth and hastened loss of bone tissues. Individuals who took dipeptidyl peptidase-4 inhibitors or glucagon-like peptide-1 analogs had no risk of falling.[33]

Insulin and glycemic management induced falling

Insulin-treated DM individuals were more likely to fall throughout the events of hypoglycemia, according to R.Kennedy. It was also discovered that insulin-treated individuals were more likely to break a bone as a result of falling.[34]

When non-insulin-dependent individuals were compared with insulin-dependent patients, the accident rates in insulin-dependent individuals were considerably higher. Insulin-treated individuals had the following characteristics: predominance of women, the elderly, and persons who had spent more time in the hospital. The fractures were mostly found around the periphery of the body. When compared to males, females also had a greater possibility of getting bone fractures during falling.[35] DM individuals were shown to have a substantially greater rate of repeated falls. The number of drugs used, increasing intensity of discomfort, impaired grip strength, physical exercise, and disturbed cognition were all issues that contributed to increased falling risks. The relevance of glycemic control has been debated as a risk factor for falling.[18]

Researchers have investigated factors associated with DM individuals' falling and found that those who were poorly controlled were more susceptible to falling.[8] Falling in older individuals with DM, whether they have hypoglycemia or not, is prevalent, accounting for up to 31% of all recorded occurrences each year. Hemoglobin A1c (HbA1c) levels had no effect on the risks of falling in individuals who did not use insulin therapy. Individuals with HbA1c of less than 6% and those on insulin, on the other hand, were shown to be at a higher risk of falling. The importance of controlling HbA1c levels in DM management cannot be overstated. Regulating HbA1c could aid in delaying the long-term consequences linked with DM, resulting in weight loss.[19]

Diabetic microcirculation problems

One of the associated reasons for falling is identified as peripheral neuropathy. In diabetics with peripheral neuropathy, postural instability has been proven to cause falls.[36,37] Poor clearance of creatinine was established as an indirect, associated reason for falling.[38] Also associated with falling in DM individuals are microcirculation problems, poor kidney performance, peripheral nerve dysfunction, and visual disturbance.[38]

It was also discovered that falling-induced bone fractures occurred in older women even when they did not have incontinence of urine. It was theorized that prompt detection and incontinence management may assist older persons to avoid falling. Urinary frequency in older people is commonly known to be difficult to manage, because they tend to hurry to bathrooms to avoid incontinence disorder, making them prone to falling.[39]

Neuropathy and sensory dysfunction

Falling in DM individuals is caused mostly by sensory dysfunction-related neuropathy. Diabetic neuropathy affects around half of all diabetics, impairing their quality of life and raising their mortality risk.[40] Neuropathy affects many sections of the autonomic and somatic nervous systems, whether large or small nerve fibers, in DM individuals. Age, poor microcirculation, persistent hyperglycemia, and oxidative stress are all common risk factors for neuropathy.[41]

In 40% to 100% of diabetic individuals, distal symmetric polyneuropathy develops, resulting in peripheral motor and sensory dysfunctions. Diabetic polyneuropathy can affect balance and walking, increasing the risk of falling in people with DM.[40,42] Controlling balance is critical for improving mobility, preventing impairment, and regaining independence in the elderly.[37]

Balance system dysfunction

Because the balance system is complex, pinpointing the source of the problem can be challenging because the dysfunction could be produced by one or more motor systems, as well as sensory systems, such as somatosensory, vestibular, and visual. In order to give an appropriate diagnosis and treatment choices, it is necessary to analyze the motor and sensory systems that affect balance. Muscle strength and balance are primarily affected by neuropathy in the ankle and foot, where myelinated sensory and motor nerve fibers terminate. In DM individuals with sensory dysfunctions, impairment of brain functioning can cause significant changes in standing and gait characteristics,[43] such as a decrease in gait speed, an increase in postural motion, and a longer step duration. Also, whether standing or walking on a rough surface, the effect of this impairment is magnified.[44]Non-fixed carpets and rugs and a slight difference in step height can slow reaction time; dorsiflexors weaken, and the capacity to prevent falls is lost,[45] increasing the risk of falling threefold.

Cognitive dysfunction

Falling can be triggered by a variety of causes, including motor and sensory dysfunctions, as well as a disruption in cognitive function, which impairs functional performance and bodily stability. According to the American Geriatrics Society, over 60% of older persons with cognitive dysfunction are at risk of falling every year.[46] Falling in older people is linked to brain changes (pre-frontal cortex) that disrupt the executive control network.[47] When assessing gait performance in individuals with cognitive impairment, it was discovered that changes in stride time are frequently linked to executive dysfunctions.[48]

Cognitive impairment can alter a person's perceptions of environmental risks and induce instability, which can lead to a fear of falling and a reduction in everyday activities.[49] Fear of falling lowers self-esteem, limits movement and aerobic

capacity, and increases the risk of falling. Despite the fact that many older persons are aware of the benefits of physical activity for boosting health and reducing the risk of falls, a significant number of those who have a high fear of falling avoid physical activities even if they are free of ailments or medical concerns. As a result, fear of falling is regarded as a crucial predisposing factor when assessing the risk of falling.[50]

Physical exercise and falling among elderly with DM

Repeated and sustained action of large muscle groups is required for aerobic exercise.[51] Aerobic energy-producing systems are generally used in activities like walking, cycling, running, and swimming. Elastic resistance therabands, weight devices, and free weights are all examples of resistance (strength) training. Joint range of motion is improved by flexibility exercises.[41] Exercises that improve balance and avoid falls are beneficial to one's gait. Flexibility, balance, and resistance are all combined in physical activities such as yoga and tai chi.[52]

Aerobic exercise

Aerobic exercise improves oxidative enzymes, insulin sensitivity, mitochondrial density, blood vessel compliance and responsiveness, cardiac output, pulmonary functions, and immunological performance.[53-56] In both types of DM, T1DM and T2DM, moderate to high levels of aerobic activity are linked to significantly decreased cardiovascular and overall mortality risks.[57] Aerobic training improves cardiorespiratory fitness, insulin resistance, endothelial functions, and levels of lipids in T1DM individuals.[58] Regular physical exercises lower insulin resistance, lipids, blood pressure, and HA1C in T2DM individuals.[53] High-intensity interval exercise leads to rapid improvements in insulin sensitivity, oxidative capacity of skeletal muscles, and control of glycemia in T2DM individuals,[59,60] and could be completed with no effects on blood glucose level.[61]

Resistance exercise

Impaired muscle strength and a rapid loss in muscular performance are also risk factors for DM.[62] Improvement in insulin sensitivity, blood lipids, body composition, bone density, blood pressure, muscle strength and mass, physical and mental functions, and cardiovascular well-being are all strong healthy points of resistance exercise.[63] It is uncertain how resistance exercise affects glucose control in T1DM individuals.[61] Resistance exercise, on the other hand, can help people with T1DM reduce their risk of hypoglycemia caused by exercise. When aerobic and resistance exercises are combined in a single workout, resistance training is performed first, which results in less hypoglycemia than aerobic activity.[64] Insulin resistance, blood glucose levels, blood pressure, muscle strength, lean mass, and fat mass are all improved with resistance exercise for T2DM individuals.[65]

Balance and flexibility exercise

Balance and flexibility training are likely to be beneficial for DM seniors. The production of advanced glycation end products, which accumulate throughout the aging process and are increased with increased blood glucose levels, is one of the most common causes of limited joint mobility. Stretching improves joint flexibility and range of motion but has little effect on blood sugar management.[66] Even if you have peripheral neuropathy, balance training can help you avoid falling by improving your balance and stride. Mixed exercise therapies (balance and resistance exercises, tai chi sessions) have been shown to prevent falls by 229%. Alternative exercise forms, such as tai chi and yoga, are less well-studied, though yoga has been shown to enhance the management of body composition, hyperglycemia, and blood lipids in T2DM individuals. While studies on balance and flexibility exercises are limited, tai chi exercise could enhance good balance, reduce diabetic neuropathy, and improve life quality in neuropathic DM individuals.[66,67]

A large clinical study compared a lifestyle modification to DM support and educational groups in T2DM elderly individuals. In the lifestyle modification group, a minor nutritional energy shortage and unsupervised exercise for a minimum of 175 min a week were the targets of the intervention, which aimed for a weight loss of at least 7%. Both groups had a similar number of major cardiovascular events, presumably because the diabetic support and education group used more cardioprotective drugs.[68]

Lifestyle modification

The focused lifestyle modification showed higher satisfactory progress in weight loss, blood lipids, blood pressure, cardiorespiratory condition, and blood glucose level with less medical treatment, as well as decreasing sleep disturbance, knee pain, DM retinopathy, kidney dysfunction, sexual problems, depression, and urinary incontinence, and improving the preservation of bodily flexibility and quality of life. Pi-Sunyer discovered very significant evidence of increased health advantages from a strict change in lifestyle.[69]

A systematic review on T2DM individuals found that aerobic exercise reduced HA1C more (by 20.18%) than resistance exercise, but heart disease risk biomarkers were not lowered.[70] Furthermore, physical activity enhanced glycemic control in diabetics, especially when done for at least 150 minutes per week. In persons with T2DM, strength training (free weights or weightlifting) improved physical infrastructure by around 50%[24] and lowered HA1C by 0.57%.[71] Combination training was preferable to either training program when it comes to glucose control.[72] T2DM older individuals should preferably engage in both resistance and aerobic exercises to achieve the best glycemic control and health results.

Conclusion

Both DM and aging have an effect on gait behavior, balance, muscle performance, and other medical complications related to the development of diabetic neuropathy, hypoglycemia, hypotension, cognitive impairment, pain, disturbed proprioceptions, and polypharmacy. Because falls are so common during activities, it is critical to figure out what elements influence balance and walking activity. Multi-medications, cognitive dysfunction, dementia, urinary incontinence, depression status, and hypoglycemia are just some of the issues that can affect the elements of controlling balance directly during motion. Others, such as multi-medications, cognitive dysfunction, dementia, urinary incontinence, depression status, and hypoglycemia, can affect balance control indirectly by disrupting posture mobility.

Physiological concerns, such as muscle strength and balance control, can also be effectively addressed through a variety of exercise programs. Exercise training has been shown to increase body performance and reduce joint discomfort, as well as improve psychological status and quality of life, muscular strength and balance, lower the chance of falling, and improve overall health in the aged and older adults.[73,74]

Aerobic exercise should be paired with therapeutic nutrition, according to studies on frailty in the aged population.[75] Despite the fact that many studies have shown that balance training can reduce the risk of falling in older people,[76] only a few studies have shown that this sort of exercise can enhance balance and gait pattern and lower the risk of falling in DM individuals. In addition, balance exercise may enhance gait and posture.[52,77]

Furthermore, physical activity has been shown to increase reaction speed, sensory perceptions, muscular strength, and sensory responses in the distal extremities, sympathetic/parasympathetic balance, neuropathy symptoms, nerve function, and cutaneous innervations.[77,78]

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Competing Interests

The authors declare that they have no competing interests.

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BRIEF REVIEW

PCOS and Hyperprolactinemia: Conflicting Conditions or Comorbidities?

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Abstract

Currently, hyperprolactinemia is considered as a condition to be excluded during the diagnosis of polycystic ovarian syndrome (PCOS), because it often demonstrates clinical signs similar to PCOS. However, some publications have reported "the prevalence of hyperprolactinemia in PCOS," which does not agree with the statement above statement. The publications presented in this review demonstrate conflicting approaches to considering the association of hyperprolactinemia and PCOS. On the one hand, the current consensus on the diagnosis of PCOS assumes the exclusion of patients with hyperprolactinemia, and on the other hand, some authors consider hyperprolactinemia as an acceptable condition and estimate its prevalence in PCOS. Based on the analysis of the literature, we have demonstrated a contradictory attitude towards the association between hyperprolactinemia and PCOS. To overcome the contradiction, we consider it appropriate to use the term "potential PCOS" before the final assessment of the contribution of hyperprolactinemia to the development of symptoms similar to PCOS. The final diagnosis of PCOS in the presence of hyperprolactinemia is possible only after its correction and reassessment of all symptoms.(**International Journal of Biomedicine. 2021;11(4):480-483.**)

Key Words: obstetricians-gynecologists • stress • professional burnout • biomarkers

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Polycystic ovarian syndrome (PCOS) is one of the most common endocrine disorders in the female population.[1] Currently, hyperprolactinemia is considered as a condition to be excluded during the diagnosis of PCOS, because it often demonstrates clinical signs similar to PCOS. However, some publications have reported "the prevalence of hyperprolactinemia in PCOS," which does not agree with the statement above statement.

The purpose of this brief review was to systematize the available conflicting data on the associations between hyperprolactinemia and PCOS.

The information search was conducted using Internet resources (PubMed, EMBASE) and literature sources for the years 2000-2021.

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The epidemiology of PCOS is well studied, but the prevalence of the syndrome varies from 2.2% to 15%,[2-6] depending on the diagnostic criteria used and the characteristics of the population sample. In women with an irregular menstrual cycle, the incidence of PCOS ranges from 16.5% to 46%,[7,8] and with anovulatory infertility - from 55% to 91%.[9,10] Diagnosis of PCOS is based on the registration of clinical and laboratory manifestations of hyperandrogenism, assessment of menstrual and ovulatory function, as well as ovarian morphology using ultrasound. The Harmonized Criteria of the European Society for Human Reproduction and Embryology and the American Society for Reproductive Medicine (ASRM/ESHRE), adopted in Rotterdam (2003),[11] are currently used to diagnose PCOS. According to this consensus, the diagnosis of PCOS can be confirmed by the presence of at least two of the following three criteria: oligo- or anovulation, clinical or biochemical signs of hyperandrogenism, polycystic ovarian morphology on ultrasound. This approach to the diagnosis of PCOS is confirmed in the international recommendations for

the assessment and treatment of PCOS, presented in 2018.[12] Clinical signs of hyperandrogenism include hirsutism, acne, and androgenetic alopecia. According to various sources, hirsutism occurs in the majority of women with PCOS[13-16] and acne in 15%-25% of them;[17,18] however, their diagnostic significance as a marker of hyperandrogenism is debatable.[19.20] Although androgenic alopecia is considered a sign of PCOS, the relationship between these states has not been definitively determined.[21] Among the reproductive disorders associated with PCOS, the most important are infertility[22,23] and miscarriage, as well as failures with IVF.[24,25] At the same time, several scientists consider PCOS as a factor that does not affect the frequency of miscarriages.[26] In patients with PCOS, the risk of developing preeclampsia and hypertension during pregnancy is up to four times higher, and the risk of premature birth is almost twice as high.[27] Along with reproductive disorders, PCOS is associated with insulin resistance, impaired glucose tolerance, and diabetes mellitus, as well as cardiovascular diseases, all of which determine the long-term consequences of this disease.[28]

The previous and current consensuses on the diagnosis of PCOS require the exclusion of other diseases with similar symptoms, including hyperprolactinemia.

Hyperprolactinemia is found in almost 30% of women with secondary amenorrhea and is a common cause of amenorrhea and infertility. There are many reasons for the development of this condition: physiological (pregnancy, lactation, exercise), taking medications that disrupt the production of dopamine (for example, antipsychotics, antidepressants, sequential contraceptives, antihypertensives, and others), primary hypothyroidism, and chronic renal failure.[7] Like PCOS, hyperprolactinemia is often the cause of reproductive loss.[29] Hyperprolactinemia often demonstrates clinical signs similar to PCOS, and it is associated with excess androgen production by the adrenal glands in vivo and in vitro,[30,31] suggesting a potential mechanism by which it may contribute to hyperandrogenism. Elevated prolactin levels are found in 6% of women with hirsutism.[32] Therefore, when diagnosing PCOS, it is necessary to exclude hyperprolactinemia as a disease with similar symptoms.

However, some authors evaluated "the prevalence of hyperprolactinemia in PCOS," which contradicts the approved approaches to the diagnosis of PCOS. Thus, Filho et al.[33] detected hyperprolactinemia in 16% of patients with PCOS. The authors showed that hyperprolactinemia is not a clinical manifestation of PCOS but is caused by other etiological factors, which logically requires further examination for other causes of hyperprolactinemia. Hayashida et al.[34] found macroprolactinemia in 6% of cases in a group of 227 women with PCOS. In 2018, a study by Kyritsi et al.[35] reported that the prevalence of idiopathic hyperprolactinemia in a group of 76 PCOS patients reached 26%. A clinical case with twin sisters with signs of PCOS associated with idiopathic hyperprolactinemia was described by Goyal et al.[36] Several authors have suggested that PCOS causes hyperprolactinemia due to a relative hyperestrogenemia in anovulatory women.[37,38] This hypothesis is supported by various experimental studies that have shown an increase in the secretion of prolactin under the influence of estrogens.[39] Nevertheless, there are few studies on this topic, and none of them has convincingly demonstrated a clear pathogenetic link between PCOS and hyperprolactinemia.

Thus, the publications presented in this review demonstrate conflicting approaches to considering the association of hyperprolactinemia and PCOS. On the one hand, the current consensus on the diagnosis of PCOS assumes the exclusion of patients with hyperprolactinemia, and on the other hand, some authors consider hyperprolactinemia as an acceptable condition and estimate its prevalence in PCOS.

Conclusion

Hyperprolactinemia and PCOS are known as conditions with similar clinical manifestations, and current guidelines on PCOS diagnosis consider hyperprolactinemia as exclusion criteria. However, some authors indicate a proportion of patients with hyperprolactinemia and PCOS that does not correspond with the previously mentioned consensuses.

In our opinion, the data on the prevalence of hyperprolactinemia in women with signs of PCOS are of interest. However, it is better to use the term "potential PCOS" in such cases, and diagnose PCOS only when clinical signs of this syndrome occur after correction of hyperprolactinemia. According to this point of view, our brief review summarizes current research data assessing the frequency of hyperprolactinemia in "potential PCOS." Nowadays, the literature regarding the prevalence of hyperprolactinemia in women with symptoms of PCOS is still unclear, and more studies are needed, including those designed to estimate asymptomatic hyperprolactinemia with the evaluation of macroprolactin.[40,41] Several studies have shown that approximately 40% of patients with hyperprolactinemia have macroprolactinemia.[42,43] Because macroprolactinemia is a common cause of hyperprolactinemia, routine screening for macroprolactin could eliminate unnecessary diagnostic testing and treatment.[44,45]

Based on the analysis of the literature, we have demonstrated a contradictory attitude towards the association between hyperprolactinemia and PCOS. To overcome the contradiction, we consider it appropriate to use the term "potential PCOS" before the final assessment of the contribution of hyperprolactinemia to the development of symptoms similar to PCOS. The final diagnosis of PCOS in the presence of hyperprolactinemia is possible only after its correction and reassessment of all symptoms.

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Competing Interests

The authors declare that they have no competing interests.

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BRIEF REVIEW

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The Role of Physical Aerobic Activity in Controlling Exercise-Induced Bronchoconstriction in Children and Adolescents with Asthma

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Abstract

Asthma, one of the major widespread chronic disorders among children and adolescents, has become more prevalent recently. The common manifestations of this disorder are caused by inflammatory airways that lead to airway restriction and lung hypersensitivity causing dry coughing, wheezing, and shortness of breath, all of which are combined with sleep disturbance, impaired physical activity, and reduced quality of life. The main goal of this brief review was to identify the associated variables that affect the management of asthma disease in children and young adolescents and to identify the role of physical aerobic exercise in the treatment of asthmatic children. The current review was based on prior research published in English databases such as Google Scholar, PubMed, and Embase in scientific articles published between January 2010 and October 2021 with the keywords "asthma," "children," "adolescents," "breathing episodes," "physical activity," and "physical exercise." Regular physical aerobic exercise training with moderate intensity has been shown to improve pulmonary functions, life quality, psychological conditions, and reduce asthma symptoms and EIB in children and adolescents with bronchial asthma.(**International Journal of Biomedicine. 2021;11(4):484-487.**)

Key Words: asthma • children • adolescents • physical activity • exercise-induced bronchoconstriction

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Abbreviations

EIB, exercise-induced bronchoconstriction**; CPET**, cardiopulmonary exercise testing.

Asthma is a chronic airway inflammatory disorder that causes repeated sensitive and persistent bouts of pulmonary issues involving shortened breath, wheezing, and cough.(1) These issues may occur as mild, moderate, or severe breathing episodes that require admission to intensive or critical care units and cause a high rate of mortality and morbidity. Numerous young asthmatics suffer disturbed quality of life, poor pulmonary functions, and impaired exercise tolerance.(2)

It has been documented that regular physical activities is one of the main elements that improve self-esteem, life quality, and psychological status in asthmatic children.(3-5) It has also been reported that, compared with their peers, asthmatic children's levels of physical activities are restricted. Asthmatics had a lower physical activity level than healthy peers in a study of physical activity in urban school-aged children with asthma in America;(6) however, other studies

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reported that asthma disorder does not affect the children's participation in general exercise.[7,8]

A recent systematic review of all available studies using a control group identified 11 studies **(**asthma sample = 32,606) reporting less physical activity in children with asthma, and 6 studies (asthma sample = 7824) reporting no difference, thus leading to the conclusion that people with asthma engage in less activity than do controls.[9]

The main goal of this brief review was to identify the associated variables that affect the management of asthma disease in children and young adolescents and to identify the role of physical aerobic exercise in the treatment of asthmatic children. The current review was based on prior research published in English databases such as Google Scholar, PubMed, and Embase in scientific articles published between January 2010 and October 2021 with the keywords "asthma," "children," "adolescents," "breathing episodes," "physical activity," and "physical exercise."

Exercise-induced bronchoconstriction

Asthma, one of the major widespread chronic disorders among children and adolescents, has become more prevalent recently.[10] The common manifestations of this disorder are caused by inflammatory airways that lead to airway restriction and lung hypersensitivity causing dry coughing, wheezing, and shortness of breath, all of which are combined with sleep disturbance, impaired physical activity, and reduced quality of life.[11] Although regular physical activity is a vital component of well-being during childhood and adolescence,[2] exercise-induced bronchoconstriction (EIB) can develop when airways narrow as a result of physical activity.[12] EIB is the preferred term for what was known for years as exercise-induced asthma. As many as 90% of people with asthma also have EIB, but not everyone with EIB has asthma. This condition may occur also in 5% to 20% of healthy individuals.[13] EIB is globally prevalent in 9% of adolescents and children, 12% in Asia and America, and 8% in European countries.[14]

EIB symptoms (shortness of breath or wheezing, decreased endurance, tightness in the chest, cough) typically appear within a few minutes after starting exercises and may continue for 10 to 15 minutes after ending the workout.[15] Wheezing in children after physical activity is often the first symptom of asthma. EIB is defined as a forced expiratory volume in one second ≥ 10% lower than the baseline value at 5, 10, 20, and 30 minutes after cardiopulmonary exercise testing (CPET).[16,17] No clear mechanism has been documented for exercise-induced asthma; however, some associated variables, including dried and cooled airways during exercise, indicate the status of EIB.[18] While physical exercise is the generating factor of bronchospasm, regular exercise training is documented as a primary element in asthma management. Previous meta-analysis and systematic reviews have shown that practicing physical exercise has improved life quality, increased cardiorespiratory performance, and reduced dyspnea in young asthmatics.[19-22]

Because bronchial asthma is a common occurrence in adolescents and children, and EIB is identified as a great barrier to participating in physical and sports activities,[23] explaining the effects of physical activity on EIB may help in the development of assessment and management guidelines for therapeutic exercise intervention in adolescents and children experiencing bronchial asthma.

Aerobic exercise training and asthma

Haskell et al.[24] found that moderate-intensity physical exercise at least 30 min 5 times a week or high-intensity physical exercise at least 20 min 3 times a week are required to control and prevent diseases. It was documented that the most beneficial modality of physical activity is moderate-intensity physical exercise for a longer time than high-intensity physical exercise.[25,26]

Aerobic exercise, sometimes known as "cardio" exercise, stimulates the heart to pump oxygenated blood to deliver oxygen to working muscles. The oxygen inhaled and demanded is needed to maintain vital physiological mechanisms in the human body during activities. It was documented that moderate-intensity aerobic training improves pulmonary inflammatory markers in an asthmatic mouse model.[27] A six-minute walk test in school-aged asthmatics found that moderate-intensity physical exercise improves life quality, lung functions, and exercise tolerance.[2]

Different studies have demonstrated that various intensities of aerobic and active play exercises may improve life quality, lung volumes, functional capacity, sleep disturbances, cardiac output, immunity, and psychological condition in different respiratory and cardiovascular diseases at different age stages.[28-32]

Children and adolescents with asthma should be encouraged to engage in regular physical activity.[33] Recent reviews have endorsed the positive influences of physical exercise on asthma symptoms, maximal oxygen uptake, cardiorespiratory fitness, and life quality in adults experiencing bronchial asthma, without adverse impacts on pulmonary functions.[21,34-36] Regular aerobic exercise improved asthma symptom management, lung function, physical capacity, body composition, and mental health in children with asthma. [37,38]

In addition, it was observed that aerobic exercise alone or combined with resistance exercise can improve the exercise tolerance of abdominal muscles in obese adolescents.[39] Similarly, it was reported that airway obstructions have been reduced and inspiratory muscle strength has been increased in asthmatic adults following an aerobic exercise program.[40]

In a population-based study conducted in Greece by Anthracopoulos et al.,[41] free-running exercise challenge tests were employed in the evaluation of children 10-12 years of age. The authors found that the prevalence of EIB and the total energy expenditure were higher in the children who were moderately active or inactive than in those who were active, regardless of body mass index or asthma symptoms.

Lu et al. found that increased sedentary time is associated with worse asthma outcomes.[42] In a study performed by Sousa et al.,[43] the level of moderate physical activity was found to be comparable between children with and without asthma, even when those with severe asthma were included, although EIB was not evaluated in that study.

Vahlkvist et al.[44] showed that poorly controlled asthma was associated with reduced physical activity and cardiovascular fitness. Faleiro et al.[33] evaluated 20 patients with severe

refractory asthma and 19 controls. In the sample as a whole, the mean age was 12.9±0.4 years. Among the patients, authors observed isolated EIB in 30%, isolated physical deconditioning in 25%, physical deconditioning accompanied by EIB in 25%, and exercise-induced symptoms not supported by the CPET data in 15%. The authors concluded that physical deconditioning (alone or accompanied by EIB) was the determining factor in reducing exercise tolerance in patients with severe refractory asthma and was not therefore found to be associated with a lack of asthma control.

A meta-analysis of 17 randomized controlled trials, including 599 children and adult asthmatics, also reported that exercise training led to a significant improvement in days without asthma symptoms.[45] A clinical review performed by Panagiotou et al.[16] showed that higher adherence to physical activity was associated with favorable clinical outcomes, such as improved lung function, asthma control, exacerbation rate, and healthcare use.

Conclusion

In general, regular physical aerobic exercise training with moderate intensity may improve pulmonary functions, life quality, psychological conditions, and reduce asthma symptoms and EIB in children and adolescents with bronchial asthma. Regular physical aerobic exercise training should be conducted in combination with pharmacological asthma treatment to achieve significant asthma control in children and adolescents.

Acknowledgments

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Competing Interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

Internal Medicine

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Major Inflammatory Markers and Their Significance in Predicting Severity of COVID-19 Disease Pattern

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Abstract

The unanticipated outbreak of the COVID-19 pandemic has shocked the world in terms of both lives and livelihood. SARS-CoV-2 virus primarily affects the respiratory system, although other organ systems are also involved. Early diagnosis followed up by a retrospective analysis and tracking of a few markers relevant to the immunological status of the individual may aid in determining the state of the patient's disease prognosis. The aim of the present study was to evaluate immunological parameters such as neutrophil to lymphocyte ratio (NLR), C-reactive protein (CRP), and D-dimer, taking into account the patient's age and oxygen saturation level.

Our retrospective analysis of clinical data revealed that such parameters as CRP, D-dimer, and NLR should be taken into consideration to predict severe COVID-19-related complications. The data obtained indicate that patients over age 60 are especially vulnerable to severe COVID-19.(**International Journal of Biomedicine. 2021;11(4):488-492.**)

Key Words: COVID-19 • neutrophilia • D-dimer • C-reactive protein

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Introduction

The unanticipated outbreak of the COVID-19 pandemic has shocked the world in terms of both lives and livelihood. The origin of the SARS-CoV-2 virus is itself a debatable mystery; however, its severity is highly unpredictable, as several countries in the world have already experienced serious consecutive waves of infection. As the virus mutates, it becomes more infectious and aggressively hits the host, leading to a higher rate of mortality.[1] Globally, this virus has been considered a major threat in both developed and under-developed countries as it mainly halts advancement of the socio-economic status of the country directly.

SARS-CoV-2 establishes residence in the host and multiplies, primarily focusing on the respiratory system and causing a respiratory disease. It enters the epithelial cells of the nasal cavity by engaging the ACE2 receptor with the viral receptor-binding domain (RBD) and begins replicating.[2-4] In the stage of established pulmonary disease, there are viral multiplication and localized inflammation in the lungs that may lead to devastating damage. The most severe stage of the illness manifests as an extrapulmonary, systemic hyperinflammation syndrome.[5]

It is highly important to block the viral entry at its initial phase. Therefore, early diagnosis and monitoring are necessary in order to control the disease progression and its clinical complications.[6] Early diagnosis followed by the retrospective analysis and follow-up of few markers could even predict

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the immunological status of the individual. The heightened immunological response and the consecutive transmission of this virus colonizing in the lungs are the two critical factors that determine the severity of the disease.[7]

Miriam Merad and Jerome C. Martin highlight that current models of COVID-19 propose three distinct immune stages that are crucial for the ultimate disease course:[8] "In the first stage, early activation of the immune system through the induction of a potent interferon response is important to control the virus. In the second stage, a delayed interferon response may lead to progressive tissue damage. This may ultimately lead to the third stage, a deleterious hyperinflammation characterized by the excessive macrophage activation and coagulation that is seen in patients with severe disease, possibly followed by dysregulation of tissue repair mechanisms and fibrosis."

According to Huertas et al., endothelial cell dysfunction and impaired microcirculatory function contribute markedly to life-threatening complications of COVID-19, such as venous thromboembolic disease and multiple organ involvement.[9] Endothelial dysfunction, complement activation, thrombin generation, platelet and leukocyte recruitment, and the initiation of innate and adaptive immune responses culminate in immunothrombosis, ultimately causing (micro)thrombotic complications, such as deep vein thrombosis, pulmonary embolism, and stroke. In this regard, the activation of coagulation and thrombocytopenia has emerged as a prognostic marker in COVID-19.[10,11]

Coagulopathy and D-dimer elevations were seen in 3.75%–68.0% of the COVID-19 patients.[12,13] Yao et al.[14] showed that a D-dimer level of >2.14 mg/L predicted in-hospital mortality in COVID-19 patients with a sensitivity of 88.2% and specificity of 71.3% (AUC=0.85; 95%CI=0.77-0.92).[14] High levels of CRP have also been used as an indicator of COVID-19 disease severity. Stringer et al. showed that a threshold cut-off of CRP ≥ 40 mg/L was associated with mortality in COVID -19 patients.[15]

As COVID-19 progresses to the lethal final phase, it is necessary to monitor the surge levels of inflammatory markers in order to prevent the development of cytokine storm and further complications. The aim of the present study was to evaluate immunological parameters such as neutrophil to lymphocyte ratio (NLR), C-reactive protein (CRP), and D-dimer, taking into account the patient's age and oxygen saturation level.

Materials and Methods

The study was investigated at Najran Armed Forces Hospital. Patients suspected of having COVID-19 were confirmed by qRT-PCR analyzing the fold change expression of SARS-CoV-2 viral-specific genes, such as the *N* gene and *E* gene in an oro-nasal throat swab. Strong COVID-19 positive patients with qRT-PCR cycle threshold values of 20-25 consented to enroll in this study. Nearly 121 strong positives volunteered for this clinical investigation. This study included the patients' complete data from the date of admission until the date of discharge or death, information that was available from the hospital administrative records.

We analyzed plasma NLR, CRP, and D-dimer values. All these parameters are closely correlated with immune status and internal microvasculature clotting, which is associated with the lethal phase of the disease. This basic diagnostic profile is usual for the typical multi-specialty hospital.

The case files of the patients were retrospectively reviewed, the required information was extracted, and patients with a co-morbidity were excluded. Treatment with azithromycin, hydroxychloroquine, lopinavir-ritonavir, steroids, or oxygen support was considered as dependent upon the patients' requirements. Asymptomatic patients, were given multivitamins and zinc tablets. The sample size was determined by the time window of the study.

Statistical analysis was performed using the IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.).

Results

A scatter plot (Fig.1) shows a moderate positive linear relationship between NLR and patient's age. The COVID-19 patients above 60 were more sensitive to increased NLR levels. We found a moderate positive linear relationship between CRP and patient's age (Fig.2).

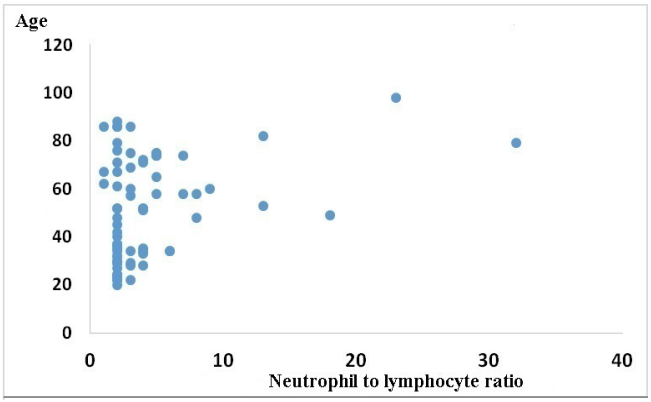

Fig. 1. *The relationship between NLR and patient's age (n=121).*

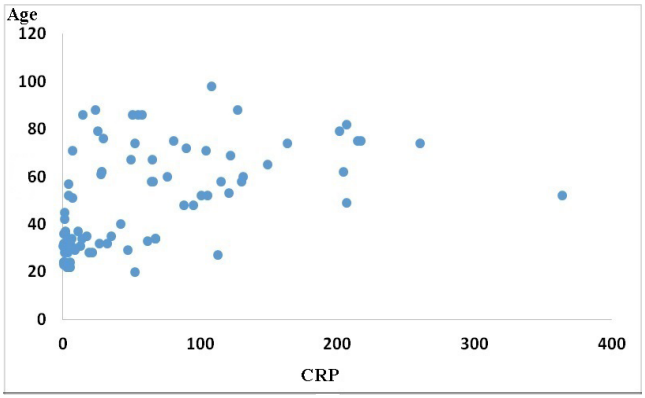

Fig. 2. *The relationship between CRP and patient's age (n=121).*

The COVID-19 patients above 60 developed higher CRP values. Figure 3 shows a moderate positive linear relationship between D-dimer and patient's age. The COVID-19 patients above 60 were more sensitive to increased D-dimer values.

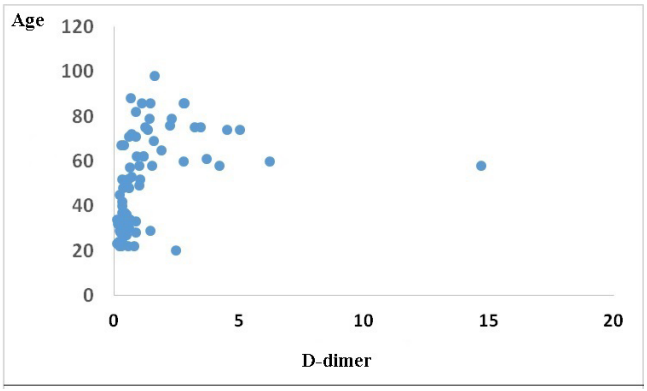

Fig. 3. *The relationship between D-dimer and patient's age (n=121).*

Figure 4 shows a strong negative linear relationship between patient's age and SaO_2: SaO_2 levels decrease in patients above 60 years. In contrast, the age groups ranging between 20-40 and 40-60 maintain consistent SaO_2 levels.

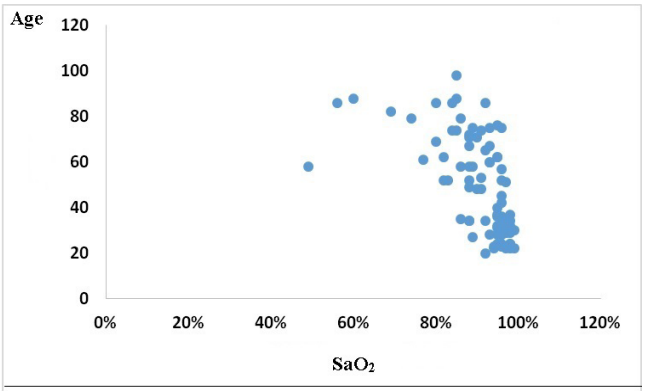

Fig. 4. *The relationship between patient's age and SaO_2 (n=121).*

Discussion

COVID-19 has quickly spread around the world with high mortality worldwide. The pathological pulmonary damage it has caused may be directly related to the viral destruction of alveolar and bronchial epithelial cells or mass production of proinflammatory cytokines (cytokine storm).[16] In COVID-19, the cytokine storm may result in an uncontrolled systemic inflammatory response, ARDS, multiple organ failure, and death in severe cases.[17,18]

Currently, it is clear that hyperinflammation and coagulopathy contribute to disease severity and death in patients infected with SARS-CoV-2.[8] It is believed that higher values of proinflammatory markers are related to extensive lung injury.[19] The neutrophils are known to develop a sophisticated network of extracellular fibers composed of DNA containing histones, called neutrophil extracellular traps (NETs). There is some evidence to suggest that NETosis is conditional on the production of reactive oxygen species.[20]

It has been shown that neutrophilia predicts a poor outcome in patients with severe COVID-19 cases, and NLR may be an independent risk factor for the severity of this disease.[21] In a recent article, Shivakumar, with associates from India, found that the neutrophil-to-lymphocyte-to-monocyte ratio and the platelet-to-lymphocyte ratio were significantly prognostic in COVID-19.[22]

The high mortality associated with thromboembolic disorders in COVID-19 has prompted clinicians to use D-dimer as a useful marker for assessing the severity of the disease.[23] According to a review by Harvard Medical School researchers, in critically ill patients with COVID-19, elevated levels of D-dimer were found in 100% of participants, elevated fibrinogen in 74%, and factor V11 in 100%.[24]

Nalbant et al.[25] found that the risk of COVID-19 was 20.3-fold greater when NLR was ≥2.4 in the logistic regression (P=0.007). The authors concluded that NLR is an independent predictor for the diagnosis of COVID-19. In a study by Seyit et al.,[26] the CRP (P=0.0001) and NLR (P=0.001) remained significantly higher in the patients with positive SARS-CoV-2 PCR test results.

The biological changes linked to aging and morbidity are one of the reasons deaths have been concentrated among older persons around the world. In addition to having less ability to fight off a novel virus, several aspects of immune functioning also may be worse for older people. Hyperinflammation has been linked to poor outcomes with COVID-19 due to "cytokine storms," or an out-of-control immune reaction. The average number of dysregulated cytokines doubles in the age group from the 50s to the 80s.[27]

In our analysis, this NLR ratio was observed to be more in cases of patients belonging to the age group of 60 and above. Our analysis was in agreement with the previously reported data; therefore, it is well understood that not only monitoring the immunological profiles but also the patient's age should be taken into consideration in the case of COVID-19.

Conclusion

Our retrospective analysis of clinical data revealed that such parameters as CRP, D-dimer, and NLR should be taken into consideration to predict severe COVID-19-related complications. The data obtained indicate that patients over age 60 are especially vulnerable to severe COVID-19.

Competing Interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

Hematology

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The Evaluation of CD3, CD 5, CD10, CD 19, and CD20 Markers in the Differential Diagnosis of the Lymphoma Subtypes in Sudanese Patients

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Abstract

The objective of this paper was to evaluate the use of CD3, CD5, CD10, CD19, and CD20 markers in the differential identification of lymphoma subtypes.

Methods and Results: This was a retrospective cross-sectional study included 82 patients with palpable lymphadenopathies. The formalin-fixed paraffin block sections immunostained with the Dako flex were investigated. CD3, CD5, CD10, CD19, and CD20 staining was performed on sections. The current study found that the two main types of lymphoma, Hodgkin's lymphoma, amd non-Hodgkin's lymphoma, have a significant association with CD3, CD10, and CD19, and a highly significant association with CD20, implying that these CD markers are crucial for general classification and diagnosis of lymphoma. CD3 had a highly significant relationship with gender. CD3 and CD20 were demonstrated to have a significant relationship with the lymphoma subtypes. The CD20 marker is the most consistent and useful marker for differentiating lymphoma subtypes.(**International Journal of Biomedicine. 2021;11(4):493-497.**)

Key Words: Hodgkin's lymphoma • non-Hodgkin's lymphoma • immunohistochemistry • CD markers

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Introduction

Lymphomas are a group of disorders produced by malignant cells that aggregate in lymph nodes, resulting in the clinical symptoms of lymphadenopathy. They occasionally spread into the blood ('leukemic phase') or invade organs other than lymphoid tissue. The characteristic multinucleated, polyploidy Reed–Sternberg (RS) cells are essential for distinguishing the four classic subtypes, and mononuclear Hodgkin (H) cells are also found in the malignant clone. RS cells and their mononuclear variants (H cells) exhibit inconsistency in antigen expression.[(1)] The use of CD markers in medicine has improved our knowledge of a variety of hematological disorders. CD markers are monoclonal antibodies that bind leukocyte cell-surface molecules as well as antigens from other cells. CD5 is found in chronic lymphocytic leukemia/ small lymphocytic lymphoma (CLL/SLL) and mantle cell lymphoma (MCL), while CD10 is present in acute precursor B- and T-cell lymphoblastic leukemia/lymphoma (ALL) and lymphomas of follicular center cell origin.[(2)]

T-cell processes are typically CD3+, CD20-, and CD45+, whereas B-cell processes are CD3-, CD20+, and CD45+. Other CD markers are utilized to distinguish lymphomas. CD markers allow hematologists to combine immunophenotyping with clinical observations to make an accurate diagnosis.[(3)]

In around 35%–40% of nodular sclerosis and mixed cellularity cases, RS-H cells express the B-cell

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antigens CD19 and CD20.[4,5] Hodgkin's lymphoma (HL) immunohistochemistry reveals that nodular lymphocyte-predominant HL (NLPHL) is a distinct HL subtype. This subtype's RS-H cells, defined as lymphocyte and histiocyte (L&H) variants, have a distinct polylobated, "popcorn" look and regularly express B-cell markers such as CD20 and CD45 (leukocyte common antigen).[6]

There have been multiple occurrences of HL coexisting with non-Hodgkin's lymphoma (NHL), either as sequential occurrences or in the same site, when they have been termed as composite. The most frequent link is lymphocyte predominance and large cell lymphoma.[7] Follicular lymphoma is frequently associated with other subtypes of HL.[8] The objective of this paper was to evaluate the use of CD3, CD5, CD10, CD19, and CD20 markers in the differential identification of lymphoma subtypes.

Materials and Methods

This was a retrospective cross-sectional study. The research was carried out at Rick Hospital in Khartoum (Sudan), from April to July 2019. Sudanese patients with lymphomas who attended histopathology laboratories agreed to participate in the study after providing written consent.

The sample size was calculated using a convenient sampling technique, and 82 patients with palpable lymphadenopathies were chosen.

Inclusion criteria. All Sudanese patients with lymphadenopathy and diagnosed with any type of lymphomas were enrolled in the study.

Exclusion criteria Participants apparently healthy without signs and symptoms of lymphadenopathy were excluded.

Sample collection and processing

Tissue sections were prepared on a slide, dewaxed in xylene for two 10-minute shifts, and then hydrated in graded alcohol for two minutes each. After hydration, the slides were stained for 10 minutes with H&E. Following staining, the slides were rinsed with tap water for 8 minutes before being moved to a coupling jar containing a counterstain, such as Eosin solution, for 2 minutes. The slides were then washed under running water for 1 minute. The stained slides were air-dried and then mounted with DPX.[9] Lymph node paraffin block sections were placed on an adhesive-coated slide containing Poly-L-lysine. After heat-induced epitope retrieval (three minutes at 110ºC in citrate buffer with PH 6.0), de-paraffinized sections were manually stained with a standard multilink detection kit (Dako detection kit) containing endogenous peroxidase block, nonspecific binding block, horseradish peroxidase, 3,3′-diaminobenzidine as chromogen, and hematoxylin. Five primary polyclonal antibodies were used to stain the sections: The formalin-fixed paraffin block sections were immunostained with the Dako flex, ready-to-use antibody system, which has been approved by prominent experts in the field.[9] CD3, CD5, CD10, CD19, and CD20 staining was performed on sections (Fig.1). The staining reaction occurred in the nucleus, and the appropriate positive control was applied.

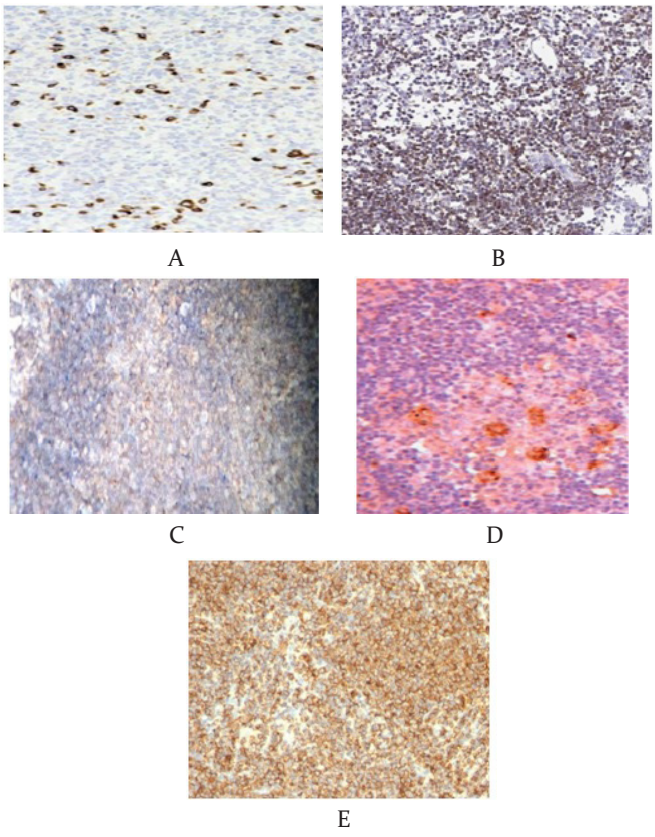

Fig. 1. *Morphological features of immunohistochmical CD3, CD5, CD10, CD19, and CD20 staining (hematoxylin counterstain) in human lymphoma:*

A - CD3+; B- CD5+; C- CD10+; D- CD19+; E- CD20+

Assessments of results for histopathology and immuno-cytochemistry

A pathologist at the histopathology department of the hospital where the study was conducted confirmed the malignant cases and their origins once they were diagnosed. H&E stains and histological immune markers were used to make the diagnosis. The level of staining on positive tumor cells was reported as negative, weak (1+), moderate (2+), or strong (3+). The intensity of strong staining was defined as staining comparable to positive control tissue. Any faint staining in tumor cells was considered weak staining. All tumors that lacked staining of tumor cells were deemed negative. Tumors with +1 staining intensity in up to 10% of tumor cells were thought to be weakly positive. A +2 intensity in 10%-30% of tumor cells was classified as moderately positive. More than 30% of tumor cells with a +3 intensity were regarded as a strong positive. To determine sensitivity and specificity, a relatively high cutoff value of 10% (1+) was used to eliminate false-positive results by using the following formulas:

Sensitivity = [True positive/True positive+False negative]×100

Specificity = [True negatives/True negative+False positive]×100

Ethical consideration

The study was conducted in accordance with ethical principles of the WMA Declaration of Helsinki (1964, ed. 2013) and approved by the Ethics Committee of University

of Medical Sciences and Technology and Rick Hospital (Khartoum, Sudan). All patients were informed about the purpose and design of the work and gave their consent to participate in the study and publish its results in the open press.

Statistical analysis was performed using the IBM SPSS Statistics for Windows, Version 20.0. (Armonk, NY: IBM Corp.). Baseline characteristics were summarized as frequencies and percentages for categorical variables. Group comparisons were performed using chi-square tests with Yates correction. A probability value of $P<0.05$ was considered statistically significant.

Results

The total number of participants in this study was 82 lymphoma patients (58.5% men and 41.5% women). The age ranges were subdivided into four categories. Group 1 included 30(36.6%) patients under age 20; Group 2 included 14(17.1%) patients aged between 21 and 40 years; Group 3 included 22(26.8%) patients between 41-60 years; Group 4 included 16(19.5%) patients over age 61 (Table 1).

Table 1. Socio-demographic characteristics of the respondents (n=82)

Socio-demographic variables	No (%)
Age, yrs	
0-20	30 (36.6%)
21-40	14 (17.1%)
41-60	22 (26.8%)
>61	16 (19.5%)
Gender	
Male	48 (58.5%)
Female	34 (41.5%)

Table 2 represents the relationship between age groups and gender, and CD markers: CD20 had a highly significant relationship with age groups, and CD3 had a highly significant relationship with gender.

Table 3 represents the relationship between HL/NHL and CD markers. We found that there is a significant association between HL/NHL and CD3, CD10, and CD19. A highly significant relationship was found between CD20 and lymphoma types.

Table 4 displays lymphoma subtypes and their relationship with CD markers. CD3 and CD20 were demonstrated to have a significant relationship with the subtypes of lymphoma.

Discussion

As is known, lymphoma is a widespread and difficult-to-diagnose disease, so highly sensitive diagnostic techniques had to be used to diagnose it. The current study focused on using CD markers in a potential diagnostic role in lymphomas as well in their subtypes.

Since each type and subtype displays specific clinicopathologic characteristics, morphological identification and classification of lymphomas depending on immunophenotyping is critical for patient management and prognosis.

Lymphomas are likely to be seen in people of all ages, ranging from 0 to more than 61 years, though they primarily affect those over age 40. According to the current study, CD20 has a significant association with age groups, whereas CD3 has a highly significant association with gender; CD3 expression is rarely abnormal in mature B-cell neoplasms such as diffuse large B-cell lymphoma, classic HL,[10] and follicular lymphoma. CD3 expression in CD20+ B-cell lymphomas is usually not a problem because co-expression of these two markers will trigger further workup with more

Table 2.

Association and frequency of each CD marker in sex and age groups (n=82)

Groups	CD3		CD5		CD10		CD19		CD20	
	- n (%)	+ n (%)	- n (%)	+ n (%)	- n (%)	+ n (%)	- n (%)	+ n (%)	- n (%)	+ n (%)
Age, yrs										
0-20	22(26.8)	8(9.8)	30(36.6)	0	22(26.8)	8(9.8)	28(31.7)	4(4.9)	8(9.8)	22(26.8)
21-40	8(9.8)	6(7.3)	14(17.1)	0	14(17.1)	0	14(17.1)	0	8(9.8)	6(7.3)
41-60	14(17.1)	8(9.8)	20(24.4)	2(2.4)	16(19.5)	6(7.3)	18(22.0)	4(4.9)	1(1.2)	21(25.6)
>61	10(12.2)	6(7.3)	16(19.5)	0	14(17.1)	2(2.4)	12(14.6)	4(4.9)	4(4.9)	12(14.6)
P- value	0.71		0.13		0.12		0.25		0.006	
Gender										
Male	40(48.8)	8(9.8)	46(56.1)	2(2.4)	42(51.2)	6(7.3)	40(48.8)	8(9.8)	13(15.9)	35(42.7)
Female	14(17.1)	20(24.4)	34(41.5)	0	24(29.3)	10(12.2)	30(36.6)	4(4.9)	8(9.8)	26(31.7)
P-value	0.000		0.34		0.053		0.38		0.46	

Table 3.

Association between CD markers and lymphoma types (n=82)

CD marker / Type of lymphoma	CD3		CD5		CD10		CD19		CD20	
	- n (%)	+ n (%)	- n (%)	+ n (%)	- n (%)	+ n (%)	- n (%)	+ n (%)	- n (%)	+ n (%)
HL	8(9.8)	0	8(9.8)	0	8(9.8)	0	6(7.3)	2(2.4)	5(6.1)	3(3.7)
NHL	46(56.1)	28(34.1)	72(87.8)	2(2.4)	58(70.7)	16(19.5)	64(78.0)	10(12.2)	16(19.5)	58(70.7)
Total	54(65.9)	28(34.1)	80(97.6)	2(2.4)	66(80.5)	16(19.5)	70(85.4)	12(14.6)	21(25.6)	61(74.4)
P-value	0.003		0.97		0.024		0.029		0.000	

Table 4.

Type of lymphoma and CD markers (n=82)

CD marker / Type of lymphoma	CD3		CD5		CD10		CD19		CD20	
	- n (%)	+ n (%)	- n (%)	+ n (%)	- n (%)	+ n (%)	- n (%)	+ n (%)	- n (%)	+ n (%)
DSBCL	1(1.2)	0	1(1.2)	0	1(1.2)	0	1(1.2)	0	0	1(1.2)
HL	8(9.8)	0	8(9.8)	0	8(9.8)	0	6(7.3)	2(2.4)	5(6.1)	3(3.7)
TCL	0	6(7.3)	6(7.3)	0	6(7.3)	0	6(7.3)	0	6(7.3)	0
BCL	8(9.8)	4(4.9)	12(14.6)	0	6(7.3)	6(7.3)	6(7.3)	6(7.3)	6(7.3)	6(7.3)
MCL	2(2.4)	0	2(2.4)	0	2(2.4)	0	2(2.4)	0	0	2(2.4)
DLBCL	29(35.4)	12(14.6)	39(47.6)	2(2.4)	35(42.7)	6(7.3)	37(45.1)	4(4.9)	2(2.4)	39(47.6)
BL	4(4.9)	4(4.9)	8(9.8)	0	4(4.9)	4(4.9)	8(9.8)	0	0	8(9.8)
FL	0	2(2.4)	2(2.4)	0	2(2.4)	0	2(2.4)	0	0	2(2.4)
ALCL	2(2.4)	0	2(2.4)	0	2(2.4)	0	2(2.4)	0	2(2.4)	0
Total	54(65.9)	28(34.1)	80(97.6(	2(2.4)	66(80.5)	16(19.5)	70(85.4)	12(14.6)	21(25.6)	61(74.4)
P-value	0.029		0.81		0.16		0.33		0.024	

DSBCL- diffuse small B cell lymphoma; TCL- T cell lymphoma; BCL - B cell lymphoma; MCL - mantle cell lymphoma; DLBCL- diffuse large B cell lymphoma; BL- Burkitt lymphoma, FL- follicular lymphoma, ALCL - anaplastic large cell lymphoma

lineage-specific markers and molecular studies.[9] Although CD3 expression in B-cell lymphoma can be a diagnostic challenge, lineage identity can be confirmed using a larger panel of immunohistochemical stains, as the CD3 expression is frequently an isolated aberrancy.

About half of all documented instances of CD3-positive DLBCL are EBV-associated large B-cell lymphoma, with plasmablastic or plasmacytic differentiation. In addition to abnormal CD3 expression, EBV-positive DLBCL frequently exhibits down-regulation of B-cell antigens, consistent with plasmablastic or plasmacytic differentiation.[11,12]

In the current study, CD5 was found to be a non-significant marker in the detection of lymphoma subtypes or even to have an association with age groups or gender, implying that CD5 in Sudanese lymphoma patients is not beneficial as a lymphoma marker. The findings in this study are consistent with previous research that found that CD5 expression abnormalities have been documented in 5%–10% of diffuse large B-cell lymphomas (DLBCLs). In the WHO classification (2008) of haematolymphoid neoplasms, CD5+ DLBCL was classified as an aggressive immunophenotypic subtype of DLBCL; however, it was removed from the list of DLBCL subgroups in the revised classification (2016). However, there is considerable debate about the clinical importance of CD5 expression, and many experts continue to believe that this subpopulation has an exceptionally poor prognosis with frequent therapy failure.[13]

The initial stage in immunophenotypic evaluation is determining if the majority cell population is B-cells, T-cells, or neither. Usually, three markers are utilized for this initial classification: CD20, CD3, and CD45. CD20 is the most consistently positive B-cell marker, CD3 is the most consistently positive T-cell marker, and CD45 is a bone marrow-derived leukocyte marker. T-cell processes are commonly CD3+, CD20- and CD45+. Normally, B-cell processes are CD3-, CD20+, and CD45+. Processes in which the invading leukocytes are either T-cells or B-cells, such as leukemia cutis, are often CD3-, CD20- and CD45+.[14] The current study found that the two main types of lymphoma, HL and NHL, have a significant association with CD3, CD10, and CD19, and a highly significant association with CD20, implying that these CD markers are crucial for general classification and diagnosis of lymphoma.[14]

This study demonstrated that the most important CD markers in this research are CD3 and CD20. CD20 has a significant association with lymphoma subtypes, as previously known, and CD20 is expressed on the surface of all B-cells (except early pro-B-cells and plasma cells). The MS4A1 gene on chromosome 11q12.2 encodes the human CD20 molecule. It is involved in B-cell development, maturation, and activation. When bound by a CD20 antibody, the CD20 molecule remains on the membrane of B-cells without dissociation or internalization. CD20 expression varies between lymphoma subtypes.[15,16]

In conclusion, the identification of CD markers not only improves the understanding of pathogenetic mechanisms in lymphomas, but also allows refining classification, improving diagnostic accuracy, and stratifying the outcomes of lymphoma patients, especially when combined with morphological classification. The CD20 marker is the most consistent and useful marker for differentiating lymphoma subtypes.

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Competing Interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

Oncology

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Pattern of Malignant Tumors in Najran, Saudi Arabia: A 5-year Retrospective Study

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Abstract

Background: The relative frequency of malignant tumors has been reported to have an association with age, gender, and location. The current study is a retrospective study to assess the pattern and relative frequency of different malignant tumors in Najran, Saudi Arabia.

Methods and Results: All patients from both genders were included in the study from June 2014 to May 2019, and data were retrieved from the records of confirmed cancer cases at the departments of pathology. For 5 years, a total of 763 cases [325(42.6%) men and 438(57.4%) women] and 37 types of malignant tumors were diagnosed in Najran. According to the histopathological diagnosis, carcinomas were the most frequent tumors (n=564, 73.9%). According to the affected organ/body system, tumors of the gastrointestinal system were the commonest malignancy, observed in 156(20.4%) of the patients (91 men and 65 women). Finally, the chi-square test revealed that the frequency of malignant tumors climbed as age increased (P=0.0005).

***Conclusion*:** The relative frequency of several cancers in Najran showed that the most common cancers in both genders are in the following order: gastrointestinal, thyroid, breast, skin and soft tissue cancers, and lymphoma. In addition, women are more affected than men, and increasing age is a risk factor to develop a malignancy.(**International Journal of Biomedicine. 2021;11(4):498-504.**)

Key Words: tumor • gender • risk factor • Najran

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Introduction

Malignant tumors are considered a leading cause of death globally and second to cardiovascular diseases in developing countries. The GLOBOCAN-database report showed that 9.6 million deaths were due to malignancy in 2018, and an estimated 18.1 million people acquired diseases caused by malignancy—cancers. The most commonly reported cancers worldwide are lung, prostate, colorectal, stomach, and liver in men; and breast, colorectal, lung, cervical, and thyroid cancers in women.[(1)] Global Cancer Statistics 2020 shows that an estimated 19.3 million new cancer cases and almost 10 million cancer deaths occurred in 2020. Female breast cancer was the most commonly diagnosed cancer, with an estimated 2.3 million new cases (11.7%), followed by lung (11.4%), colorectal (10.0%), prostate (7.3%), and stomach (5.6%) cancers.[(2)]

Both developed and developing countries have a high burden of cancer in their healthcare systems; however,

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differences exist regarding the types of cancers in several regions of the world.[3] Several factors are responsible for increasing the burden of cancer, such as population growth and aging, in parallel with other environmental, socio-economic, and cultural factors. These factors are more common in rapidly growing economies, where the cause of cancer shifted from cancers related to poverty and infections to cancers associated with lifestyle modifications.[1,4] Saudi Arabia has been undergoing extraordinary economic development since the early 1970s, which has had a deep impact on environmental factors such as quality of air, water, food consumption styles, overall lifestyle, and industrial conditions. Thus, modernization has modified the patterns of genetic and environmental risk factors responsible for cancer development.[5] The epidemiological pattern of cancer in Saudi Arabia is unique to that of Western countries. According to the Saudi Cancer Registry and GLOBCAN-2020, colorectal cancer, non-Hodgkin lymphoma, and leukemia were the most common types in males,[2,6] which is in contrast to the European and North American regions, where the solid cancers are more frequent.[7,8]

In the modern era, it is becoming possible to detect cancer and perform reliable diagnosis. This is due to the availability of modern medical facilities and research, and people are getting more conscious and aware of their health. Additionally, one of the important fields for researchers to study is the different types and frequencies of each type of cancer, which may help in taking proper public health measures for the prevention, diagnosis, and treatment of cancer. Saudi Arabia is a vast country with many regions of variable climatic and cultural backgrounds. Therefore, the frequency of several cancer types differs from one place to another,[7] and the actual incidence rate can only be assessed from population-based registries of the country.

Najran is a populous city of Saudi Arabia with more than 500 new cancer cases reported in the last 5 years. It was also reported that among cancer cases in Najran, breast cancer was the most common (42%), second - liver cancer (22%), and third - leukemia (17%).[9] The present paper is a retrospective study based on the records of pathology departments at all hospitals in Najran. This study was conducted to investigate the relative frequency of different malignant tumors in the region, which may provide a balanced estimate of the incidence of different cancers and give a solid background for further studies for the sake of improving and developing the diagnostic and therapeutic tools.

Methods

A retrospective study was conducted after getting approval from the ethical committee at the College of Medicine, Najran University. All patients from both genders were included in the study from June 2014 to May 2019, and data were retrieved from the records of confirmed cancer cases by histopathology and cytopathology at the departments of pathology in King Khalid Hospital, Najran General Hospital, Maternity & Child Hospital, and Najran University Hospital. King Khalid Hospital is a reference hospital and the main hospital in the region to date. It is well equipped and provides all major medical and surgical facilities. The clinical laboratory has facilities for all routine and specialized tests. Its radiology department is the best in the region and equipped with modern machinery for ultrasonography, mammography, and CT scanning. Similarly, the pathology laboratory is modern and operational to perform frozen sections, routine stains, and sophisticated immunoperoxidase studies.

The information collected on a data sheet included file number, age, gender, type of tissue, malignancy, and histology. Data were carefully entered in an excel file and analyzed, and statistics were performed using GraphPad Prism 6. Baseline characteristics were summarized as frequencies and percentages for categorical variables. Group comparisons with respect to categorical variables are performed using chi-square tests We used the Chi-square test to compare observed data.A probability value of $P<0.05$ was considered statistically significant.

This study was approved by the Ethical Committee at the College of Medicine, Najran University.

Results

For 5 years, a total of 763 cases [325(42.6%) men and 438(57.4%) women] and 37 types of malignant tumors were diagnosed in Najran. The mean age of all patients was 52.6 years; the mean age of men and women was 53.5 and 51.7 years, respectively.

According to the histopathological diagnosis (Figure 1), carcinomas were the most frequent tumors (n=564, 73.9%) – 203(36%) cases in men and 361(64%) cases in women. Lymphomas were the second most frequent tumor (n=83, 10.9%) – 51(61.4%) cases in men and 32(38.6%) in women.

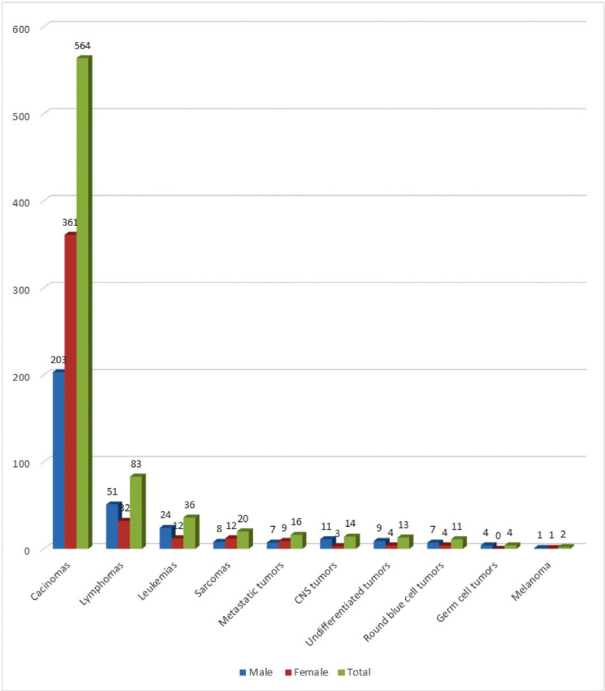

Fig. 1. *Most frequent tumors in men and in women according to the histopathological diagnosis.*

Leukemia ranked third (n=36, 4.7%), and men [24(66.7%)] were affected more than women [12(33.3%)]. These diseases were followed by sarcomas [20(2.6%)], metastatic tumors [16(2.1%)], CNS tumors [14(1.8%)], undifferentiated tumors [13(1.7%)], and round blue cell tumors [11(1.4%)]. Germ cell tumors were exclusively in men [4(0.5%)], and the least common tumor was melanoma [2(0.3%)], which was diagnosed in one man and one woman.

According to the affected organ/body system (Table 1), tumors of the gastrointestinal system were the commonest malignancy, observed in 156(20.4%) of the patients (91 men and 65 women). The thyroid gland was the second most affected organ – in 130(17%) patients (35 men and 95 women). The breast tumors were at the third position (16.3%) and exclusively in women (n=124). Skin and soft tissue tumors accounted for 8.5% (29 men and 36 women). Tumors of lymphoid tissues were at the fifth position at 7.7% (34 men and 25 women). The genital tract tumors were at the sixth position (41 women and 14 men). This was followed by bone marrow tumors [42(5.5%)], tumors of the urinary system [40(5.2%)], oral cavity [25(3.3%)], respiratory system [(22(2.9%)], hepatobiliary system and pancreas [22(2.9%)], CNS [15(2%)], and bone [8(1%)]. Noteworthy, all the last observations were more common in men than women (Table 1).

Table 1.

Distribution of different malignant tumors according to the affected organ/body system

Type of tumor	Total	%	Male	%	Female	%
Tumors of gastrointestinal tract	156.0	20.4	91.0	58.3	65.0	41.7
Thyroid tumors	130.0	17.0	35.0	26.9	95.0	73.1
Breast tumors	124.0	16.3	0.0	0.0	124.0	100.0
Skin and soft tissue tumors	65.0	8.5	29.0	44.6	36.0	55.4
Lymphoid tumors	59.0	7.7	34.0	57.6	25.0	42.4
Tumors of genital tract	55.0	7.2	14.0	25.5	41.0	74.5
Bone marrow tumors	42.0	5.5	27.0	64.3	15.0	35.7
Urinary system	40.0	5.2	32.0	80.0	8.0	20.0
Tumors of oral cavity	25.0	3.3	20.0	80.0	5.0	20.0
Tumors of respiratory system	22.0	2.9	13.0	59.1	9.0	40.9
Tumors of hepatobiliary system and pancreas	22.0	2.9	14.0	63.6	8.0	36.4
CNS tumors	15.0	2.0	12.0	80.0	3.0	20.0
Bone tumors	8.0	1.0	4.0	50.0	4.0	50.0
Total	763.0		325.0		438.0	

According to gender (Table 2), the most frequent tumors in men were found in the following order: tumors of gastrointestinal tract [91(28%)], thyroid tumors [35(10.8%)], lymphoid tumors [34(10.5%)], tumors of the urinary system [32(9.8%)], skin and soft tissue tumors [29(8.9%)], bone marrow tumors [27(8.3%)], tumors of the oral cavity [20(6.2%)], tumors of the genital tract [14(4.3%)], tumors of hepatobiliary system and pancreas [14(4.3%)], tumors of the respiratory system [13(4%)], CNS tumors [12(3.7%)], and finally bone tumors [4(1.2%)].

The most frequent tumors in women (Table 2) were found in the following order: breast tumors [124(28.3%)], thyroid tumors [95(21.7%)], tumors of gastrointestinal tract (GIT) [65(14.8%)], tumors of genital tract [41(9.4%)], tumors of skin and soft tissues [36(8.2%)], lymphoid tumors [25(5.7%)], bone marrow tumors [15(3.4%)]; tumors of the respiratory system [9(2.1%)], tumors of the urinary system [8(1.8%)], tumors of hepatobiliary system and pancreas [8(1.8%)], tumors of the oral cavity [5(1.1%)], tumors of the CNS [4(0.9%)], and bone tumors [3(0.7%)].

Table 2.

Common malignant tumors according to gender

Type of tumor	Total	Male	%	Type of tumor	Total	Female	%
Tumors of GIT	156.0	91.0	28.0	Breast tumors	124.0	124.0	28.3
Thyroid tumors	130.0	35.0	10.8	Thyroid tumors	130.0	95.0	21.7
Lymphoid tumors	59.0	34.0	10.5	Tumors of GIT	156.0	65.0	14.8
Urinary system tumors	40.0	32.0	9.8	Tumors of genital tract	55.0	41.0	9.4
Skin and soft tissue tumors	65.0	29.0	8.9	Skin and soft tissue tumors	65.0	36.0	8.2
Bone marrow tumors	42.0	27.0	8.3	Lymphoid tumors	59.0	25.0	5.7
Tumors of oral cavity	25.0	20.0	6.2	Bone marrow tumors	42.0	15.0	3.4
Tumors of genital tract	55.0	14.0	4.3	Tumors of respiratory system	22.0	9.0	2.1
Tumors of hepatobiliary system and pancreas	22.0	14.0	4.3	Urinary system tumors	40.0	8.0	1.8
Tumors of respiratory system	22.0	13.0	4.0	Tumors of hepatobiliary system and pancreas	22.0	8.0	1.8
CNS tumors	15.0	12.0	3.7	Tumors of oral cavity	25.0	5.0	1.1
Bone tumors	8.0	4.0	1.2	Bone tumors	8.0	4.0	0.9
Breast tumors	124.0	0.0	0.0	CNS tumors	15.0	3.0	0.7

According to the subtype of tumors (Table 3), the most common carcinomas were adenocarcinoma [30.7% (n=173: M=86, F=87)], representing 42.2% of carcinomas in men (the most common carcinoma subtype in men), and 24.2% in women. Papillary thyroid carcinoma was the second most common carcinoma [22.2% (n=125: M=33, F=92)], with 25.6% of carcinomas in women and 16.2% in men. In-situ and invasive ductal carcinoma of the breast were the third most common carcinomas (21.1%, n=119) with no registered cases in men and the most common carcinomas in women (33.1%).

Table 3.

Distribution of different malignant tumors in males and females according to the subtypes of tumors

Type of tumor							
Carcinomas		N	%	Male	%	Female	%
	Adenocarcinoma	173	30.7	86	42.2	87	24.2
	Thyroid papillary carcinoma	125	22.2	33	16.2	92	25.6
	In-situ and invasive ductal carcinoma of breast	119	21.1	0	0	119	33.1
	Squamous cell carcinoma	63	11.2	34	16.7	29	8.1
	Basal cell carcinoma	25	4.4	10	4.9	15	4.2
	Transitional cell carcinoma	25	4.4	22	10.8	3	0.8
	Renal cell carcinoma	14	2.5	9	4.4	5	1.4
	Hepatocellular carcinoma	6	1.1	5	2.5	1	0.3
	Thyroid follicular carcinoma	5	0.9	2	1	3	0.8
	In-situ and invasive lobular carcinoma of breast	4	0.7	0	0	4	1.1
	Carcinosarcoma	3	0.5	0	0	3	0.8
	Small cell carcinoma	2	0.4	2	1	0	0
Total		564		203		361	
Sarcoma		N	%	Male	%	Female	%
	Leiomyosarcoma	6	30	1	12.5	5	41.7
	Osteosarcoma	4	20	2	25	2	16.7
	Dermatofibrosarcoma	3	15	1	12.5	2	16.7
	Ewing sarcoma	2	10	1	12.5	1	8.3
	Rhabdomyosarcoma	2	10	1	12.5	1	8.3
	Kaposi Sarcoma	1	5	1	12.5	0	0
	Angiosarcoma	1	5	1	12.5	0	0
	Liposarcoma	1	5	0	0	1	8.3
Total		20		8		12	
Leukemias		N	%	Male	%	Female	%
	Acute myelogenous leukemia	12	33.3	9	37.5	3	25
	Multiple myeloma	12	33.3	7	29.2	5	41.7
	Myeloproliferative neoplasm	7	19.4	4	16.7	3	25
	Chronic myelogenous leukemia	3	8.3	3	12.5	0	0
	Myelodysplastic syndrome	2	5.6	1	4.2	1	8.3
Total		36		24		12	
Lymphomas		N	%	Male	%	Female	%
	Non-Hodgkin's lymphoma	48	57.8	34	66.7	14	43.8
	Hodgkin's lymphoma	35	42.2	17	33.3	18	56.3
Total		83		51		32	
Round blue cell tumors		N	%	Male	%	Female	%
	Neuroendocrine tumor	8	72.7	7	100	1	25
	Small round blue cell tumor	3	27.3	0	0	3	75
Total		11		7		4	
Germ cell tumors		N	%	Male	%	Female	%
	Germ cell tumor	3	75	3	75	0	0
	Seminoma	1	25	1	25	0	0
Total		4		4		0	
CNS tumors		N	%	Male	%	Female	%
	Glioblastoma multiforme NOS	6	42.9	5	45.5	1	33.3
	Malignant meningioma	4	28.6	2	18.2	2	66.7
	Oligodendroglioma NOS	2	14.3	2	18.2	0	0
	Glioma NOS	1	7.1	1	9.1	0	0
	Astrocytoma NOS	1	7.1	1	9.1	0	0
Total		14		11		3	

Squamous cell carcinoma was at the fourth position [11.2% (n=63: M=34, F=29)], accounting for 16.7% of carcinomas in men and 8.1% in women. Basal cell carcinoma and transitional cell carcinoma were the fifth most common carcinoma subtypes – 4.4% (n=25) for each subtype. Other subtypes of carcinoma were found in the following order: renal cell carcinoma (2.5%), hepatocellular carcinoma (1.1%), thyroid follicular carcinoma (0.9%), in-situ and invasive lobular carcinoma of the breast (0.7%), carcinosarcoma (0.5%), and small cell carcinoma (0.4%).

Regarding sarcoma subtypes (Table 3), leiomyosarcoma was the most common [30% (n=6: M=1, F=5)], constituting 41.7% of sarcomas in women and 12.5% in men. After that, the following sarcoma subtypes were in the following order: osteosarcoma [20% (n=4: M=2, F=2)], dermatofibrosarcoma [15% (n=3: M=1, F=2)], Ewing sarcoma [10% (n=2: M=1, F=1)], rhabdomyosarcoma [10% (n=2: M=1, F=1)], Kaposi sarcoma 95% [n=1: (M=1, F=0)], angiosarcoma [5% (n=1: M=1, F=0)], and liposarcoma [5%: (n=1: M=0, F=1)]. When it comes to leukemia subtypes, acute myelogenous leukemia [33% (n=12: M=9, F=3)] and multiple myeloma [33% (n=12: M=7, F=5)] were the most common ones. Myeloproliferative neoplasm was the second most common leukemia subtype [19.4% (n=7: M=4, F=3)]. The last two leukemia subtypes were chronic myelogenous leukemia [8.3% (n=3: M=3, F=0)] and myelodysplastic syndrome [5.6% (n=2: M=1, F=1)].

The most common lymphoma subtype (Table 3) was non-Hodgkin's lymphoma [57.8% (n=48: M=34, F=14)] and the second most common lymphoma subtype was Hodgkin's lymphoma [42.2% (n=35: M=17, F=18)]. Regarding the round blue cell tumors, neuroendocrine tumor was the most common [72.7% (n=8: M=7, F=1)], followed by the small round blue cell tumors [26.3% (n=3: M=0, F=3)]. Germ cell tumors were reported only in men [3(75%)], and there was only one case of seminoma, comprising 25% of the germ cell tumor subtypes.

Of the CNS tumor subtypes (Table 3), glioblastoma multiforme was the most common [42.9% (n=6: M=5, F=1)] and malignant meningioma was the second most common [28.6% (n=4: M=2, F=2)]. Oligodendroglioma not otherwise specified (NOS) was diagnosed in 2(14.3%) men while glioma NOS (7.1%) and astrocytoma NOS (7.1%) were found in one case each.

According to age, the patients were categorized into five groups (Table 4): Group 1 (1-15 years), Group 2 (16-30 years), Group 3 (31-45 years), Group 4 (46-60 years), and Group 5 (over 60 years). The most affected age group was Group 5, representing 31.8% of cases (n=243); Group 4 formed 30.1% of the cases (n=230). Group 3 was the third (25.4%) most affected age group (n=194), then the Group 2, which constituted 11.5% of all cases (n=88). The least affected age group was Group 1, which made up only 1.0% of cases (n=8). Most of the carcinomas(n=183), sarcomas(n=7), metastatic tumors(n=6), and round blue cell tumors (n=5) were diagnosed in Group 4. Meanwhile, most of lymphomas(n=27), leukemias(n=18), undifferentiated tumors(n=5), and the only two cases of melanoma, were in Group 5. The majority of the CNS tumors were diagnosed in Groups 4 (n=6) and 5 (n=6). There were only four cases of germ cell tumors, diagnosed in Group 2 and 3, equally. Finally, only eight cases (1.0%) were discovered in Group 1; those cases were carcinomas (n=4), lymphomas (n=3), and only one case of sarcomas. Finally, the chi-square test revealed that the frequency of malignant tumors climbed as age increased (P=0.0005).

Table 4.
Distribution of different malignant tumors according to age groups

Type of tumor	Age group (year)					
	Group 1	Group 2	Group 3	Group 4	Group 5	Total
	1-15	16-30	31-45	46-60	Over 60	
Carcinomas	4	49	150	183	178	564
Lymphomas	3	19	21	13	27	83
Leukemias	0	4	8	6	18	36
Sarcomas	1	6	5	7	1	20
Metastatic tumors	0	3	2	6	5	16
CNS tumors	0	2	0	6	6	14
Undifferentiated tumors	0	2	2	4	5	13
Round blue cell tumors	0	1	4	5	1	11
Germ cell tumors	0	2	2	0	0	4
Melanoma	0	0	0	0	2	2
Total	8	88	194	230	243	763
%	1.0	11.5	25.4	30.1	31.8	

Discussion

A few previous studies were conducted in Saudi Arabia to report the relative frequency of different cancers. However, some of these studies are old now, and others were conducted in a single referral hospital or institution, which may have several limitations. Accordingly, this study was planned to include all patients from different hospitals in Najran. Another important aspect is the referral bias[10] since patients with cancers are usually referred to specialized and tertiary centers due to the unavailability of certain services such as radiotherapy for a certain type of cancer and sometimes for the sake of advanced surgical intervention. Hence, true figures might be underrepresented sometimes at the general hospitals or overestimated at the specialized-tertiary centers. For the same reason, it was speculated that the actual figures might be higher than what the current study reported. However, since the incidence rate of cancer in the whole population is not available, the current study has revealed important conclusions about malignancies in Najran. Another advantage of the current study is the involvement of all cases in different age groups and in both males and females.

In this study, there were a total of 763 confirmed cases of malignant tumors. According to the histopathological diagnosis, carcinomas were the most frequent tumors, and they affected females more than males. The common subtype of

carcinomas was adenocarcinoma (30.7%), representing 42.2% of carcinomas in men (the most common carcinoma subtype in men), and 24.2% in women. Moreover, most of the diagnosed adenocarcinomas in men (28%) affected the gastrointestinal system. These observations are consistent with the Saudi Cancer Registry report and GLOBCAN-2020 report, which revealed that gastrointestinal cancer was the most common one, and Najran was at the top of the list of regions that have the highest rate of gastrointestinal cancer.[2,11] However, in a study conducted in the North of Saudi Arabia, stomach cancer and gastrointestinal tumors were among the common cancers, with 9.6% frequency, but not the most common compared to breast cancer (24.7%) and leukemia (18.7%).[12]

Although papillary thyroid carcinoma was the second common subtype of carcinomas (22.2%) in both men and women; in-situ and invasive ductal carcinoma of the breast were the most common carcinomas in females (33.1%). The last observation is in agreement with different studies during the last three decades that showed breast cancer is the most common cancer type and accounted for 24%-29% of all female cancers.[2,6,13,14] In the same context, a review article that considered all cities of Saudi Arabia stated that the crude frequency of breast cancer was 15.9% from 2001 to 2014. Furthermore, the highly frequent morphology for this cancer was infiltrating duct carcinoma (78.7%), and then lobular carcinoma.[7] These results regarding breast cancer are found not only in Saudi Arabia since it has been reported as the most common type of women`s cancer in 140 of 184 countries.[15]

The frequency of papillary thyroid carcinoma indicated that women are affected with a higher frequency than men with an approximate ratio of 3:1. A previous study from all regions of Saudi Arabia showed that the relative frequency of thyroid cancer was 3.7% for both genders and 5.6% for women.[16] Another study was conducted in Riyad Armed Forces Hospital in 1994, and the results were similar to the present study; thyroid cancer was quite high in Riyadh with a case fatality rate of 5.0%, and the female to male ratio was 2.4:1.[14] Furthermore, the percentage of thyroid cancer among total cancer cases has increased by 1.7% from 2001 to 2014, making thyroid cancer the second most common cancer in women in Saudi Arabia.[7] Thyroid cancer is a rare tumor worldwide, but thyroid cancer frequency in Saudi Arabia has always been surprisingly high.[17] The associated risk factor may include industrialization and deficiency of iodine in vast desert regions of Saudi Arabia. In the GLOBCAN-2020 report, thyroid malignancies were the second most common in women, accounting for 14.3%, but in men – the fourth most common cancer, representing 6.2%.[13]

Oral cancer is the 10th most common cancer in the world and the third most common cancer in Saudi Arabia.[18] In the present study, the relative frequency of oral cavity cancer, mainly squamous cell carcinoma, was 3.3% in Najran. The main cause of this cancer may be regarded as chewing smokeless tobacco-like substances called Shamma and Quat in Najran. Another study from Saudi Arabia also showed a high relative frequency of oral cavity cancer for the same reason, and the majority of the patients presented with lesions in the tongue.[14]

According to GLOBOCAN-2020, lung cancer has a worldwide prevalence of 11.4%, making it the second most commonly diagnosed cancer in the world among all other types.[2] In the present study, the frequency of malignant tumors of the respiratory system is extremely lower (2.9%) than that of the other countries, which may be due to the use of smokeless tobacco. Interestingly, although the Saudi Cancer Registry in 2014 reported a low incidence rate of lung cancer in Saudi Arabia, as compared to other countries, still it was high and ranked fourth.[19] Moreover, the same conclusion was reported in the GLOBCAN-2020 report.[2,13]

Lymphomas (10.9%) were the second common frequent tumor after carcinomas, and leukemia was in the third place (4.7%). Both lymphomas and leukemia were more frequent in males than in females. Our results are in agreement with studies that used data from the Saudi Cancer Registry up to 2014 and reported that the incidence of lymphoma was 8.4%.[6,7] However, the most common lymphoma subtype in the current study was non-Hodgkin's lymphoma, which is in agreement with GLOBCAN-2020 report,[2] while it was reported that Hodgkin lymphoma is relatively more frequent in Saudi Arabia than in countries in the West, and it might be due to the high consanguineous marriage rate (38.9%) in the population.[7]

Leukemia was also found to be among the frequent cancers in Najran (4.7%), and 33% of leukemias were acute myelogenous leukemia as the most common subtype. Saudi Cancer Registry ranked leukemia as the fifth most common cancer among both genders during 1999-2013, and the Precursor B-cell lymphoblastic leukemia was highly frequent, then precursor cell lymphoblastic leukemia with almost the same male to female ratio.[20]

Next to carcinomas, lymphomas, and leukemia, the following tumors came in order: sarcomas (2.6%), metastatic tumors (2.1%), CNS tumors (1.8%), undifferentiated tumors (1.7%), and round blue cell tumors (1.4%). Finally, germ cell tumors were exclusively in men, and the least common tumor was melanoma (0.3%), which was diagnosed in one man and one woman. The latter observation is supported by previous reports.[21-23]

It is noteworthy that the number of diagnosed tumors in the pediatric group (Group 1) was only eight cases (1%). The most affected age groups were Groups 5 and 4. The chi-square test revealed that the frequency of malignant tumors climbed as age increased, indicating age as a risk factor for developing cancer (P=0.0005). This supports the previously reported data that the incidence of cancer in adolescents and young adults is lower than that of the elderly in Saudi Arabia.[24]

In conclusion, the relative frequency of several cancers in Najran showed that the most common cancers in both genders are in the following order: gastrointestinal, thyroid, breast, skin and soft tissue cancers, and lymphoma. In addition, women are more affected than men, and increasing age is a risk factor to develop a malignancy.

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Competing Interests

The authors declare that they have no competing interests.

Disclaimers

The views expressed in this article are the *author's* own and do not reflect the official position of the institutions.

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ORIGINAL ARTICLE

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The Frequency Detection of Opportunistic Sexually Transmitted Infections among HIV-Infected Women Planning Pregnancy

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Abstract

The purpose of our study was to determine the frequency of detection of opportunistic sexually transmitted infections (*Chlamydia trachomatis*, *Trichomonas vaginalis*, *Ureaplasma spp*, *Human papillomavirus*) in HIV-infected women planning pregnancy.

***Methods and Results*:** We examined 31 HIV-positive Caucasian women. They sought pregnancy planning advice at the Scientific Center for Family Health and Human Reproduction Problems in Irkutsk during 2014-2015. The average age of the women was 30.9±4.5 years (20-39 years). A clinical diagnosis of HIV was made at Irkutsk Regional AIDS Center. All HIV-infected women were tested for the presence of DNA of pathogens of bacterial and viral sexually transmitted infections in the epithelium of the cervical canal. *Chlamydia trachomatis* was detected in 1(3.2%) participant, *Trichomonas vaginalis* in 1(3.2%), *Ureaplasma spp*. in 14(45.2%), and HPV in 22(71%). Co-infection of HPV and *Ureaplasma spp*. was observed in 35.5% of HIV-positive women.

Conclusion: the prevention and detection of sexually transmitted infections in HIV-infected individuals remain a public health priority and an integral component of HIV primary care.(**International Journal of Biomedicine. 2021;11(4):505-510.**)

Key Words: HIV • HPV • *Chlamydia trachomatis* • *Trichomonas vaginalis* • *Ureaplasma spp*.

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Abbreviations

AIDS, acquired immunodeficiency syndrome; **ASCUS,** atypical squamous cells of undetermined significance; **cART**, combination antiretroviral therapy; **HAART**, highly active antiretroviral therapy; **HIV**, human immunodeficiency virus; **HPV**, human papillomavirus; **HSIL**, high-grade squamous intraepithelial lesion; **LSIL**, low-grade squamous intraepithelial lesion; **OpIs**, opportunistic infections; **PCR**, polymerase chain reaction; **PID**, pelvic inflammatory disease; **STDs**, sexually transmitted diseases; **STIs**, sexually transmitted infections.

Introduction

Human immunodeficiency virus (HIV) is a critical public health problem.[1,2] By July 2020 at the global level, there were 38 million people infected with HIV; of these, more than 19 million were women.[3] Russia has the highest HIV incidence rate in Europe with more than 1.2 million HIV-infected people.[4] Among the regions of the Russian Federation, the Irkutsk Region (Eastern Siberia) has an unfavorable epidemiological situation for HIV; there were 29,200 people living with HIV by July 2021.[5] About 1204 new cases of HIV infection were detected in the 6 months of 2021. Of these, 98.3% were persons of reproductive age; the share of women among them was 44.3%.[5]

The use of combination antiretroviral therapy (cART) in HIV-infected patients led to an increase in their life expectancy, and as a result, an increased risk of

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developing concomitant diseases.[6] HIV infection progresses slowly, and its distinctive feature is the suppression of the immune system functions, which contributes to the risk of opportunistic infections (OpIs). Among them, a special place is occupied by STIs caused by bacteria and viruses since they have the most aggravating effect on reproductive health.[7,8] In addition, patients with HIV co-infection have more severe oxidative stress than HIV-monoinfected patients, which can also contribute to the development of reproductive system disorders.[2,9,10]

Sexually transmitted diseases (STDs) lead to the formation of undesirable pathological conditions in young women of reproductive age.[11,12] Most women know about STDs and AIDS and how to prevent them; however, just 40% use condoms.[13] Curable STIs, including *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis*, are associated with adverse pregnancy outcomes.[14] Therefore, it is important to conduct screening for specific STDs during pregnancy and among women planning pregnancy.[15,16]

The purpose of our study was to determine the frequency of detection of opportunistic STIs (*Chlamydia trachomatis, Trichomonas vaginalis, Ureaplasma spp, Human papillomavirus)* in HIV-infected women planning pregnancy.

Materials and Methods

We examined 31 HIV-positive Caucasian women. They sought pregnancy planning advice at the Scientific Center for Family Health and Human Reproduction Problems in Irkutsk during 2014-2015. The average age of the women was 30.9±4.5 years (20-39 years). We found that 22(71%) and 9(29%) women had secondary special education and secondary education, respectively; 7(22%) women were married, 13(42%) had an unregistered marriage, 2(6%) were single, 9(30%) were divorced; 22(71%) women had regular sex and 9(29%) women had irregular sex (less than 4 times per month); 19(61%) women used condoms, 9(29%) had interrupted sexual intercourse, 3(10%) did not use any contraception, and 20(64%) women had a permanent sexual partner - male with HIV infection.

A clinical diagnosis of HIV was made at Irkutsk Regional AIDS Center. HIV stage 4-A was found in 13(42%) women, HIV stage 4-B - in 18(58%) women. The average duration of HIV infection was 8±2.5 years; 15(48%) patients received HAART**.** HIV was mainly transmitted sexually in 80% and through the parenteral route of transmission in 20% of cases.

All HIV-infected women were tested for the presence of DNA of pathogens of bacterial and viral STDs in the epithelium of the cervical canal: *Chlamydia trachomatis, Trichomonas vaginalis, Ureaplasma species*, HPV. We used the PCR method to detect the DNA of bacteria and viruses. We used reagents manufactured by the Central Research Institute of Epidemiology ("AmpliSens *Chlamydia trachomatis*-FL," "AmpliSens *Trichomonas vaginalis*-FL," "AmpliSens *Ureaplasma spp.*-FL," "AmpliSens HPV HCR screen-Eph," AmpliSens HPV HCR genotype-EPh") and followed the manufacturer's instructions. Biological material was sampled with cervix brushes that were placed in vials with a transport medium (isotonic aqueous saline buffer solution with preservative). DNA was isolated from the obtained samples by sets of "DNA–Sorb–AM" reagents.

The PCR was done on a thermocycler "Tertsik" (Russia). The 4-channel rotor fluorimeter "ALA-1/4" (BioSan, Latvia) was used to detect end-point fluorescence after PCR runs with work reagents ("AmpliSens *Chlamydia trachomatis*-FL," "AmpliSens *Trichomonas vaginalis*-FL," "AmpliSens *Ureaplasma spp.*-FL"). A 3% agarose gel electrophoresis with ethidium bromide was used to detect DNA fragments after PCR runs with reagents ("AmpliSens HPV HCR screen-Eph," "AmpliSens HPV HCR genotype-Eph").

Statistical analysis was performed using the statistical software STATISTICA (v10.0, StatSoft, USA). Group comparisons with respect to categorical variables are performed using chi-square tests or, alternatively, Fisher's exact test when expected cell counts were less than 5; z-test was used to analyze the differences in proportions. A value of $P<0.05$ was considered significant.

The study was carried out in compliance with Ethical Principles for Medical Research Involving Human Subjects, Adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, and amended by the 64th WMA General Assembly, Fortaleza, Brazil, October 2013. The study was approved by the Ethics Committee of the Scientific Center for Family Health and Human Reproduction Problems. Written informed consent was obtained from each patient.

Results and Discussion

In our study, *Chlamydia trachomatis* was detected in 1(3.2%) participant, *Trichomonas vaginalis* in 1(3.2%), *Ureaplasma spp.* in 14(45.2%), and HPV in 22(71%). Only 6(19.4%) HIV-infected women did not have opportunistic STIs. HPV and Ureaplasma spp. were observed most often, so we assessed infection with *Ureaplasma spp*. in two groups of patients with HIV infection. In Group 1 (22 women with HPV), *Ureaplasma spp*. was detected in 50% of cases, and in Group 2 (9 women without HPV) only in 33.3% of cases, but the differences were not statistically significant.

Earlier, we published that in a group of HIV-infected women, HPV type 16 was found in 16(51.6%) and ranked the first among 12 types of HPV.[17] Only 2(12.5%) patients had HPV 16 mono-infection, and 14(87.5%) had HPV type 16 combined with other types of HPV. Most HPV-positive women were infected with several (on average three) types of HPV. Also, we compared abnormal colposcopy results in two groups of HIV patients. ASCUS was detected in all patients of Group 1 (100%) and in 6(66.7%) patients of Group 2. In Group 1, 2(9.1%) women were diagnosed with LSIL, 10(45.5%) with HSIL.

Currently, OpIs are a serious medical and social problem due to their widespread and adverse impact on the level of general and reproductive health of the population.[12] In people without abnormalities of the immune system, such infections usually do not lead to the disease, but against the background

of a reduced immune status, OpIs manifest, especially, in HIV-infected people.[18,19] OpIs are peculiar markers of immunological problems and develop as a result of the progressive course of HIV infection.[17,19,20]

A study conducted in Ethiopia showed that the overall prevalence of OpIs among the HIV-infected people was 33.6%, the majority being females - 206(53.6%).[21] Opportunistic diseases can be caused by various bacteria, viruses, and fungi, but among all their diversity, the leading role, according to the degree of influence on reproductive health, is assigned to STIs. In 2015, about 1.1 billion people had STIs other than HIV/AIDS.[22] About 500 million people have been infected with *Treponema pallidum*, *N. gonorrhoeae*, *Chlamydia trachomatis* or *Trichomonas vaginalis*; at least an additional 530 million people have genital herpes, and 290 million women have HPV.[22] STIs other than HIV resulted in 108,000 deaths in 2015.[23]

Some STIs in women often cause the serious condition of PID,[24] which can lead to infertility, chronic pain or death.[25,26] In addition to the fact that PID itself is a serious disease, STIs can also increase the risk of both acquiring and transmitting HIV by 10 times. A systematic review of the prevalence of STIs among persons living with HIV demonstrated a mean point prevalence of STI co-infection of 16.3% (SD=16.4, median=12.4%).[27] In a study conducted in Tanzania among pregnant women, it was shown that the prevalence of STIs was greater in HIV-positive than in HIV-negative women.[28]

There are more than 20 types of pathogens that can be transmitted sexually.

Chlamydia is one of the most common STIs, caused by the bacterium *Chlamydia trachomatis*. In women, symptoms may include abnormal vaginal discharge, burning during urination, and bleeding in between periods, although most women do not experience any symptoms.[29] Chlamydia can cause PID. In turn, PID can cause serious problems during pregnancy and even has the potential to cause infertility. In a study by Waung et al., the prevalence of chlamydia in HIV-infected patients was 5%,[30] which was comparable with our data - 3.2%. Chlamydia screening should be performed on all women and men upon entry into care for HIV and then annually if sexually active.[31]

Trichomoniasis is a protozoal infection caused by *Trichomonas vaginalis* that infects the genital tract of both men and women. Women may present with vaginal discharge and men may present with urethritis, but both may also be asymptomatic. *Trichomonas vaginalis* affects 3.1% of women of reproductive age.[31] However, among HIV-infected people, trichomoniasis is observed more often. Recent surveillance data obtained in a study by Meites et al. showed that the prevalence of *Trichomonas vaginalis* in HIV-infected women was 29.3%.[32] In a study by Kalichman et al.,[27] the prevalence of Trichomoniasis in HIV-infected patients was 18.8%. A study conducted in pregnant women showed that trichomoniasis was present in 18.6% of HIV-positive and 10.2% of HIV-negative women.[33] In our study, trichomoniasis was detected in 3.2% of HIV-infected women, which was less than in other studies. It should be noted that trichomoniasis is associated with adverse birth outcomes, such as premature delivery or rupture of the membranes and low birth weight; therefore, it is important to conduct a study for trichomoniasis for all women planning pregnancy.[33]

Ureaplasma infection is an infectious disease caused by a membrane parasite that occupies an intermediate position between unicellular microorganisms and viruses. *Ureaplasma spp*. has 14 known serotypes and is divided into two biovars - *Ureaplasma parvum* and *Ureaplasma urealyticum*.[34] *Ureaplasma spp* belongs to opportunistic microflora, that is, a small amount of *Ureaplasma spp*. can inhabit the urogenital tract of healthy people without causing any pathological processes. However, in cases of favorable conditions for itself, Ureaplasma multiplies and becomes the cause of the development of inflammation. Ureaplasma infection in women most often affects the vagina, uterus, fallopian tubes, and ovaries. Besides genital tract infections and infertility, Ureaplasma infection is also associated with adverse pregnancy outcomes and diseases in the newborn.[25]

In our study, *Ureaplasma spp*. was found in 45.2% of HIV-infected women; co-infection of HPV and *Ureaplasma spp*. was observed in 35.5% of cases. This is consistent with the results of other studies. In a study in Ghana, 36.5% of HIV-infected women were infected by *Ureaplasma urealyticum*, and 30.21% were co-infected with HPV.[35] In other studies conducted among HIV-positive women, *Ureaplasma urealyticum* was detected in 16.3% persons from West Africa,[36] 29.4% from Israel,[37] 41% from Italy,[38] and 51.4% from Tanzania.[39] However, among HIV-positive women from Brazil, *Ureaplasma urealyticum* was identified in only 2.1% of cases.[40] Thus, all HIV-infected pregnant women should be screened to decrease the transmission of these pathogens and to protect their own health.[41]

HPV infection is one of the most widespread STIs.[42] On average, the frequency of HPV in the world is 10%. The highest frequency level of HPV is observed in Africa – 22.1%, and Central America and Mexico – 20.4%. In North America, Europe, and Asia the frequency of HPV is 11.3%, 8.1%, and 8.0%, respectively.[43] Infection with HPV among HIV-positive women is 2 times higher than in women without HIV infection.[44] In our study, HPV infection was detected in 71% of HIV-positive women. Our data is comparable with other authors from Russia. So, in St. Petersburg, HPV was detected in 80.5% of HIV-infected women.[45] Other authors confirm data on the higher frequency of HPV infection in groups of HIV-infected women. Thus, in a study by Shipulina et al.,[46] HPV was detected in 38.7% of HIV-infected women and in only 14.8% of women without HIV. In a study performed by Marochko et al.,[47] HPV was detected in 58.2% of HIV-infected women, and in 23% of women without HIV. Thus, the results obtained in these studies showed that the prevalence of HPV infection in the group of HIV-infected women was 2.5 times higher than in the groups of women without HIV. At the same time, HPV infection in HIV-infected women has increased pathogenicity and significantly increases the risk of cervical lesions and cancer.[48] Therefore, all HIV-infected women should be screened for cervical cancer beginning within 1 year of HIV diagnosis and continue throughout life.[31]

In conclusion, it should be noted that the prevention of STIs is an important part of the care of an HIV-infected person.[31] As the overall health of persons living with HIV has improved in the last two decades, sexual health, including prevention and detection of STIs, has become an important component of HIV primary care.[49] Most women with HIV are of reproductive age.[50] Previously, it was believed that HIV-infected women should avoid pregnancy and be given the highest priority for family planning services.[51] However, modern antiretroviral drugs against HIV infections, by suppressing the amount of virus in the body, can provide a fulfilling life. Strategies for reducing STI risk include vaccination, mutual monogamy, reducing the number of sexual partners, and abstinence. Comprehensive sex education may also be useful. In this way, the prevention and detection of STIs in HIV-infected individuals remain a public health priority and an integral component of HIV primary care.

Conclusion

The principal findings of this study are that HIV-infected women of reproductive age have a high frequency of detection of such opportunistic STIs as high-risk HPV and *Ureaplasma spp*., which are detected in 71% and 45.2% of women, respectively. Co-infection of HPV and *Ureaplasma spp*. is observed in 35.5% of HIV-positive women. Since HPV and *Ureaplasma spp*. are highly pathogenic, our results reiterate the need for routine screening in HIV-infected patients for these infections.

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Competing Interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

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Microbiological Study of Vaginal Microbiota and Endometrium in Women with Chronic Endometritis

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Abstract

The research objective was to study the spectrum of the vagina and endometrium microorganisms in women with chronic endometritis (CE) in order to take adequate therapeutic measures.

Methods and Results: We did a cross-sectional study in 47 women (average age of 35.38±5.19 years) with histologically confirmed CE. The vaginal microbiota and endometrial biopsies were assessed using microbiological research methods in accordance with the medical technology "Integral assessment of the state of the vaginal microbiota." To identify the share of different types of microorganisms in the structure of the biocenosis, the coefficient of constancy of the species (C) was used.

Only 19% of patients had a titer of *Lactobacillus* spp. within the age norm, while the deficit was observed in 80% of women. Among the representatives of Enterobacteriaecae, *Escherichia coli* and *Klebsiella aerogenes* were sown, which are considered to be random species (C=11% and C=2.1%, respectively). The average titer for *E. coli* was 3.6±1.3 lg CFU/swab and for *K. aerogenes* - 2.14 lg CFU/swab. An atypical variant of *E. coli* with hemolytic properties was found in only one sample. All isolates of the genus *Staphylococcus* were also random species (C did not exceed 25%). Coagulase-negative staphylococci (CoNS) were detected in 7 patients (C=15%), while the average titer was 2.1±0.4 lg CFU/swab. *S. aureus* was isolated from only one patient at a titer of 5 lg CFU/swab. *Corynebacterium* spp. were isolated in 11% of cases (C=11% - random species), in a titer of 3.2±0.8 lg CFU/swab. *Enterococcus* spp. also belonged to random species (C=23.4%). At the same time, *E. faecalis* was inoculated in 19% of cases and *E. faecium* was sown in 4.3%, the average titer of which was 3.1±0.9 and 5 lg CFU/swab. *Streptococcus* spp. were recorded in only one case at a concentration of 5 lg CFU/swab. Fungi of the *Candida* were isolated as a random species in 8.5% of cases. The growth of microorganisms in endometrial samples was obtained only in 3 examined women with CE (6.4% of cases). The endometrial microbiota were represented only by random species, for which the C index ranged from 2.1% to 4.3%.

***Conclusion*:** The microbiological study of the microbiota of vaginal discharge showed the presence of dysbiotic disorders with a significant deficiency of lactobacilli (80%) without the dominance of representatives of the *Lactobacillus* spp. In the structure of opportunistic microflora, *Escherichia coli*, coagulase-negative staphylococcus, *Enterococcus* spp., and *E. faecalis* prevailed as random species. Representatives of the microbiota in endometrial biopsies were identified only in 6.4% of cases, and are represented by random species. (**International Journal of Biomedicine. 2021;11(4):511-514.**)

Key Words: chronic endometritis • microbiota • vagina • endometrium

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Abbreviations

CE, chronic endometritis; **CPB,** conditionally pathogenic bacteria; **CFU**, colony-forming units; **CoNS**, coagulase-negative staphylococci; **STIs,** sexually transmitted infections.

Introduction

Chronic endometritis (CE) is a clinical and morphological syndrome, in which, as a result of persistent damage to the endometrium by an infectious agent, multiple secondary morphofunctional changes occur, disrupting cyclic biotransformation and receptivity of the mucous membrane of the uterine body.[1] CE is detected in about 10% to 21%

of women of reproductive age, and its presence is associated with infertility and miscarriage.[2-10]

For a long time, the uterine cavity was considered a sterile biotope.[2] However, at present, the use of molecular genetic research methods makes it possible to identify associations of difficult-to-cultivate and uncultured microorganisms on the surface of the endometrium in women of reproductive age.[11-16] There are no concurrent views on the influence of certain groups of conditionally pathogenic bacteria (CPB) on the development of the endometrium inflammatory pathology, and this raises doubts about the advisability of prescribing antimicrobial therapy for CE.[4,17-19] For a more complete assessment of the state of the vaginal microbiota, it is necessary not only to assess the total quantitative characteristics of *Lactobacillus* spp., but also to determine the species composition and functional parameters of lactobacilli in various gynecological diseases.[20]

The research objective was to study the spectrum of the vagina and endometrium microorganisms in women with CE in order to take adequate therapeutic measures.

Materials and Methods

From 2020 to the present, we have been conducting a cross-sectional study under the auspices of the Scientific Centre for Family Health and Human Reproduction Problems. Our study included 47 women with histologically confirmed CE. The average age of the patients was 35.38±5.19 years. Inclusion criteria were the presence of histologically verified CE, reproductive age (18-45 years). Exclusion criteria were the use of antibacterial, hormonal, or immunomodulatory drugs, and the presence of STIs.

The patients were included in the study after signing a written informed consent. All women who agreed to participate answered a questionnaire survey and underwent general clinical, gynecological, and laboratory-instrumental examination. The sampling of material from the uterine cavity was made on the fourth to ninth day of the menstrual cycle (middle proliferative phase) using a pipelle biopsy of the endometrium with a disposable intrauterine probe (Taizhou Kechuang Medical Apparatus Co., Ltd, China), followed by pathological examination of the endometrial tissue for verification of CE signs.

The vaginal microbiota and endometrial biopsies were assessed using microbiological research methods in accordance with the medical technology "Integral assessment of the state of the vaginal microbiota."[21] Vaginal discharge and endometrial biopsy homogenate were inoculated on standard culture media; CFU were counted. Microbiota were identified through the use of standardized bacteriological algorithms, taking into account morphological, cultural and biochemical properties.

To identify the share of different types of microorganisms in the structure of the biocenosis, the coefficient of constancy of the species (C) was used according to the formula: $C = p \times 100 / P$, where p is the number of observations containing the studied species, P is the total number of observations. At values of C≥50%, microorganisms were considered constant; at values of 25%≤C≤50% they were considered additional; at values of C<25% they were considered random.[22]

All data have been entered into the REDCap system. Statistical processing was carried out using the STATISTICA Version 6.1 (StatSoft USA). Baseline characteristics were summarized as frequencies and percentages for categorical variables and as mean±SD for continuous variables.

The study was carried out in compliance with Ethical Principles for Medical Research Involving Human Subjects, Adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, and amended by the 64th WMA General Assembly, Fortaleza, Brazil, October 2013. The study was approved by the Ethics Committee of the Scientific Center for Family Health and Human Reproduction Problems. Written informed consent was obtained from each participant.

Results and Discussiom

According to the results of the microbiological study of vaginal discharge in women with CE, *Lactobacillus* spp. were identified as permanent representatives of the vaginal microbiota (C=79%). Moreover, only 19% of patients had a titer of *Lactobacillus* spp. within the age norm, while the deficit was observed in 80% of women. Bacterial vaginal communities dominated by *Lactobacillus* spp. have been considered for a long time a sign of vaginal health.[2,23,24] Among the representatives of the genus *Lactobacillus*, *L. crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii* dominate in the vaginal microbiota of healthy women. The prevalence of *L. crispatus* indicates the stability of the vaginal microbiota, as this species produces lactic acid, hydrogen peroxide and bacteriocins. *L. iners* is a marker of vaginal dysbiotic disorders. This species does not produce lactic acid and hydrogen peroxide, which are necessary for maintaining eubiosis and cannot provide a transition to the prevalence of *L. crispatus*.[25] Due to the structure of the genome, *L. iners* is able to quickly adapt to changing environmental conditions, switching its metabolism and using other substances, not glycogen, as food resources. Thus, under conditions of dysbiosis, *L. iners* produces cholesterol-dependent cytolysin, which destroys cell walls, and uses glycerol of destroyed cell membranes as a new food substrate. This leads to the death of other species of *Lactobacillus* spp., a decrease in the concentration of lactic acid and an increase in the pH of the vaginal environment.[26] The significance and role of *L. gasseri* and *L. jensenii* species in maintaining the normal state of the vaginal microbiota still remain controversial.[20]

Among the representatives of Enterobacteriaecae, *Escherichia coli* and *Klebsiella aerogenes* were sown, which are considered to be random species (C=11% and C=2.1%, respectively). The average titer for *E. coli* was 3.6±1.3 lg CFU/swab and for *K. aerogenes* - 2.14 lg CFU/swab. An atypical variant of *E. coli* with hemolytic properties was found in only one sample. Vaginal discharge of such intestinal biotope residents as enterobacteria confirms the translocation of microorganisms from the intestine against the background of immunodeficiency in women with chronic endometrial inflammation.[27]

All isolates of the genus *Staphylococcus* were also random species (C did not exceed 25%). Coagulase-negative

staphylococci (CoNS) were detected in 7 patients (C=15%), while the average titer was 2.1±0.4 lg CFU/swab. S. aureus was isolated from only one patient at a titer of 5 lg CFU/swab. CoNS are part of the normal vaginal microbiota; however, under certain conditions, lactobacilli are not able to suppress the growth and production of toxins by staphylococci. The increase in pH created by the violation of the microbiota ratio promotes the production of *S. aureus* toxins.[20]

Corynebacterium spp. were isolated in 11% of cases (C=11% - random species), in a titer of 3.2±0.8 lg CFU/swab. *Enterococcus* spp. also belonged to random species (C=23.4%). At the same time, *E. faecalis* was inoculated in 19% of cases and *E. faecium* was sown in 4.3%, the average titer of which was 3.1±0.9 and 5 lg CFU/swab. *Streptococcus* spp. were recorded in only one case at a concentration of 5 lg CFU/swab. Streptococci are glycogen-dependent bacteria, which ensures their successful colonization of the vaginal epithelium, thereby creating competition for lactobacilli. Despite the fact that these microorganisms are part of the normal vaginal microbiota, their presence, even as an accidental one, can contribute to the development of dysbiotic disorders. Also, changes in the composition of the vaginal microbiota can lead to the spread of opportunistic microflora in the upper genital tract and contribute to the development of infertility.[28]

Fungi of the *Candida* were isolated as a random species in 8.5% of cases. The average titer was 4.0±0.8 lg CFU/swab.

The growth of microorganisms in endometrial samples was obtained only in 3 examined women with CE (6.4% of cases). The endometrial microbiota were represented only by random species, for which the C index ranged from 2.1% to 4.3%. Moreover, *Lactobacillus* spp. titer was 4 lgCFU/swab, for *E. coli* (one isolate) - 3 lg CFU/swab, *E. faecalis* (2 isolates) - 3.5±0.7 lg CFU/swab. Only one case, *E. faecalis*, was sown both from the vagina and from endometrial samples. It is possible to assume that the presence of these types of CPB probably indicates an upward spread of the infectious process.

Conclusion

Thus, in women with CE, a microbiological study of the microbiota of vaginal discharge showed the presence of dysbiotic disorders with a significant deficiency of lactobacilli (80%) without the dominance of representatives of the *Lactobacillus* spp. In the structure of opportunistic microflora, *Escherichia coli*, coagulase-negative staphylococcus, *Enterococcus* spp., and *E. faecalis* prevailed as random species. Representatives of the microbiota in endometrial biopsies were identified only in 6.4% of cases, and are represented by random species.

Our study is a pilot. The limitation of our study is that the sample is not large enough to obtain more significant results and understand the role of changes in the microbiota of the vagina and endometrium in the development of CE.

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Conflict of Interests

The authors declare that they have no conflicts of interest.

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ORIGINAL ARTICLE

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Prevalence of Uterine Fibroids in Women in Eastern Siberia: A Cross-Sectional Study

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Abstract

The objective of this study was to determine uterine fibroids (UF) prevalence in the unselected (medically unbiased) female population in the Eastern Siberia region, Russia, and to evaluate the significant risk factors.

Methods and Results: The study included 2389 women aged from 18 to 80 yrs (mean age of 42.8±11.9 yrs). Subjects were evaluated consecutively by means of questionnaires, anthropometry, vital signs, gynecological examination, and pelvic ultrasound. We demonstrated 26.41% UF prevalence in the unselected female population from Eastern Siberia. We found that the single nodules predominate among all fibroids, with the types 3–5 and the size of either ≤1 cm or ≥4 cm as the most frequent variants. Our study confirmed that the prevalence of fibroids increases with age. The incidence of fibroids is significantly lower in women with the age at menarche of 15 years. We also have found that a BMI of more than 25 kg/m^2, more than 4 pregnancies, and late menopause are risk factors for the development of fibroids.(**International Journal of Biomedicine. 2021;11(4):515-518.**)

Key Words: uterine fibroid • epidemiology • risk factors

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Introduction

The most common neoplasms affecting women's health are uterine fibroids (UFs), which can cause significant morbidity and may adversely impact fertility.[1-10] Currently, only limited data are available concerning the prevalence and clinical issues of UFs in Russia.[11,12]

The most relevant studies on the risk of UFs consider age, race, endocrine disruptors, obesity, and lifestyle, as well as genetic, reproductive, and hormonal factors, as significant predictors. Nevertheless, more studies are needed to further understand UF biology and risk factors to clarify the etiopathogenesis of this disease. [1,13-15]

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The objective of this study was to determine UF prevalence in the unselected (medically unbiased) female population in the Eastern Siberia region, Russia, and to evaluate the significant risk factors.

The primary study aim was to collect data concerning the prevalence of UFs in the female population of the Eastern Siberia region, and the type, quantity, and size of the myomas. The secondary study aim was to analyze the association between the patient's age and the type, quantity, and size of UFs, as well as between the frequency of the UFs and BMI, parity, age at menarche, menopause presence and age.

Material and Methods

We performed a multicenter, institution-based, cross-sectional study in Irkutsk Region and the Burjat Republic (Russia) during 2016-2019. The study included 2389 women aged from 18 to 80 yrs (mean age of 42.8±11.9 yrs). All

women were stratified by age groups (≤25, 26–30, 31–35, 36–40, 41–45, 46–50, 51–55, 56–60, 61–65, and ≥66 years). The participants for this study were recruited during an obligatory, early, medical employment assessment.

Exclusion criteria were as follows: the subject is not willing to comply with all study procedures and be available for the duration of the study; anything that would place the individual at increased risk or preclude the individual's full compliance with or completion of the study; unwillingness to participate or difficulty understanding the consent processes or the study objectives and requirements; history of hysterectomy, bilateral oophorectomy, endometrial ablation, or uterine artery embolization.

Subjects were evaluated consecutively by means of questionnaires, anthropometry, vital signs, gynecological examination, and pelvic ultrasound. Pelvic ultrasound was performed by 3 experienced specialists with the appropriate intra/inter-observer variations, using Mindray M7 (MINDRAY, China), a transvaginal probe (5,0-8,0 MHz) or transabdominal probe (2,5-5,0 MHz).

The following parameters were evaluated: (1) gynecological history (menarche, parity, live-births, abortions, missed abortions, use of hormonal or other contraceptives, gynecological operations); (2) height and weight, body mass index (BMI); (3) ultrasound measurement of the myomas: location, number, size. The UF volume was determined by the following formula: (length × width × height × 0.457)/1000.

We used the initial data derived from the electronic data capture system REDCap.[16] The data were processed in a manner enabling the evaluation utilizing the statistical software STATISTICA version 12 (StatSoft, USA). The frequencies of categorical variables were compared using Pearson's chi-squared test or Fisher's exact test, when appropriate. A probability value of $P<0.05$ was considered statistically significant.

The study was approved by the Ethics Committee of the Scientific Center for Family Health and Human Reproduction Problems. Written informed consent was obtained from each participant.

Results and Discussion

The main results of our study population are shown in Table 1. As presented, UFs were found in 631(26.41 %) of the total 2389 women included in the present study.

In the majority of patients with fibroids (57.9%), ultrasound examination revealed only one single nodule, 19.1% had 2 fibroids, 11.5% had 3, and the same number (11.5%) had 4 or more (Fig.1).

In the present study, fibroids were classified according to their type.[17] The nodules types 3–5 predominated in all age groups (76.2%), subserous fibroids (types 6–7) were found in 41.4% and only 6.3% of cases had submucous (types 0–2) (Fig.2). For the analysis of the size of myomas, the maximum node in the case of multiple myomas was taken into account. An equal number (35% each) of all fibroids was <1cm and >4cm; 14.5% of the nodules were from 1 cm to 2 cm, 7.9% from 2 cm to 3 cm, and 7.1%, from 3 cm to 4 cm (Fig.3).

Table 1.
Characteristics of the study population

	Frequency N=2389	Percentage	Fibroids		P-value	OR, 95% CI
			Yes N=631	No N=1758		
Age, years	2389/0*				<0.001	
≤25	161	6.74	2 (1.24)	159	<0.001	0.032 [0.008;0.129]
26-30	235	9.84	5 (2.13)	230	<0.001	0.053 [0.022;0.129]
31-35	349	14.61	33 (9.46)	316	0.001	0.252 [0.174;0.365]
36-40	361	15.11	73 (20.22)	288	0.004	0.668 [0.507;0.879]
41-45	331	13.86	104 (31.42)	227	0.026	1.331 [1.034;1.713]
46-50	292	12.22	124 (42.47)	168	<0.001	2.315 [1.798;2.980]
51-55	246	10.30	113 (45.93)	133	<0.001	2.665 [2.036;3.490]
56-60	242	10.13	101 (41.74)	141	<0.001	2.185 [1.662;2.873]
61-65	103	4.31	46 (44.66)	57	<0.001	2.347 [1.573;3.499]
≥66	69	2.89	30 (43.48)	39	0.001	2.200 [1.355;3.573]
BMI	2386/3*				<0.001	
<25	940	39.40	163 (17.34)	777	<0.001	0.441 [0.361;0.540]
25-29.9	772	32.36	236 (30.57)	536	0.001	1.368 [1.130;1.655]
30-34.9	428	17.94	145 (33.88)	283	<0.001	1.560 [1.246;1.954]
≥35	246	10.31	85 (34.55)	161	0.002	1.549 [1.170;2.050]
Age at menarche, years	2385/4*				0.045	
≤11	206	8.64	55 (26.70)	151	0.934	1.014 [0.734;1.400]
12	474	19.87	134 (28.27)	340	0.317	1.121 [0.896;1.404]
13	661	27.71	193 (29.20)	468	0.060	1.211 [0.992;1.478]
14	689	28.89	172 (24.96)	517	0.292	0.897 [0.732 1.098]
15	208	8.72	38 (18.27)	170	0.005	0.597 [0.415;0.859]
≥16	147	6.16	39 (26.53)	108	0.983	1.004 [0.688;1.465]
Menopause	2375/14*					
yes	669	28.17	281(42.00)	388	<0.001	2.836 [2.337;3.442]
no	1706	71.83	347(20.34)	1359		
Age at menopause, years	667/2*				0.010	
≤44	85	12.74	19 (22.35)	66	<0.001	0.352 [0.206;0.601]
45-50	197	29.54	74 (37.56)	123	0.122	0.764 [0.543;1.075]
50-52	235	35.23	102 (43.40)	133	0.623	1.084 [0.786;1.495]
53-55	107	16.04	60 (56.07)	47	0.001	1.958 [1.290;2.974]
≥56	43	6.45	26 (60.47)	17	0.012	2.213 [1.177;4.163]
Parity	2386/3*					
yes	2058	86.25	575 (27.94)	1483	<0.001	1.925 [1.418;2.612]
no	328	13.75	55 (16.77)	273		
Parity/ number	2058				<0.001	
1	321	15.60	62 (19.31)	259	0.002	0.631 [0.470;0.846]
2	363	17.64	91 (25.07)	272	0.531	0.921 [0.712;1.191]
3	346	16.81	79 (22.83)	267	0.103	0.800 [0.611;1.047]
4	281	13.65	68 (24.20)	213	0.372	0.877 [0.656;1.171]
5	242	11.76	83 (34.30)	159	0.003	1.524 [1.149;2.022]
6	173	8.41	60 (34.68)	113	0.010	1.531 [1.103;2.123]
7	108	5.25	39 (36.11)	69	0.019	1.613 [1.078;2.416]
≥8	224	10.88	93 (41.52)	131	<0.001	2.148 [1.619;2.851]
Live-births	2058					
yes	1978	96.11	561 (28.36)	1417	0.034	0.536 [0.299;0.962]
no	80	3.89	14 (17.50)	66		
Live-births/ number	1978					
1	682	34.48	197 (28.89)	485	0.708	1.040 [0.847;1.277]
2	907	45.85	275 (30.32)	632	0,075	1.194 [0.982;1.453]
3	333	16.84	75 (22.52)	258	0.009	0.693 [0.525;0.916]
≥4	56	2.83	14 (25.00)	42	0.571	0.838 [0.454;1.547]
abortions	2051/7*					
yes	1397	68.11	448 (32.07)	949	<0.001	1.978 [1.580;2.476]
no	654	31.89	126 (19.27)	528		
abortions/ number	1397				<0.001	
1	434	31.07	111 (25.58)	323	0.001	0.638 [0.496;0.822]
2	361	25.84	102 (28.25)	259	0.071	0.785 [0.604;1.022]
3	225	16.11	79 (35.11)	146	0.286	1.178 [0.872;1.590]
4	126	9.02	46 (36.51)	80	0.263	1.243 [0.849;1.820]
>5	251	17.97	110 (43.82)	141	<0.001	1.865 [1.410;2.467]
Missed abortion	2049/10*					
yes	60	2.93	16 (26.67)	44	0.814	0.933 [0.522;1.666]
no	1989	97.07	558 (28.05)	1431		

** - number of missing data*

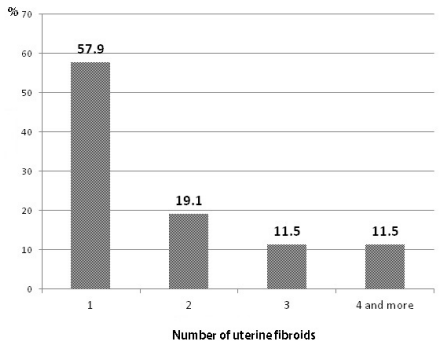

Fig. 1. *Number of UFs per patient (n = 631).*

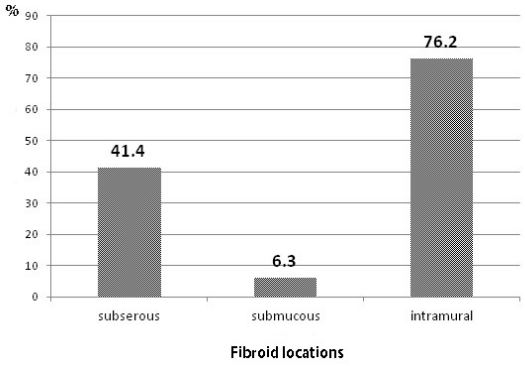

Fig. 2. *Fibroid locations in all age groups (n = 782).*

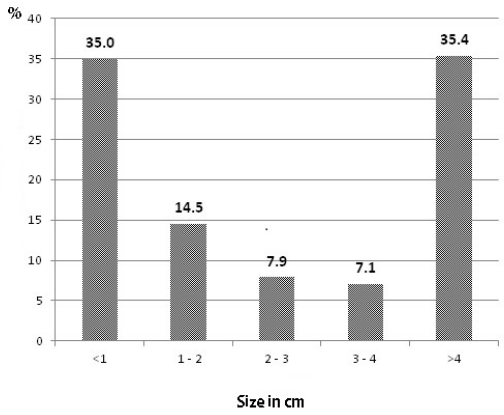

Fig. 3. *Size of UFs in all age groups (n = 631).*

With increasing age, the prevalence of uterine myomas rose from 1.24% (18–25 years) to 45.93% (51–55 years). The prevalence of fibroids at the age of 56–60 years was comparable to those at the age of 46–50 years and remained high in the older age groups of 61–65 years and over. The risk estimates were as follows: the age of ≤40 years was associated with a significant decrease in UF risk, whereas the age ≥41 years – with an increased risk (Table 1).

By the age of 36–40 years, the number of multiple nodules increased statistically significantly and then remained stable, while the proportion of women with single UF tended to decrease by 41–45 years (Fig.4).

When analyzing the impact of BMI on the UF prevalence, we found that OR for UF was lower in slim women and higher in women with BMI≥25 kg/m^2.

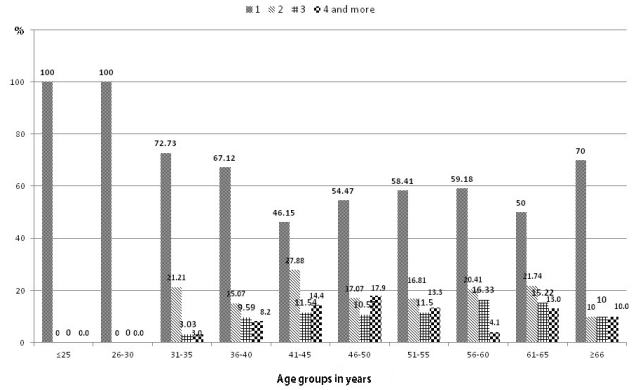

Fig. 4. *Number of UFs in study age groups.*

The relationship between age at menarche and the UF prevalence has been discussed by different authors.[13] In our study, the mean onset of menarche was between 9 years and 20 years (13.3±1.4 years). In most cases, no associations were found between the age at the first menstrual cycle and the age of fibroid diagnosis. In almost all age groups, except the group with the age of menarche equal to 15 years, the frequency of fibroids was similar, and ranged from 24.96% to 29.20% for age at menarche of 14 and 13 years, respectively. However, for the menarche age of 15 years, the incidence of fibroids is significantly lower (18.27%).

Menopausal women have a 2 times higher prevalence of fibroids than women of reproductive age, and the presence of menopause is a significant risk factor for fibroids (OR=2.8 [2.3;3.4]). The risk for UF rose significantly in women with the age of menopause at ≥53 years, whereas early menopause (≤44 years) was shown as a protective factor. However, both of these effects may be influenced by age as a possible impact factor concerning UF prevalence.

In our study, over 86% had at least one pregnancy. The prevalence of fibroids among this category of study participants was 27.94%. If there were more than 4 pregnancies, the prevalence of fibroids tended to increase from 34.30% to 41.52%. In our study, we found that among women with a history of abortions, the frequency of fibroids was significantly higher, as compared to that in women without a history of abortions (32% vs. 19.27%).

In conclusion, we demonstrated 26.41% UF prevalence in the unselected female population from Eastern Siberia. We found that the single nodules predominate among all fibroids, with the types 3–5 and the size of either ≤1 cm or ≥4 cm as the most frequent variants. Our study confirmed that the prevalence of fibroids increases with age. The incidence of fibroids is significantly lower in women with the age at menarche of 15 years. We also have found that a BMI of more than 25 kg/m^2, more than 4 pregnancies, and late menopause are risk factors for the development of fibroids.

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Competing Interests

The authors declare that they have no competing interests.

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The Effect of Low Alcohol Consumption during Pregnancy on the Metabolic Processes of Women and Their Alcohol-Exposed Babies

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Abstract

The aim of this study was to examine the effect of low or very low amounts of alcohol consumption on the LPO-AOD systems of pregnant women and their infants after birth, and the effect of that exposure on infant, growth, health, and development.

Methods and Results: A sample of 201 pregnant women (mother-child dyads) was recruited for the study. Pregnant women were categorized into three groups according to the amount of alcohol they consume: 1) non-drinking, 2) very low drinking, and 3) low drinking. Small amounts of alcohol consumption caused dysfunction of the LPO-AOD system and the development of OS in women, and had negative effects on infants.

The biomarkers of potentially harmful LPO, such as thiobarbituric acid reactants (TBARs), were higher in very low and low drinking mothers. The activity of the AOD system was lower among mothers who drank alcohol. Alcohol consumption decreased levels of retinol, SOD activity, GSH, and GR activity. Higher rates of pathological conditions, delayed development, and slower growth were observed among infants who were prenatally exposed to alcohol.

Conclusion: Identification and preventive interventions are needed for pregnant women who use alcohol in any amount. (**International Journal of Biomedicine. 2021;11(4):519-525.**)

Key Words: lipid peroxidation • antioxidant defense • pregnant women • infant • alcohol

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Abbreviations

AOD, antioxidant defense; **CDs**, conjugated dienes; **GPO**, glutathione peroxidase; **GSH**, reduced glutathione; **GSSG**, oxidized glutathione; **GST**, glutathione-S-transferase; **GR**, glutathione reductase; **LPO**, lipid peroxidation; **OS**, oxidative stress; **SOD**, superoxide dismutase; **TBARs**, thiobarbituric acid reactants.

Introduction

The period of organogenesis in embryonic development is critical because alcohol exposure during this time has long-term effects. One of the reasons for the teratogenic effect of alcohol is its rapid penetration through the placenta and blood-brain barrier, where alcohol can have effects that are more serious than those of many other substances, including exposure to maternal smoking and illicit drug use.[1-5]

There is evidence that the fetus is exposed to the same alcohol level as the mother. The effects of alcohol on the fetus are extremely destructive and damaging. The severity of the damage depends on many factors: maternal age, social environment, amount of alcohol consumed, frequency of alcohol consumption, duration of maternal alcohol abuse, and other factors.[6-17]

The biotransformation of ethanol is a typical reaction of toxification, which yields metabolites that are more toxic than

ethanol.[4,17,18] As shown in numerous studies, even small doses of ethanol and its metabolites, especially acetaldehyde, lead to congenital malformations of the child, directly or indirectly, through disruption of maternal biochemical mechanisms, and result in fetal alcohol syndrome.[1,4,8]

It is known that an imbalance in the LPO-AOD system leads to the development of OS, which is accompanied by a decrease in the body's resistance to adverse responses to the external and internal environment.[19-26] This imbalance can be assessed by measuring oxidized and reduced forms of glutathione, the activity of enzymes affecting glutathione metabolism (SOD, GR, GPO, and GST), substances that protect from OS (α-tocopherol and retinol), and products that result from oxidation (CDs and TBARS).

Few studies have reported the relationship between alcohol consumption and activities of components of the LPO-AOD system among mothers and infants, and how prenatal alcohol exposure affects infant growth, health, and development by the age of one year.

The aim of this study was to examine the effect of low or very low amounts of alcohol consumption on the LPO-AOD systems of pregnant women and their infants after birth, and the effect of that exposure on infant, growth, health, and development.

Materials and Methods

Study participants

Pregnant women consecutively enrolled in prenatal care at the Irkutsk Regional Perinatal Center (IRPC) between June 2012 and June 2014 were recruited to participate. Those women who initiated prenatal care at ≤10 weeks of gestation were eligible to participate. Since the study included mother-child dyads, if pregnancy was terminated or did not result in a live birth, the participant was removed from the study.

A total of 204 pregnant women were approached about participating in the study. Two refused to participate, resulting in a sample of 202 pregnant women (mother-child dyads) recruited for the study. All participants were admitted for delivery to IRPC. One delivery resulted in the neonate's death due to severe fetal distress and hypoxia within 24 hours of delivery, resulting in a final sample of 201 mother-child dyads.

Procedures and data collection

At the first prenatal visit, the women underwent standard medical procedures, including a prenatal care medical exam, were prescribed folic acid, and were provided with information about a healthy lifestyle during pregnancy (risks of alcohol use, tobacco use, and secondhand smoke). All participants completed a face-to-face interview with a study investigator and a brief intervention recommended for alcohol use.[27] At 30-32 weeks of gestation, maternal laboratory tests were completed. At delivery, a neonatologist examined neonates and samples of cord blood were collected. One-to-three days after delivery, mothers completed a face-to-face interview with the study investigator. Follow-up data on infant development were collected from child medical charts.

Collected data included maternal medical history and birth outcomes. Infant medical information noted by a developmental pediatrician at 6 and 12 months was extracted from the hospital's patient records. Characteristics of interest included socio-demographic characteristics, such as maternal age at the time of the birth and marital status; maternal medical history, including number of previous deliveries (parity) and chronic conditions; infectious diseases, such as HIV; pregnancy complications and mode of delivery (vaginal delivery or cesarean section); sex of the neonate, and; the neonate's birth weight, gestational age at birth, and evaluation (APGAR Score) at birth by a neonatologist.

Two face-to-face structured interviews were conducted with each participant by a study investigator. The interviews utilized measures developed by the Prevent FAS Research Group.[28] The first 20- to 30-minute interview was conducted at the time of the first prenatal visit to collect socio-demographic information and to assess women's drug use, alcohol use, smoking, and other risk factors prior to pregnancy. The second interview was conducted on the first to the third day after delivery to collect information about alcohol use and smoking during pregnancy, and took approximately 20 minutes to complete. The postnatal timing of reports was selected due to recent research data suggesting that pregnant women's reports about their consumption during pregnancy are more affected by biases than are their retrospective, after-pregnancy reports about drinking during pregnancy.[29] Several additional measures were implemented to further improve accuracy and elicit truthful self-reports. All interviews were conducted in a clinical setting in private. Participants were alcohol-free when interviewed and were reassured of confidentiality. Questions were worded clearly.

Alcohol exposure

Participants were asked to provide detailed reports about their alcohol consumption during 40 weeks of pregnancy. Following guidance from Sobell & Sobell (2003), participants were provided with a calendar and asked to memorize personal events that might be associated with alcohol use, such as holidays and birthdays. Although self-reports about alcohol consumption may be affected by desirability bias, they are considered to be reasonably accurate among volunteers recruited in health care settings when confidentiality is protected.[30-33]

The concept of "one drink" as a unit of consumption was not familiar to women in Siberia. Therefore, similar to beverage- and container-specific approaches that have been used in Russia[8] and other countries,[34,35] a beverage-specific approach was used to determine standardized alcohol content and volume of alcohol consumption. Participants were provided with a card that showed pictures of alcoholic beverages and containers that are common in Russia, and were asked about the type of beverage, type of container, and a number of containers consumed during each month of pregnancy. This information was converted into ethanol volume. The total amounts of alcohol consumed during the first half (1-20 weeks of gestation) and the second half (21-40 weeks) of pregnancy were calculated for each participant and utilized in data analysis. For reporting clarity, these data were then converted to U.S. standard drink units (i.e., 14 grams of pure alcohol).[36] The women were categorized as having very

low alcohol consumption if they drank less than 750 ml of beer, dry wine, or champagne, and were categorized as having low alcohol consumption if they consumed between 750 ml and 3850 ml of beer, dry wine, or champagne. The control group was women who never consumed alcohol during pregnancy.

Parameters of LPO-AOD system

Between 30 and 32 weeks gestation, fasting blood was drawn to measure indicators of LPO-AOD system function among pregnant women.

The SOD, GR, GST, and GPO activity was measured in erythrocytes using a commercially available kit. Fluorometry was used to measure GSH and GSSG levels in hemolysate.[37] Retinol and α-tocopherol levels were detected in plasma by fluorometry.[38] The concentration of CDs absorbance was detected on plasma heptane extracts at 232 nm.[39] The coefficient of molar absorption ($K=2.2 \cdot 10^5\ M^{-1}\ S^{-1}$) for conversation of absorption units of μmol/L was used. TBARs levels were detected by fluorometry in μmol/L.[40]

Umbilical cord blood samples were obtained from 140(69.7%) of 201 infants; 66(70.1%) of 93 infants in Group 1; 53(70.1%) of 75 infants in Group 2, and; 21(63.6%) of 33 infants in Group 3. We examined the same markers of the LPO-AOD system in infants as we did in their mothers.

Child developmental evaluation

Child development data, including infant height, weight, head and chest circumferences, congenital malformations, rickets, and developmental milestones, were extracted from the children's medical charts. Following routine medical procedures and standard protocols, neonates were evaluated at birth (n=201) by their clinic's neonatologists. At 6 and 12 months of age, infants (n=201) were evaluated by a pediatrician and a pediatric neurologist at the infant's local pediatric clinic. Anemia was diagnosed based on blood hemoglobin (≤12 g/dL for newborns; <10 g/dL for infants aged between 6 and 12 months). Rickets was diagnosed by clinical evaluation by a pediatrician. Congenital malformations included congenital heart disease, retroperitoneal tumor, adrenal gland tumor, malformations, fetal alcohol syndrome spectrum disorders, and cerebral palsy. Pediatric neurologists evaluated all children in the first 2-4 days of life and at 6 and 12 months to evaluate psychomotor development according to developmental milestones.

Statistical processing was carried out using the STATISTICA Version 10 (StatSoft, USA). The normality of distribution of continuous variables was tested by Shapiro-Wilk test. For descriptive analysis, results are presented as mean±standard deviation (SD), median (Me) (interquartile range [IQR]). Multiple comparisons were performed with one-way ANOVA and Tukey's HSD Post-hoc Test. Kruskal-Wallis test was used to compare means of 3 groups of variables not normally distributed. Categorical variables were analyzed using the chi-square test with the Yates' correction. A value of P<0.05 was considered significant.

The study was carried out in compliance with Ethical Principles for Medical Research Involving Human Subjects, Adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, and amended by the 64th WMA General Assembly, Fortaleza, Brazil, October 2013. The study was approved by the Ethics Committee of the Scientific Center for Family Health and Human Reproduction Problems. Written informed consent was obtained from each patient.

Results

Clinical characteristics of pregnant women and infants at birth

We analyzed the clinical characteristics of the surveyed women and their newborns (Table 1). A total of 201 pregnant women aged 15 to 42 (29.1±6.0) enrolled in the study at their initiation of prenatal care at 7-to-10 weeks (7.1±0.5) of gestation. There were no differences in age, marital status, or other socio-demographic characteristics between women who reported consuming alcohol and those who reported no alcohol use during pregnancy. Among participants, 69.2% were married and 41.8% had a higher education than a school diploma or secondary education. A total of 37.3% and 34.8% reported smoking prior to pregnancy and during pregnancy. None were positive for HIV.

Table 1.
Characteristics of the surveyed pregnant women

Variable	Group 1 (n=93)	Group 2 (n=75)	Group 3 (n=33)	Statistics
Age, yrs	29.7±0.6	28.3±0.7	29.0±1.0	P=0.0000 P_{1-2}=0.0000 P^{1-3}=0.0000 P_{2-3}=0.0000
Weight, kg	68.2±1.2	69.1±0.3	68.8±0.4	P=0.0000 P_{1-2}=0.0000 P^{1-3}=0.0018 P_{2-3}=0.2144
Height, cm	166.3±0.7	166.1±0.3	166.7±0.6	P=0.0000 P_{1-2}=0.0610 P^{1-3}=0.0017 P_{2-3}=0.0000

In this sample (n=201), 93 participants reported no alcohol consumption during pregnancy (Group 1), and 108 reported consuming alcohol in some amount. Based on the total amount of alcohol reported during pregnancy, participants were categorized into three groups: no alcohol use during pregnancy (Group 1, n=93), very low (≤2 drinks) alcohol use (Group 2, n=75), and low (>2 drinks) alcohol use (Group 3, n=33). The average amounts of alcohol consumed were 1.28±0.48 drinks for Group 2 and 5.91±2.67 drinks for Group 3.

With regard to tobacco use, 15.1% of Group 1, 42.7% of Group 2, and 72.7% of Group 3 women reported smoking daily or occasionally, less than 10 and not more than 20 cigarettes a day, during pregnancy. Women with higher alcohol consumption during pregnancy smoked more often than did those with lower alcohol consumption during pregnancy (Group 1 vs. Group 2, P=0.005; Group 1 vs. Group 3, P=0.001; Group 2 vs. Group 3. P=0.04).

There were no significant differences between the heights, weights, and head and chest circumferences of the infants in any of the three groups (Table 2).

Table 2.

Characteristics of infants who were prenatally exposed to alcohol and infants with no alcohol exposure.

Variable	Group 1 (n=93)	Group 2 (n=75)	Group 3 (n=33)	Statistics
	M±SD; Me (IQR)			
Height, cm	50.9±2.9	50.1±4.3	49.7±4.4	P=0.1937 P_{1-2}=0.3532 P^{1-3}=0.2541 P_{2-3}=0.8652
Weight, kg	3.3(2.9-3.7)	3.2(2.8-3.7)	3.04(2.6-3.6)	P>0.05
Head circumference, cm	33.9±1.5	33.1±3.7	33.6±2.2	P=0.1494 P_{1-2}=0.1259 P^{1-3}=0.8405 P_{2-3}=0.6357
Chest circumference, cm	32.7±2.6	31.7±3.6	31.9±3.1	P=0.0973 P_{1-2}=0.0952 P^{1-3}=0.4090 P_{2-3}=0.9484

Development of infants at 6 and 12 months of age

Congenital malformations were significantly more common in children with low prenatal alcohol exposure at both ages, compared with the children with very low exposure (Table 3). Rickets was also significantly more common at 12 months in Group 3 children than in Group 2 children. Hypoxic-ischemic central nervous system damage in newborns was noted in 2.2% of infants from Group 1, 8% of infants from Group 2, and 24.4% of infants from Group 3.

Table 3.

Congenital malformations and other pathological conditions of infants at 6 and 12 months of age

Variable		Group 1 (n=93) n (%)	Group 2 (n=75) n (%)	Group 3 (n=33) n (%)	Statistics
6 months	Congenital malformations	4(4.3)	7(9.3)	10(30.3)	P=0.0001
	Rickets	18(19.4)	16(21.3)	7(21.2)	P=0.9436
	Anemia	22(23.7)	18(24.0)	8(24.2)	P=0.9970
12 months	Congenital malformations	4(4.3)	7(9.3)	10(30.3)	P=0.0001
	Rickets	10(10.8)	8(10.7)	7(21.2)	P=0.2477
	Anemia	12(12.9)	14(18.7)	8(24.2)	P=0.2881

Children in Groups 2 and 3 had significantly lower weights, heights, and head circumferences at 6 and 12 months of age, and significantly larger fontanelles at 12 months of age than did children in Group 1 (Table 4). Infants with very low exposure had significantly lower heights and weights than did infants with no exposure at 6 and 12 months.

In analyzing the physical and psychomotor development of children 6 and 12 months of age, those with low prenatal alcohol exposure were more likely to have psychomotor development delayed for their age. Thus, they had significantly more motor development delay at 6 and 12 months than did children with no exposure (Group 1 vs. Group3 at 6 months. *P*=0.023; Group 1 vs. Group 3 at 12 months, *P*=0.015).

Table 4.

Anthropometric data in the infants at the first year of life

Variable		Group 1 (n=93)	Group 2 (n=75)	Group 3 (n=33)	Statistics
		M±SD; Me (IQR)			
6 months	Weight, kg	7.7±0.5	6.9±0.5	6.8±0.6	P=0.0000 P_{1-2}=0.0000 P^{1-3}=0.0000 P_{2-3}=0.6251
	Height, cm	70.2±1.1	67.8±1.1	67.6±1.6	P=0.0000 P_{1-2}=0.0000 P^{1-3}=0.0000 P_{2-3}=0.7027
	Head circumference, cm	43.7±1.4	45.9±0.42	40.7±0.9	P=0.0000 P_{1-2}=0.0000 P^{1-3}=0.0000 P_{2-3}=0.0000
	Large fontanelle, cm	1.5(1.5-1.5)	1.5(1.5-1.5)	1.5(1.5-1.5)	NaN
12 months	Weight, kg	12.3±0.9	10.3±1.2	10.0±1.0	P=0.0000 P_{1-2}=0.0000 P^{1-3}=0.0000 P_{2-3}=0.3510
	Height, cm	81.2±2.0	78.1±1.8	77.6±2.0	P=0.0000 P_{1-2}=0.0000 P^{1-3}=0.0000 P_{2-3}=0.4301
	Head circumference, cm	48.8±0.9	51.4±0.5	44.5±1.3	P=0.0000 P_{1-2}=0.0000 P^{1-3}=0.0000 P_{2-3}=0.0000
	Large fontanelle, cm	1.0(0.5-1.0)	1.0(0.5-1.0)	1.0(0.5-1.0)	NaN

Characteristics of LPO-AOD process

The level of CDs was significantly lower in women of Group 2 than in women of Group 1 (*P*=0.003). The concentrations of TBARs in Groups 2 and 3 were significantly higher than those in Group 1 (Table 5).

There were no statistically significant differences in α-tocopherol levels between the groups. However, retinol concentrations were significantly lower in both drinking groups than in the non-drinking group. Dysregulation in the glutathione system was indicated by significantly lower GSH values and GR activity, and higher GSSG and GST levels, among women of Group 1 than in women of Group 3.

Table 6 indicates the levels of markers related to the LPO-AOD system in infants who were prenatally exposed to alcohol and infants with no alcohol exposure. CDs were statistically significantly higher in infants from Groups 2 and 3 than in infants from Group 1. In the AOD system, the values of α-tocopherol and SOD activity were significantly lower in Groups 2 and 3 than in Group 1. In terms of changes in the glutathione system, there were significantly lower levels of

GSH in Group 2 and GR in Groups 2 and 3 than in Group 1. Lower GR in Groups 2 and 3 was accompanied by a decrease in the activity of the other glutathione-dependent enzymes, GST and GP.

Table 5.

LPO-AOD system components in the pregnant women of the study groups

Variable	Group 1 (n=93)	Group 2 (n=75)	Group 3 (n=33)	Statistics
	M±SD; Me (IQR)			
CDs, µmol/L	2.12 (1.61-2.68)	1.19 (1.26-2.50)	1.84 (1.50-2.40)	P_{1-2}=0.003 P^{1-3}=0.088 P_{2--3}=0.989
TBARs, µmol/L	0.83 (0.61-1.12)	1.19 (0.7-1.57)	1.18 (0.87-1.54)	P_{1-2}<0.001 P^{1-3}<0.001 P_{2--3}=0.951
α-tocopherol, µmol/L	6.49 (5.48-7.77)	5.46 (4.10-8.07)	6.33 (4.77-9.88)	P_{1-2}=0.243 P^{1-3}=0.167 P_{2--3}=0.053
Retinol, µmol/L	0.75 (0.59-0.92)	0.58 (0.431-0.70)	0.57 (0.48-0.67)	P_{1-2}<0.001 P^{1-3}<0.001 P_{2--3}=0.769
SOD, U/mgHb	1.69±0.15	1.63±0.19	1.64±0.19	P=0.0658 P_{1-2}=0.0670 P^{1-3}=0.3275 P_{2--3}=0.9585
GSH, mmol/L	2.11 (1.85-2.41)	1.96 (1.81-2.24)	2.09 (1.72-2.39)	P_{1-2}=0.014 P^{1-3}=0.412 P_{2--3}=0.334
GSSG, mmol/L	1.81 (1.5-2.13)	1.92 (1.75-2.26)	1.97 (1.78-2.25)	P_{1-2}=0.023 P^{1-3}=0.025 P_{2--3}=0.717
GR, µmol/min/L	951 (752-1168)	764 (459-965)	855 (541-1097)	P_{1-2}<0.001 P^{1-3}=0.068 P_{2--3}=0.434
GST, µmol/min/L	809 (678-951)	966 (852-1450)	852 (457-1423)	P_{1-2}<0.001 P^{1-3}=0.068 P_{2--3}=0.434
GPO, µmol/min/L	279 (216-316)	256 (197-308)	275 (206-321)	P_{1-2}=0.260 P^{1-3}=0.895 P_{2--3}=0.364

Table 6.

LPO-AOD components in infants who were prenatally exposed to alcohol and infants with no alcohol exposure

Variable	Group 1 (n=66)	Group 2 (n=53)	Group 3 (n=21)	Statistics
	M±SD; Me (IQR)			
CDs, µmol/L	1.34 (1.12-1.67)	1.73 (0.98-2.13)	1.68 (1.52-2.13)	P_{1-2}=0.006 P^{1-3}<0.001 P_{2--3}=0.396
TBARs, µmol/L	1.32 (1.06-1.61)	1.35 (1.16-1.64)	1.31 (0.90-1.44)	P_{1-2}=0.976 P^{1-3}=0.764 P_{2--3}=0.771
α-tocopherol, µmol/L	6.98 (5.84-8.17)	5.88 (5.22-7.05)	6.08 (5.21-7.15)	P_{1-2}=0.006 P^{1-3}=0.029 P_{2--3}=0.823
Retinol, µmol/L	0.41±0.10	0.43±0.11	0.42±0.10	P_{1-2}=0.385 P^{1-3}=0.876 P_{2--3}=0.460

Table 6 (continued).

LPO-AOD components in infants who were prenatally exposed to alcohol and infants with no alcohol exposure

Variable	Group 1 (n=66)	Group 2 (n=53)	Group 3 (n=21)	Statistics
	M±SD; Me (IQR)			
SOD, U/mg Hb	1.74±0.08	1.64±0.14	1.69±0.13	P_{1-2}<0.010 P^{1-3}=0.030 P_{2--3}=0.109
GSH, mmol/L	2.26±0.40	2.04±0.28	2.15±0.29	P_{1-2}=0.014 P^{1-3}=0.223 P_{2--3}=0.139
GSSG, mmol/L	1.97 (1.66-2.20)	1.96 (1.78-2.29)	1.96 (1.59-2.06)	P_{1-2}=0.781 P^{1-3}=0.197 P_{2--3}=0.151
GR, µmol/min/L	857.5 (731-985)	652 (456-851)	699 (456-1085)	P_{1-2}<0.001 P^{1-3}<0.001 P_{2--3}=0.277
GST, µmol/min/L	1244 (1056-1478)	995 (852-1431)	1133 (813-1455)	P_{1-2}=0.012 P^{1-3}=0.041 P_{2--3}=0.961
GPO, µmol/min/L	280 (231-318)	231 (199-291)	245 (233-316)	P_{1-2}=0.002 P^{1-3}=0.031 P_{2--3}=0.396

Discussion

Our findings indicate that even a small amount of alcohol consumed during pregnancy can cause serious metabolic changes in mothers and their newborn babies. In particular, alcohol exposure leads to an imbalance of redox exchange and dysfunction of the LPO-AOD system. The concentrations of TBARs were 60.4% higher in the very low and low drinking groups than in the control group of non-drinkers. The increase of TBARs indicates the decline of AOD that can be described as the OS development.[22]

Retinol concentration decreased in both drinking groups, compared with the control group**.** This reduction may result in a corresponding reduction in the antioxidant effect of retinol. The role of retinol as a prohormone may also be affected by oxidation, prompting its development into retinoic acid, a true hormone, which is involved in the regulation of gene expression.[21,41] Further, retinoic acid has morphogenetic action, and its deficiency can lead to fetal malformations.

SOD activity was significantly lower in very low drinkers than in non-drinkers. Even a small reduction in SOD activity is an important signal of the metabolic shift in the direction of prevailing pro-oxidant processes, because of the high content of the enzyme in the red blood cell.[21] Women who drank very low amounts of alcohol had lower GSH and GR values, and higher GSSG and GST values, than did those who drank no alcohol. The reduced form of glutathione participates in neutralizing oxidants and in transporting substances across membranes, and has an antitoxic effect.[24,42-44] The decreased GSH and GR levels in women who consumed alcoholic beverages had a negative effect on their health.[45] Reduced activity of GR in women who consumed even very low doses of alcohol, in turn, indicates the protective function of the enzyme, conversion of GSSG to GSH.

Infants prenatally exposed to small and moderate amounts of alcohol had higher rates of pathological conditions, smaller heights and weights, larger fontanelles, and smaller head circumferences at birth and 6 and 12 months of age, as well as delayed psychomotor development than those with no alcohol exposure. In terms of the LPO-AOD system of newborns, changes similar to those that occurred in their mothers were found, especially for SOD, GSH, and GR. The decrease in GSH concentration and activity of the enzymes involved in its metabolism, which were found in infants of Group 2, has a negative effect on the balance in the LPO-AOD system. It is reported that α-tocopherol activated γ-glutamyl cysteine synthetase, which leads to regulation of glutathione biosynthesis. In this case, α-tocopherol has an indirect effect on the AOD system.[1]

The assessments of the LPO-AOD system for women who consumed alcoholic beverages in the prenatal period indicate increased OS, even when consuming low doses. Thus, the results suggest that even a small amount of alcohol drunk by women during pregnancy can cause serious metabolic changes in the newborn body, leading to an imbalance of redox exchange and dysfunction of the LPO-AOD system.

Conclusion

The use of even small doses of alcohol during pregnancy can cause dysfunction of the LPO-AOD system and the development of OS and can have negative effects on infant growth, health, and development. Specialists and researchers in the medical and social fields must address the prevention of any amount of alcohol use in pregnant women.

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Conflict of Interests

The authors declare that they have no conflicts of interest.

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Endometrial Cytokines in Women with Reproductive Disorders

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Abstract

The purpose of this research was to study changes in endometrial cytokine concentrations in women suffering from reproductive disorders with and without chronic endometritis (CE) to justify pathogenetic treatment.

Methods and Results: The study included 100 women of reproductive age with reproductive disorders. Group 1 included 50 patients with reproductive disorders and CE; Group 2 included 50 patients with reproductive disorders and without CE. Later on, all patients were divided into the following subgroups: Sub1A (n=31), and Sub2A (n=16) with an isolated bacterial flora, Sub1B (n=19) and Sub2B (n=34) with the absence of bacterial flora. The control group consisted of 31 fertile women.

Endometrial aspiration pipe biopsy was performed on days 4-9 of the menstrual cycle (middle proliferative phase) using a disposable intrauterine probe (Taizhou Kechuang Medical Apparatus Co., Ltd, China) followed by histological examination of endometrial tissue. Laboratory diagnostics for sexually transmitted infections (STIs) was performed using the bacterial culture method. For the diagnosis of viral infection (HPV, HSV, CMV), cervical samples were studied using PCR. If STIs were detected, the patients were excluded from further research. Ultrasound examination of the pelvic organs was performed using the Aloka-5500 device with a 7MHz vaginal probe in two-dimensional visualization mode. The concentration of cytokines (IL-1β, INF-γ, TNF-α, ILs-4,6,8,10) in the endometrium was determined using the Protein Contour test systems (Saint Petersburg) and Multiskan EX ELISA Analyzer (Germany).

In both groups, reproductive disorders were accompanied by hypoprogesteronemia and relative hyperestrogenemia, significantly apparent in CE. We found a 3-fold increase in the level of tissue pro- and anti-inflammatory cytokines (IL-1β, IL-4,6,10, INF-γ), and a 4-fold increase in the level of TNF-α and IL-8 in Group 1, compared to the CG. In Group 2, we found a 1.4-fold increase in the levels of IL-1β and INF-γ, compared to the CG. In Sub1A, the levels of IL-6 and IL-8 were significantly higher than in the control group. In Sub1A, the isolated bacterial flora caused a cytokine inflammatory response characterized by a significant increase in the concentration of INF-γ and TNF-α, compared to Sub2A and Sub2B ($P<0.05$). We also found a tendency towards a decrease in the tissue levels of IL-4 in Sub1A, compared to Sub1B and Sub2B; the IL-10 level was significantly lower than in Sub2B ($P=0.0009$)

Conclusion: The results obtained in the present study showed the peculiarities of changes in cytokines at the level of endometrial tissue both in chronic inflammation of the endometrium and in its absence in women with reproductive disorders. The severity of the immune response is significantly higher in patients with CE, with the most significant change in the role of IL-10. The results obtained may be useful for the diagnosis and treatment of CE and immunological correction in women with reproductive disorders. (**International Journal of Biomedicine. 2021;11(4):526-531.**)

Key Words: reproductive disorders • chronic endometritis • cytokines • endometrium

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Abbreviations

CE, chronic endometritis; **FSH**, follicle-stimulating hormone; **IL**, interleukin; **INF**, interferon; **LH**, luteinizing hormone; **Ops**, opportunistic microbes; **PRL**, prolactin; **PID**, pelvic inflammatory disease; **STIs,** sexually transmitted infections; **TNF-α**, tumor necrosis factor alpha.

Introduction

The state of innate immunity of the uterus, the mechanisms of the response of uterine endometrial cells to the presence of bacterial flora, are ambiguous and considered to be a poorly studied area. The presence of a bacterial infection in the genital tract favors the development of pelvic inflammatory diseases, complications of pregnancy, and reproductive disorders,[1-4] which definitely indicates the interaction between reduced fertility, pelvic organs, and immune status.[5]

Regulation of the decidual process is strictly controlled by various cell structures, cytokines, and growth factors generated by various cellular constituents of the endometrium, including epithelial cells, stromal cells, local immune cells, and the vasculature.[6]

Cytokines are referred to as cellular messengers playing a key role in many biological conditions, such as immune defense and reproduction. It is known that the endometrium endothelial cells can play an active role in the innate immunity of the uterus.[7] This is because Toll-like receptors are expressed in endometrial endothelial cells, and the stimulation of endometrial endothelial cells by LPS induces a highly specific inflammatory cytokine/chemokine response characterized by the secretion of IL-6, IL-8, and G-CSF (granulocyte colony-stimulating factor).[7,8]

Studying the state of the cytokines level in endometrial tissue will not only expand diagnostic capabilities but also, thanks to the growing knowledge of immune processes, it can boost new therapeutic methods for improving the quality of endometrial tissue and increasing reproductive potential.

The purpose of this research was to study changes in endometrial cytokine concentrations in women suffering from reproductive disorders with and without chronic endometritis (CE) to justify pathogenetic treatment.

Materials and Methods

A cross-sectional study was performed. The patients (n=327) were recruited from outpatient visits in the period from 2012 to 2014. According to the results of the questioning, 223(68.2%) women were found to have infertility: among them were 125(38.2%) women with primary infertility and 98(30.0%) women with secondary infertility; 104(31.8%) women had experienced miscarriage.

The criteria for inclusion in the main group were the absence of pregnancy in regular sex life without contraception for a year or more, or miscarriage during the last year, or failure in assisted reproductive technology programs. Exclusion criteria were the presence of causes for reproductive disorders: endocrine, genetic, hemostasiological, and immunological disorders, including male infertility.

The patients were examined according to the standards of infertility examination, including questionnaires, as well as general clinical, gynecological, and laboratory instrumental examinations. Ultrasound examination of the pelvic organs was performed using the Aloka-5500 device with a 7MHz vaginal probe in two-dimensional visualization mode.

Endometrial aspiration pipe biopsy was performed on days 4-9 of the menstrual cycle (middle proliferative phase) using a disposable intrauterine probe (Taizhou Kechuang Medical Apparatus Co., Ltd, China) followed by histological examination of endometrial tissue.

According to the results of the previous stage, 100 women were finally included in the study. All women were divided into two groups: Group 1(1) included 50 patients (average age of 30.5±0.6 years)with reproductive disorders and CE; Group 2(2) included 50 patients (average age of 30.2±0.7 years) with reproductive disorders and without CE.

Later on, all patients were divided into the following subgroups: Sub1A (n=31), and Sub2A (n=16) with an isolated bacterial flora, Sub1B (n=19) and Sub2B (n=34) with the absence of bacterial flora.

The control group (CG) consisted of 31 fertile women. The criteria for inclusion in the group were a regular menstrual cycle, the absence of neuroendocrine disorders and severe somatic pathology, and a pregnancy that ended in childbirth within the last year.

Laboratory diagnostics for STIs (N. gonorrhoeae, T. vaginalis, Ur. Urealyticum, M. hominis, M. Genitalium. Chl. Trachomatis) was performed using the bacterial culture method. For the diagnosis of viral infection (HPV, HSV, CMV), cervical samples were studied using PCR. If STIs were detected, the patients were excluded from further research. Microbiological studies of the vaginal biotope were carried out in accordance with the guidelines for research methods used in clinical and diagnostic laboratories of medical and preventive institutions. The concentration of cytokines (IL-1β, INF-γ, TNF-α, ILs-4,6,8,10) in the endometrial tissue was determined using the Protein Contour test systems (Saint Petersburg) and Multiskan EX ELISA Analyzer (Germany). The percentages and absolute counts of blood lymphocytes were determined by the method of indirect immunofluorescence with monoclonal antibodies using the BD FACSCalibur flow cytometer (USA).

Statistical processing was carried out using the STATISTICA Version 10 (StatSoft, USA). The normality of distribution of continuous variables was tested by Shapiro-Wilk test. The mean (M) and standard deviation (SD) were calculated. Multiple comparisons were performed with one-way ANOVA and Tukey's HSD Post-hoc Test. Pearson's Correlation Coefficient (r) was used to determine the strength of the relationship between the two continuous variables. A value of $P<0.05$ was considered significant.

The study was carried out in compliance with Ethical Principles for Medical Research Involving Human Subjects, Adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, and amended by the 64th WMA General Assembly, Fortaleza, Brazil, October 2013. The study was approved by the Ethics Committee of the Scientific Center for Family Health and Human Reproduction Problems. Written informed consent was obtained from each patient.

Results

According to the medical history, Group 2 women, compared to CG and Group 1 women, had diseases of the

ENT organs, gastrointestinal diseases, kidney diseases, and allergic diseases significantly more often (P<0.05).

We found (Table 1) an increase in the levels of testosterone, estradiol, and a decrease in the progesterone level in Group 1, compared to the CG. In Group 2, a significant increase in the levels of PRL and testosterone, and a decrease in the progesterone level were revealed, compared to the CG. The differences between Groups 1 and 2 was characterized by an increase in the concentration of estradiol and a decrease in the level of progesterone (both hormones within the reference values) more in Group 1 than in Group 2. Thus, reproductive disorders were accompanied by hypoprogesteronemia and relative hyperestrogenemia, significantly apparent in CE.

Table 1.

The levels of pituitary hormones and sex hormones in study groups

Variable	Group 1 (n=50)	Group 2 (n=50)	Control group (n=31)	Statistics
	1	2	3	
Prolactin, mIU/L	368.9±185. 65	424.1±213.22	297.81±100.14	P=0.0541 P_{1-2}=0.4450 P_{1-3}=0.3587 P_{2-3}=0.0425
LH. mIU/ml	5.12±2.23	4.67±2.84	4.2±1.43	P=0. 2254
FSH. IU/ml	6.47±2.18	6.46±1.77	6.92±1.77	P=0.5222
Estradiol. pmol/L	419.58±186.86	354.1±225.57	276.19±157.58	P=0.0130 P_{1-2}=0.2679 P_{1-3}=0.0095 P_{2-3}=0.2404
Progesterone. nmol/Ll	39.79±31.94	43.05±19.69	74.19±13.17	P=0.0000 P_{1-2}=0.7773 P_{1-3}=0.0000 P_{2-3}=0.0000
Testosterone, pmol/L	2.0±1.06	1.98±1.07	1.41±0.88	P=0.0250 P_{1-2}=0.9948 P_{1-3}=0.0345 P_{2-3}=0.0429

We found a 3-fold increase in the level of tissue pro- and anti-inflammatory cytokines (IL-1β, IL-4,6,10, INF-γ), and a 4-fold increase in the level of TNF-α and IL-8 in Group 1, compared to the CG. In Group 2, we found a 1.4-fold increase in the levels of IL-1β and INF-γ, compared to the CG.

In Sub1A, the levels of IL-6 and IL-8 were significantly higher than in the control group. In Sub1A, the isolated bacterial flora caused a cytokine inflammatory response characterized by a significant increase in the concentration of INF-γ and TNF-α, compared to Sub2A and Sub2B (P<0.05) (Table 2). In Sub1A, we found a tendency towards a decrease in the tissue levels of IL-4 compared to Sub1B and Sub2B; the IL-10 level was significantly lower than in Sub2B (P=0.0009)

Correlation analysis revealed a direct correlation between IL-10 and IgM (r=+0.34), IgA (r=+0.35) and an inverse correlation between IL-10 and CD19+ (r=-0.30) in Group 1. In Group 2, we found an inverse correlation between phagocytosis indexes and IL-1 (r=-0.33), and a direct correlation with TNF-α (r=+0.33). At the same time, a inverse correlation was found between IL-10 and IL-1 (r=-0.41) and a direct correlation with TNF-α (r=+0.33). A strong direct connection between IgA and IgM (r=+0.77) was revealed.

Table. 2.

The levels of tissue cytokines in the study Subgroups

Cyto-kines	Sub1A n=31	Sub2A n = 16	Sub1B n=19	Sub2B n = 34	CG n=31	Statistics
	1	2	3	4	5	
IL-1β, pg/ml	64. 97 ±39.25	34. 22 ±37.72	62.85 ±43.54	44.46 ±44.49	23.64 ±3.37	P=0.0001 P_{1-2}=0.0536 P_{1-3}=0.9995 P_{1-4}=0.1628 P_{1-5}=0.0002 P_{2-3}=0.1468 P_{2-4}=0.8860 P_{2-5}=0.8794 P_{3-4}=0.4005 P_{3-5}=0.0030 P_{4-5}=0.1515
IL-4, pg/ml	22.38 ±20.12	14.38 ±12.98	38.22 ±13.71	41.54 ±81.22	13.71 ±1.93	P=0.0505 P_{1-2}=0.9747 P_{1-3}=0.7181 P_{1-4}=0.3879 P_{1-5}=0.9334 P_{2-3}=0.4850 P_{2-4}=0.2393 P_{2-5}=1.0000 P_{3-4}=0.9988 P_{3-5}=0.2996 P_{4-5}=0.0783
IL-6, pg/ml	85.32 ±39.91	83.85 ±45.6	87.00 ±71.15	100.56 ±98.13	39.53 ±3.81	P=0.0026 P_{1-2}=1..0000 P_{1-3}=1.0000 P_{1-4}=0.8613 P_{1-5}=0.0354 P_{2-3}=0.9998 P_{2-4}=0.9018 P_{2-5}=0.1476 P_{3-4}=0.9413 P_{3-5}=0.0733 P_{4-5}=0.0012
IL-8, pg/ml	92.82 ±48.24	99.55 ±102.56	112. 98 ±72.80	81.31 ±71.82	23 ±2.42	P=0.0000 P_{1-2}=0.9968 P_{1-3}=0.8032 P_{1-4}=0.9465 P_{1-5}=0.0002 P_{2-3}=0.9695 P_{2-4}=0.8718 P_{2-5}=0.0011 P_{3-4}=0.3974 P_{3-5}=0.0000 P_{4-5}=0.0024
IL-10, pg/ml	38.67 ±39.46	70.4 ±2.51	51.34 ±51.35	76.51 ±51.01	26.67 ±4.61	P=0.0000 P_{1-2}=0.0564 P_{1-3}=0.7806 P_{1-4}=0.0009 P_{1-5}=0.7237 P_{2-3}=0.5754 P_{2-4}=0.9839 P_{2-5}=0.0024 P_{3-4}=0.1454 P_{3-5}=0.1735 P_{4-5}=0.0000
INF-γ, pg/ml	100.65 ±76.29	45.33 ±70.01	73.46 ±65.33	44.50 ±50.21	25.75 ±4.24	P=0.0000 P_{1-2}=0.0168 P_{1-3}=0.4758 P_{1-4}=0.0011 P_{1-5}=0.0000 P_{2-3}=0.5930 P_{2-4}=1.0000 P_{2-5}=0.7974 P_{3-4}=0.3925 P_{3-5}=0.0375 P_{4-5}=0.6758
TNF-α, pg/ml	58.00 ±54.25	16.33 ±14.43	48.17 ±53.34	14.42 ±20.24	9.48 ±0.85	P=0.0000 P_{1-2}=0.0017 P_{1-3}=0.8731 P_{1-4}=0.0000 P_{1-5}=0.0000 P_{2-3}=0.0650 P_{2-4}=0.9996 P_{2-5}=0.9696 P_{3-4}=0.0093 P_{3-5}=0.0023 P_{4-5}=0.9798

Discussion

Intercellular interactions are nonspecific and regulate the processes occurring both during physiological changes and in pathological conditions in the endometrial tissue. The interactions of the cellular structures of the endometrial tissue contribute to the regulation of signaling pathways, and their change can cause impaired implantation.

During pregnancy, apically secreted cytokines by the endometrial epithelium affect the development, migration, and attachment of blastocysts, and affect the transformation of the underlying stroma. Decidualized stromal cells, as the main component of the decidual membrane in pregnant women, also produce cytokines, which in their turn control the decidualization process, and chemokines, which are chemoattractants for natural killer cells of the uterus, macrophages, and for trophoblast migration. Activated leukocytes in the developing decidua contribute to regulatory cytokines in the local microenvironment.[9]

The mechanism of an increased immune response during the presence of an infectious agent is associated with a higher level of expression of mRNA encoding TLR4 and TLR2, recognizing bacterial LPS and lipopeptides, respectively, as mechanisms of bacterial persistence,[10-14] but under conditions of the endometrium chronic inflammation, we observe the activity of all studied cytokines, regardless of the presence of bacterial flora.

It is possible to assume that the persistence of the bacterial flora is observed with a decrease in the body's colonization resistance, manifested both by the activity of opportunistic microflora and by a decrease in the number of lactobacilli.[15]

The results obtained in our study indicate changes in the local immune response that are characteristic of inflammation in women with reproductive disorders and CE on the background of opportunistic microbes. The presence of an infectious agent in the endometrium was characterized by multidirectional changes in cytokine levels, which were expressed on the background of a significant increase in the concentration of TNF-α and INF-γ ($P<0.05$).[16]

INF-γ is the most important endogenous immuno-modulator necessary for the development of a specific immune response. It is known that in the late stages of acute inflammation and in chronic inflammation, INF–γ enhances the secretion of antibodies, including autoreactive ones.[17,18]

A level of IL-10 in the CE endometrium decreases in the presence of opportunistic microbes. A decrease in IL-10 in response to the activity of an infectious agent indicates the development of an inadequate, pronounced, local inflammatory reaction in the endometrial tissue with a deficiency of anti-inflammatory cytokines, which may be one of the mechanisms of long-term persistence of the infection in the endometrial tissue.

IL-10 can directly regulate innate and adaptive Th1 and Th2 responses by limiting T cell activation and differentiation in the lymph nodes as well as suppressing proinflammatory responses in tissues, leading to impaired pathogen control and/or reduced immunopathology.[19]

The correlations between IL-10 and IgM/IgA characterize the failure in the endometrial mucosa barrier, which is the first line of immune defense against the external environment, and one major benefit resulting from the homeostatic relationship between the host and the commensal microbiota is the resistance to pathogen colonization; thus, data obtained indicate resistance to colonization by pathogenic microorganisms.[20]

The increased levels of pro- and anti-inflammatory endometrial cytokines in women with reproductive disorders and without CE, which were lower than in CE, may be explained by the interrelation of the immune and endocrine systems.

The endometrium is a hormone-dependent tissue and is dependent on the cyclic secretion of sex hormones. This is confirmed by the literature data on the insufficiency of secretory and histochemical endometrial rearrangement in various disorders of ovarian function and in the use of hormonal therapy.[21] Progesterone can regulate local and systemic inflammation. The progesterone-induced blocking factor (PIBF) is a progesterone-induced mediator, which conveys some of the immunological effects of progesterone. PIBF acts on lymphocytes in pregnancy to induce a type 1 to type 2 cytokine shift by upregulating the production of type 2 cytokines. PIBF is capable of increasing the production of IL-4 and IL-10 in peripheral blood mononuclear cells, but has no effect on the Th1 cytokines IFN-γ and TNF-α.[22] In vivo data support the effect of PIBF on NK activity.[23,24] The increased resorption rates observed in PIBF-depleted mice are corrected by treating the mice with anti-NK antibodies,[25] suggesting that PIBF contributes to the success of murine gestation by controlling NK activity.

An excessive amount of proinflammatory cytokines in progesterone deficiency, in addition to the direct embryotoxic effect, leads to local thrombus formation due to the effect on almost all links of the hemostasis system, which prevents adequate implantation and subsequent invasion of the trophoblast.[26-28]

The human endometrium contains a conspicuous number of immune cells, the number and the phenotype of which change during the menstrual cycle. It has become evident in recent years that the immune cell phenotype and function can be influenced by microbiota.[29] "Immune cells can sense the presence of microbes through their pattern recognition receptors, setting up host-microbe interaction. The microbiota exerts an appropriately controlled defense mechanism by competing for nutrients and mucosal space with pathogens."[29]

Conclusion

Our results showed the peculiarities of changes in cytokines at the level of endometrial tissue both in chronic inflammation of the endometrium and in its absence in women with reproductive disorders. The severity of the immune response is significantly higher in patients with CE, with the most significant change in the role of IL-10. Reproductive disorders are accompanied by a moderate activity of endometrial cytokines and preservation of

correlations with phagocytosis, which, possibly, allows us to judge a compensatory change in the concentration of proinflammatory endometrial cytokines against the background of a decrease in the level of progesterone. The results obtained may be useful for the diagnosis and treatment of CE and immunological correction in women with reproductive disorders.

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Competing Interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

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Molecular Predictors of Effective Implantation and Live Birth in IVF Programs

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Abstract

The aim of the study was to improve the possibilities of predicting blastocyst implantation and live birth of ART programs in women of late reproductive age with tubal-peritoneal infertility based on immunohistochemical markers of the endometrium

***Methods and Results*:** The results of IVF and IVF/ICSI programs were analyzed in 68 patients of late reproductive age (36-44 years of age) with tubal-peritoneal factor of infertility. Morphological examination of the endometrium was performed on Day 7 after confirmed ovulation in the cycle preceding ART**.** The expression of vitamin D receptors (VDR) and HOXA11 in endometrial stromal cells was assessed by immunohistochemical method. The effectiveness of using the endometrial markers VDR and HOXA11 as potential predictors of ART programs efficiency was confirmed by prognostic models. The levels of the stromal expression of VDR<8.7% and HOXA11<6.1% (probability >0.27) were determined to be favorable for successful blastocyst implantation. The expression levels of VDR<8.3% and HOXA11<6.1% in endometrial stromal cells are prognostically favorable for live birth (probability >0.19) in women of late reproductive age with tubal-peritoneal infertility who undergoing ART treatment with their own oocytes.(**International Journal of Biomedicine. 2021;11(4):532-537.**)

Key Words: assisted reproductive technology • vitamin D receptor • HOXA11 • implantation • live birth

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Abbreviations

ART, assisted reproductive technology; **AUC**, the area under the ROC curve; **IVF**, in vitro fertilization; **IHCM**, immunohistochemical markers; **ICSI**, intracytoplasmic sperm injection; **PCC**, percentage correctly classified; **RIF**, recurrent implantation failure; **VDR,** vitamin D receptor.

Introduction

A new tendency in reproductive medicine is to improve the percentage of healthy perinatal outcomes for women using assisted reproductive technology (ART).[(1)] Recurrent pregnancy losses, corresponding to the loss of three or more consecutive pregnancies up to 20 weeks, are noted in 1%–2% of couples, and their causes remain unexplained in more than half of the cases.[(2)] The use of ART has improved the rates of pregnancy and live births.[(3)] However, recurrent implantation failure (RIF), determined by the absence of fertilization after several IVF attempts, showed the need to study the effect of the endometrial pattern on the outcomes of ART programs.[(4-7)] It is reported that suboptimal endometrial susceptibility and altered embryo-endometrial dialogue are responsible for

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two-thirds of implantation failures.[8] An unknown reason for the functional inferiority of the endometrium in women with tubal-peritoneal infertility indicates the need to choose the most informative methods to predict the outcomes of ART programs. The female reproductive system is unique in relation to other tissues and systems due to predominantly postnatal differentiation and transformations during the menstrual cycle and pregnancy.

Homeobox genes (as known as *HOX* genes) are leading candidates for the regulation of endometrium differentiation in preparation for embryonic implantation. HOX genes encode proteins that act as transcription factors.[9,10] Hoxa11/HOXA11 expression is preserved in the adult endometrium of mice[11,12] and humans.[12] Some of the *Hox/HOX* genes participate in remodeling of the genitourinary tract, and regulation of endometrial proliferation and cell differentiation, with the formation of susceptibility of the uterine mucosa to implantation or apoptosis and tissue rejection during each menstrual cycle.[13-16]

Homeobox-deficient mice (*Hoxa11*) were found to be infertile due to stromal-specific endometrial defects that exclude decidual transformations.[17,18]

The prognostic potential of vitamin D to ensure female fertility is determined by its extensive receptor network in the organs of the reproductive system, including the endometrium.[19] Recent studies have confirmed the steroidogenic effect of the active form 1,25(OH)2D and participation in the modulation of cell proliferation, differentiation and apoptosis of cells of the reproductive organs.[20,21] The effect of vitamin D on the endometrium is carried out by the formation in the cell nucleus of a ligand-independent transcription factor for the regulation of target genes and communication with cytosolic and membrane receptors.[22,23]

VDR is a part of a group of transcriptional regulators providing a variety of biological effects of 1,25(OH)2D and related compounds.[24] The recognition of molecular and cellular endometrial breakdowns during the "implantation window" as one of the significant reasons for ineffective IVF attempts explains the interest in studying the mechanisms of local activity of VDR and *Hox/HOX* genes.

The aim of the study was to improve the possibilities of predicting blastocyst implantation and live birth of ART programs in women of late reproductive age with tubal-peritoneal infertility based on ICHM of the endometrium.

Materials and Methods

Our prospective cohort study included 68 women of late reproductive age (36-44 years of age) with tubal-peritoneal infertility who underwent ART programs at the Center of Obstetrics and Gynecology.

Inclusion criteria for the study were tubal factor of infertility, normal or reduced ovarian reserve with a preserved regular ovulatory menstrual cycle, normozoospermia or minor pathozoospermia of the husband (donor), use of the patient's own oocytes, embryos of good and excellent quality.

Exclusion criteria were infertility due to the absence of ovulation; endometriosis, uterine fibroids 4 cm or more, uterine factor of infertility, chronic active endometritis, HIV infection, hepatitis B and C, severe pathozoospermia, systemic diseases; somatic diseases in the stage of exacerbation or decompensation.

Depending on the outcomes of ART programs, 4 groups were formed, two of them according to the incidence of clinical pregnancy: Group 1 (n=18) – with pregnancy, Group 2 (n=50) – with a negative result; and two groups according to the indicator of live birth (frequency of deliveries with a live fetus(es) [take-home baby]): Group 3 (n=14) – with a favorable result, Group 4 (n=54) –with a negative result.

Endometrial sampling by aspiration biopsy using a Pipelle catheter during the period of the proposed "implantation window" was performed in 68 women in the cycle preceding the ART programs (on Days 17-25 of the menstrual cycle, depending on the results of ultrasound monitoring and on Day 7 after the peak of luteinizing hormone.

For histological examination, the standard method of fixation in 10% neutral formalin was used, followed by dehydration, degreasing and embedding in paraffin in a histological machine, according to the generally accepted technique. From paraffin blocks, sections with a thickness of 5μm were prepared, several (5-10) on 10-15 glass slides. After dewaxing, the sections were stained with hematoxylin and eosin; the avidin-biotin immunoperoxidase method was used for the immunohistochemical study of endometrial biopsies.

The polyclonal antibodies (PCAB) used in the study were designed to work with paraffin sections. Treatment options for dewaxed sections were selected depending on the manufacturer's instructions (Table 1). A Histophine detection system (Nichirei Corp., Japan) was used to visualize primary antibodies.

Table 1.

Panel of antibodies for immunohistochemical study.

Antibodies	Clone	Working dilution	Manufacturer
VDR	polyclonal	1:100	GeneTex, USA
HOXA11	polyclonal	1:250	GeneTex, USA

The stained samples were automatically classified by the program into 10 color channels, depending on the color and the intensity of staining. After classification, pseudo-color masks were applied to the structures of interest. In this case, the relative density (%) of the studied structures was calculated in relation to the total area of the studied frame.

The results of VDR and HOXA11 expression were calculated by counting and finding the percentage of the relative density of stained endometrial stromal cells. Morphofunctional assessment of the endometrium was performed using licensed software (Morphology 5.2., Russia). The preparations were examined and photographed using a Primo Star microscope(Carl Zeiss, Germany), with the help of a Pixera Pro 150ES digital camera (Pixera, Japan) at an operating magnification of x400.

Embryo transfer was carried out in "fresh" cycles and cycles with the transfer of thawed embryos. For the pregnancy rate indicator, only clinical pregnancy (sonographic presence

of fetal egg/eggs) was taken into account, and for live births – the number of ART programs that ended with live births/ fetuses.

All patients were informed about the purpose and design of the work and gave their consent to participate in the study and publish its results in the open press. The study was approved by the SUSMU Ethics Committee.

Statistical analysis was performed using the IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). The normality of distribution of continuous variables was tested by the Kolmogorov-Smirnov test with the Lilliefors correction and Shapiro-Wilk test. Continuous variables with normal distribution were presented as mean (standard deviation [SD]); non-normal variables were reported as median (Me) and interquartile range (Q1-Q3). Means of 2 continuous normally distributed variables were compared by independent samples Student's t test. Differences of continuous variables departing from the normal distribution, even after transformation, were tested by the Mann-Whitney U-test. Threshold values (cut-off point) were calculated by the ROC analysis method. To construct a prognostic model and estimate OR (odds ratio), we used the method of multiple logistic regression; 95% CI (confidence interval) was calculated. A value of $P<0.05$ was considered significant.

Results

The onset of clinical pregnancy was detected in 18(26.5%) patients with their own oocytes in ART programs. In Group 1 and Group 2, VDR expression in the endometrial stroma was 7.35(4.9;8.1)% and 8.56.3;9.8)% (P=0.016), respectively, and HOXA11 expression – 5.1(4.3;6.1)% and 7.4(5.4; 8.7)% (P=0.001), respectively.

The AUC was determined for implantation markers: VDR of the stroma – 0.739±0.063 (P=0.003) and HOXA11 – 0.767±0.064 (P=0.000).

The threshold value of VDR expression in endometrial stromal cells at the cut-off point was 8.7%. The sensitivity and specificity of the method were 100% and 40%, respectively (Fig.1). The threshold value of HOXA11 expression in endometrial stromal cells, calculated for successful implantation at the cut-off point, was 6.1%. The sensitivity and specificity of the method were 80% and 73%, respectively (Figure 2).

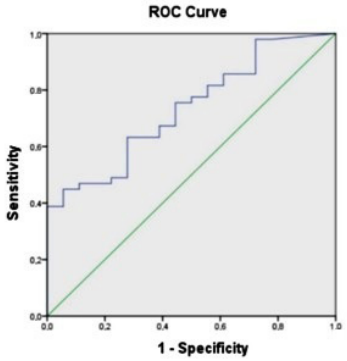

Fig.1. *ROC-curve of the relationship between successful implantation (the onset of clinical pregnancy) and the VDR expression in endometrial stromal cells*

AUC 0.739±0.063 (P=0.003); 95% CI: 0.616-0.862.

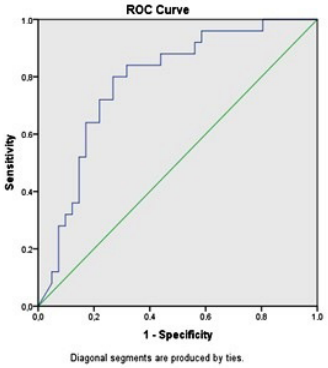

Fig.2. *ROC-curve of the relationship between successful implantation (the onset of clinical pregnancy) and the HOXA11 expression in endometrial stromal cells.*

AUC 0.767±0.064 (P=0.000); 95% CI: 0.642-0.892.

To create a mathematical model that allows predicting implantation, we used multiple regression analysis with forced inclusion of two variables: VDR, HOXA11 (Table 2).

Table 2.

Calculation of the blastocyst implantation prognosis

Parameter	B	Value	OR(Exp(в)	95% CI for OR
HOXA11	-0.487	0.003	0.615	[0.444;0.850]
VDR	-0.302	0.012	0.740	[0.585;0.935]
Constant	4.121	0.005	61.649	

The equation was as follows:

$$p = \frac{1}{1 + e^{-(4.121 - 0.487 \cdot x_1 - 0.302 \cdot x_2)}},$$

where x_1 is the value of HOXA11 expression in the endometrial stroma, x_2 is the value of VDR expression in endometrial stromal cells, p is the probability of implantation.

If the probability calculated by the model is >0.27, then the onset of implantation is predicted. The resulting model is statistically significant (P<0.001). Model sensitivity (83.3%), specificity (74.0%), and PCC (76.5%) indicate a high predictive ability.

The ROC curves for predicting blastocyst implantation, taking into account the expression of individual markers in the endometrium, are presented in Figure 3.

Live birth in ART programs occurred in 14(21.7%) women. The expression of IHCM in the endometrial stroma in Groups 1 and 2 was as follows: 7.1(4.9;8.0)% and 8.5(6.1;9.8)%, respectively, for VDR (P=0.240); 5.0(4.3;6.1)% and 7.1(5.3;8.7)%, respectively, for HOXA11 (P=0.006). In patients of Group 1, the decrease in HOXA11 expression turned out to be statistically significant (P=0.006).

The AUC of IHCM expression in women with their own oocytes was 0.728±0.067 (P=0.009) for the VDR in the stroma and 0.744±0.071 (P=0.005) for HOXA11.

The value of the threshold parameter of HOXA11 expression, prognostically favorable for live birth, was

6.1%. The sensitivity and specificity of the method were 80% and 63%, respectively. The threshold value of VDR expression in stromal cells was calculated and turned out to be prognostically favorable for live birth was 8.3%. The sensitivity and specificity of the method were 93% and 53%, respectively.

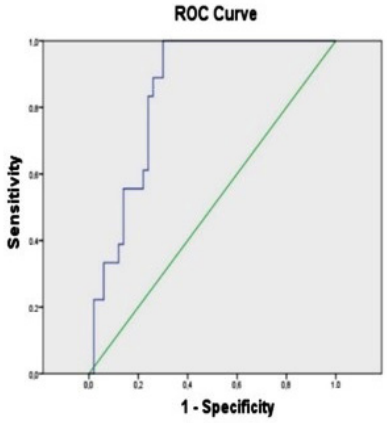

Fig.3. *AUC for the blastocyst implantation prognosis model.*

AUC= 0.846 (P=0.000); 95% CI: 0.756-0.935.

To create a mathematical model that allows predicting live birth, we used multiple regression analysis with the forced inclusion of two variables: VDR, HOXA11 (Table 3).

Table 3

Calculation of the live birth prognosis

Parameter	B	Value	OR(Exp(B)	95% CI for OR
HOXA11	-0.308	0.030	0.735	[0.556;0.971]
VDR	-0.329	0.006	0.720	[0.568;0.912]
Constant	2.825	0.033	16.860	

The equation was as follows:

$$P = \frac{1}{1 + e^{-(2.825 - 0.308 \cdot x_1 - 0.329 \cdot x_2)}},$$

where x_1 is the value of HOXA11 expression in the endometrial stroma, x_2 is the value of VDR expression in endometrial stromal cells, p is the probability of live birth.

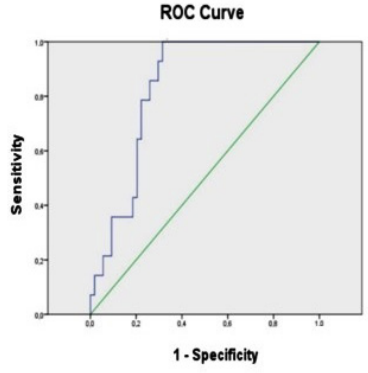

Fig. 4. *AUC for the live birth prediction model*

AUC= 0.831 (P=0.000); 95% CI: 0.736-0.925.

The probability calculated by the model of >0.19 means a favorable prognosis for live birth (P<0.001). Model sensitivity (86.0%), specificity (70.0%), and PCC (78.6%) indicate a high predictive ability. ROC curves for predicting live birth, taking into account the expression of individual markers in the endometrium, are shown in Figure 4.

Discussion

Predicting the outcomes of ART programs is possible if the "subtle" endometrial patterns of fertility regulation are taken into account. The results obtained showed that effective interaction between the endometrium and the blastocyst during the "implantation window" suggests a lower level of VDR expression in endometrial stromal cells.

These results explain the data of studies indicating a decrease in VDR expression in the middle stage of the secretion phase (during the "implantation window") in healthy women and a decrease in the antiproliferative effect at low levels of VDR expression.[25] This is probably due to the progesterone-like activity of vitamin D along with the limitation of local pro-inflammatory reactions necessary for blastocyst implantation. The susceptibility of the endometrium during the implantation period was due to a decrease in VDR activity, which probably affects not only the metabolic profile of cell subpopulations, but also the ratio of immune cells.

The data we obtained differ from the results of researchers who indicated the relationship between positive outcomes of IVF and IVF/ICSI programs and increased VDR expression. In the study by J. Guo et al.,[26] for example, the absence of significant statistical differences (P=0.083), the younger age of women with pregnancy (P=0.032), the small sample size (n=16), and the study of endometrial samples at different phases of the menstrual cycle in different women are factors that limit the significance of the results.

In earlier studies, we identified a decrease in the expression of the VDR protein in the stromal epithelium (P=0.016) of women with pregnancy, as opposed to the glandular layer.[27] It has been calculated that a 1% decrease in VDR expression in the stroma increases the chance of a favorable outcome by 1.35 times.[28]

The combination of the optimal expression level of VDR and HOXA11 has a beneficial effect on implantation and the outcomes of IVF and IVF/ICSI programs. Therefore, it is possible to assert that a lower expression of endometrial markers is crucial for the synchronized dialogue between the endometrium and the embryo, as well as for live birth.

Our research results differ from those given in the literature. Thus, Makker A. et al.[29] revealed no differences in protein expression in patients with infertility and uterine fibroids that do not deform the cavity, compared with healthy fertile women. According to other data,[30] the minimum level of HOXA11 in the endometrium was found only in infertility of unknown origin, which was significantly lower than in the control group, in contrast to women with other types of infertility (P=0.005). A decrease in HOXA11 expression in the middle of the secretory phase during implantation and in the early stages of pregnancy was determined only in the

glandular endometrium, in contrast to stromal cells, in which high expression persisted throughout the menstrual cycle.[31]

Conclusion

The effectiveness of using the endometrial markers VDR and HOXA11 as potential predictors of ART programs efficiency is confirmed by prognostic models. The levels of the stromal expression of VDR<8.7% and HOXA11<6.1% (probability >0.27) were determined to be favorable for successful blastocyst implantation. The expression levels of VDR<8.3% and HOXA11<6.1% in endometrial stromal cells are prognostically favorable for live birth (probability >0.19) in women of late reproductive age with tubal-peritoneal infertility who undergoing ART treatment with their own oocytes.

Competing Interests

The authors declare that they have no competing interests.

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Effectiveness of Cryopreservation in Patients of Young Reproductive Age with the Risk of Ovarian Hyperstimulation, "Thin" Endometrium and Previous IVF Failures

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Abstract

The article provides an analysis of clinical, anamnestic and laboratory parameters for patients of young reproductive age who participated in IVF programs and have cryopreserved embryos. The main reasons for embryo cryopreservation were prevention of OHSS, "thin" endometrium and "previous IVF failures." It has been found that the patients from the group of transfer cancellation due to prevention of ovarian hyperstimulation had a higher ovarian reserve, a larger number of eggs, fresh and frozen embryos, and shorter shelf life of frozen embryos. All embryos were of the best quality (corresponding to the day of cultivation); the "post-thaw cultivation" technique was applied. During stimulation, lower amounts of gonadotropins were used. Patients with thin endometrium and previous IVF failures demonstrated slow growth of follicles, which required a higher course dose of gonadotropins with the addition of LH-containing preparations. Regardless of the group, in most cases, frozen/thawed embryos were transferred at the blastocyst stage (Day 5). The pregnancy rate was high in patients at risk of OHSS and with thin endometrium (48.6%, 48.0%). Patients with IVF failures had a lower pregnancy rate; this is due to endometrial pathology in the medical history, a smaller number of antral follicles, oocytes, fresh and frozen embryos, and longer shelf life of frozen embryos. (**International Journal of Biomedicine. 2021;11(4):538-542.**)

Key Words: assisted reproductive technologies • in vitro fertilization • vitrification • ovarian hyperstimulation syndrome

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Abbreviations

AMH, anti-Müllerian hormone; **ART**, assisted reproductive technology; **FET**, frozen-thawed embryo transfer; **FSH**, follicle-stimulating hormone; **hCG**, human chorionic gonadotropin; **IVF**, in vitro fertilization; **LH**, luteinizing hormone; **OHSS**, ovarian hyperstimulation syndrome.

Introduction

According to the Register of the Russian Association for Human Reproduction, 44,000 protocols were conducted in 2018 using frozen/thawed embryos (28.1% of the total number of assisted reproductive technology (ART) cycles in Russia), of which more than 12,000 pregnancies ended in childbirth. The pregnancy rate per cycle was 41.5%(39.6% in 2017), per embryo transfer — 42.4%(41.0% in 2017).[(1)]

In cryoprotocols, the ultrafast freezing technique is used for ovarian hyperstimulation syndrome (OHSS) prevention if there are no conditions for "fresh" transfer — at "thin"

endometrium. In addition, this technique allows preserving and using embryos at the request of a woman.[2-5]

Prognostically adverse factors of OHSS development include the following: age under 35 years, weight deficit — under 25 kg/cm^2, enlarged ovarian size, polycystic or multi-follicular structure of ovaries, pre-existing OHSS, AMH level higher than 3.6ng/ml, a great number of basal antral follicles, increased or rapidly increasing serum estradiol levels during ovarian stimulation, the use of HCG instead of progesterone to support the luteal phase after IVF, a large number of aspirated oocytes (>20).[6,7]

Successful outcomes in cryoprotocols depend on many factors, and endometrial thickness is an important characteristic. There is no generally accepted approach to the definition of the "thin" endometrium in the literature. Decreased endometrial thickness in the IVF cycle is one of the indications to cryopreserve embryos: 7mm or less affects the effectiveness of ART programs.[6,7] With 8 mm thickness or more, one can observe high implantation and pregnancy rates.[6,7] FET cycles are possible in a modified natural cycle, stimulated cycle, a cycle of hormone replacement therapy, and a "pure" natural cycle.(8,9) In patients with thin endometrium, it is necessary to prepare endometrium using estradiol,[9] which increases the endometrial thickness and pregnancy rates. Different culture media are used for cryotransfer.[10] The quality of the transferred embryo is important, which is achieved by "post-thaw cultivation" to the blastocyst stage.[11]

Factors that reduce the pregnancy rate in ART and cryotransfers are the age of a woman older than 36 years, obesity, surgical interventions, low AMH levels, the duration of frozen embryo storage over 12 months.[6,11,12]

The purpose of the study was to investigate the effectiveness of devitrified embryo transfers in patients at risk of OHSS, "thin" endometrium, and previous IVF failures.

Materials and Methods

To serve the study's purpose, we retrospectively analyzed 300 protocols with FET performed in 2018–2020.

Criteria for inclusion in the study were age ≤35 years, tuboperitoneal infertility (N97.1), vitrified embryos available.

Exclusion criteria included a low AMH level, other infertility factors, external genital endometriosis, myoma, abnormalities of uterine development, the absence of cryopreserved embryos, male infertility, the use of any donor material (donor oocytes, sperm cells and embryos).

Depending on the cause of cryopreservation, 3 groups were formed:

Group 1 included patients (n=111) whose embryos were frozen to prevent OHSS development.

Group 2 included patients (n=100) whose embryos were cryopreserved due to "thin endometrium" in IVF program — M-echo of ≤ 7 mm.

Group 3 included patients (n=89) with previous IVF failures who had "extra" embryos stored after the IVF program.

In the compared groups, we studied clinical and anamnestic data, hormonal status, IVF program preceding the cryoprotocol, embryological stage, and pregnancy rate after the performed manipulations. Controlled ovarian stimulation in IVF cycles was performed under the protocol with gonadotropin-releasing hormone antagonists. The ovulation trigger was hCG at the dosage of 6500IU. In all cases, cryopreservation was done by vitrification using the Kitazato kit (Japan). The quality of embryos was assessed at the cleavage stage according to classification by J. Lens and co-authors. Embryos at the blastocyst stage were evaluated according to classification by D. Gardner and co-authors (1999). The thawing process was carried out pursuant to the manufacturers' recommendations. When preparing endometrium in cryoprotocol, estrogen (E2) and progesterone (P) preparations were prescribed in phases according to the day of embryo cultivation. Next, one or two excellent and good-quality embryos were transferred when the endometrial thickness (M-Echo) was ≥ 8 mm. The effectiveness of the frozen/thawed embryo transfers was evaluated based on the pregnancy rate per embryo transfer.

Statistical processing was carried out using the STATISTICA Version 10 (StatSoft, USA). The normality of distribution of continuous variables was tested by Shapiro-Wilk test. For descriptive analysis, results are presented as mean±standard deviation (SD), median (Me), interquartile range (IQR=Q1;Q3). Multiple comparisons were performed with one-way ANOVA and Tukey's HSD Post-hoc Test. Kruskal-Wallis test was used to compare means of 3 groups of variables not normally distributed. Categorical variables were analyzed using the chi-square test with the Yates' correction. A value of $P<0.05$ was considered significant.

The study was approved by the Ethics Committee of the Scientific Center for Family Health and Human Reproduction Problems. Written informed consent was obtained from each participant.

Results

Depending on the cause of embryo cryopreservation, clinical and anamnestic characteristics have shown that the patients of the studied groups were comparable in terms of age, age at menarche, and body mass index (Table 1).

Table 1.
Clinical and anamnestic characteristics of patients

Variable	Group 1 n=111 (1)	Group 2 n=100 (2)	Group 3 n=89 (3)	Statistics
	M±SD; Me (Q1;Q3)			
Age, years	31.4±3.1 31.9 (23;35.2)	31.7±2.9 31.7 (24.3;35.9)	31.8±2.8 32.3 (23.2;35.9)	P=0.5998
Age at menarche, years	13.3±1.2 13 (11;16)	13.3±1.3 13 (11;18)	13.2±1.3 13 (10;18)	P=0.8222
Body mass index, kg/m^2	23.2±3.8 21.9 (17.9;35.2)	23.2±3.9 21.9 (17.7;37.9)	23.7±3.9 23.3 (17.5;34.9)	P=0.5925
Pelvic inflammatory diseases	108(97.3%)	95(95.0%)	85(96.6%)	P=0.8724
Previous reconstructive and plastic fallopian surgery	70(63.1%)	77(77.0%)	69(77.5%)	P=0.0304

Most of them had a history of pelvic inflammatory diseases and reconstructive and plastic fallopian surgery, which caused infertility.

All patients underwent pipelle biopsy of the endometrium before IVF was planned. Histological study showed that in Group 1, the endometrium in all patients corresponded to the menstrual cycle phase, without pathology. In Group 2.19% had a normal endometrium, 69% had chronic endometritis, and 12% had endometrial polyps. In Group 3 with IVF failures, 29.2% had a normal endometrium, 19.1% — chronic endometritis, 37.1% — endometrial polyps, 4.5% — endometrial hyperplasia, 2% — myoma, and 4.5% had intrauterine synechiae. All patients with endometrial pathology underwent hysteroscopy, antibacterial therapy, and cyclic hormone therapy.

The analysis of laboratory and ultrasound parameters of the studied patients showed that all patients from all groups were comparable in FSH, LH levels, which were within the reference and acceptable range for IVF programs (Table 2). However, in Group 1, estradiol and AMH were significantly higher than in Groups 2 and 3.

Table 2.

Hormonal status and ultrasound data of patients before entering the IVF program

Variavle	Group 1 n=111 (1)	Group 2 n=100 (2)	Group 3 n=89 (3)	Statistics
	M±SD; Me (Q1;Q3)			
Basal FSH, mlU/ml	6.3±1.9 6.3 (2.6;9.1)	6.5±1.7 6.4 (1.6;9.4)	7.1±1.9 7 (2.7;9.4)	P=0.0078 P_{1-2}=0.7093 P_{1-3}=0.0067 P_{2-3}=0.0657
Basal LH, mlU/ml	6.7±4.3 6.2 (2.9;11.6)	5.9±3.5 5.5 (1.6;12.8)	5.9±2.8 5.4 (1.3;11.5)	P=0.1866
Basal E2, (pg/ml	430.5±132.2 410.2 (101.7;993)	140.3±51.9 113.5 (51.2;415)	155.4±52.2 111 (90;1016)	P=0.0000 P_{1-2}=0.0000 P_{1-3}=0.0000 P_{2-3}=0.4867
Basal P, nmol/l	24.5±6.3 16.1 (9.1;160)	30.7±6.7 25.9 (15.3;125.2)	31.2±22.1 30.6 (9.2;84)	P=0.0000 P_{1-2}=0.0021 P_{1-3}=0.0012 P_{2-3}=0.9635
Basal AMH, ng/ml	4.8±2.6 4.1 (1.6;12.9)	3.6±1.7 2.4 (1.7;8.6)	3.2±1.5 2.3 (1.8;9.6)	P=0.0000 P_{1-2}=0.0001 P_{1-3}=0.0000 P_{2-3}=0.3687
M-Echo endometrial thickness, mm	9.8±1.1 10 (8;11)	6.1±1.2 6 (5;8)	11±1.3 10 (8;12)	P=0.0000 P_{1-2}=0.0000 P_{1-3}=0.0000 P_{2-3}=0.0000
Left ovary volume, cm^3	12.9±4.3 9.6 (4.9;23.4)	7.9±3.4 7.3 (5.8;21.3)	7.4±4.1 6.7 (5.9;11.6)	P=0.0000 P_{1-2}=0.0000 P_{1-3}=0.0000 P_{2-3}=0.6619
Right ovary volume, cm^3	15.1±7.1 10.3 (5.5;24.0)	8.7±4.9 8.2 (5.8;10.7)	7.1±4.1 6.6 (5,7;18.8)	P=0.0000 P_{1-2}=0.0000 P_{1-3}=0.0000 P_{2-3}=0.1262
Number of antral follicles	13.6±2.9 9.0 (8.5;17.0)	6.8±2.5 6.5 (4.5;8.0)	6.1±2.4 5.5 (4.5;7.0)	P=0.0000 P_{1-2}=0.0000 P_{1-3}=0.0000 P_{2-3}=0.1622

Group 1 showed low progesterone levels (24.5±26.3 nmol/l), which was the reason for cancelling the fresh transfer since it reduced the probability of placentation and carrying of pregnancy.[6,7]

According to ultrasound data (Table 2), Group 1 patients had more antral follicles than women in Groups 2 and 3. In Group 2, the endometrial thickness on the day before entering the IVF program was 6.1±1.2 mm, which was the reason for cancelling embryo transfer.

In our study, we analyzed retrospectively the induced cycle preceding cryotransfer (egg collection cycle) to evaluate the stimulation profiles and gonadotropin doses (Table 3). In Group 1, FSH+LH-containing preparations were used less often in ovarian stimulation; the course dose of gonadotropin in Group 1 was lower than in Groups 2 and 3. Based on this, we concluded that in groups with "IVF failures" and "thin" endometrium, follicles were growing slower, which required the addition of LH-containing gonadotropins and a higher course dose of gonadotropins in the course of ovarian stimulation. Thus, the Group 1 patients had a better prognosis for good ovarian response in a stimulated cycle. However, in this group, higher estradiol levels on the day of transvaginal aspiration (3161.2±1712.4 pg/ml) caused the cancellation of fresh embryo transfer to prevent OHSS (Table 3).

Table 3.

Characteristics of oocyte collection cycle

Variable	Group 1 n=111 (1)	Group 2 n=100 (2)	Group 3 n=89 (3)	Statistics
	M±SD; Me (Q1;Q3)			
Day of menstrual cycle at the start of IVF stimulation	3.2±1.2 3(2;7)	2.78±0.9 3(2;5)	2.7±0.8 3(2;5)	P=0.7639
IVF stimulation with FSH+LH preparations	63(56.8%)	70(70.0%)	69(77.5%)	P=0.006
Course dose of gonadotropin in IVF stimulation, IU	2118.3±861.3 2025 (787.5;5625)	2442.2±883 2250 (1050;5850)	2292.9±818.7 2175 (1012.5;4987.5)	P=0.0239 P_{1-2}=0.0176 P_{1-3}=0.3252 P_{2-3}=0.4562
Day of menstrual cycle during follicular aspiration	14.2±1.8 14(10;20)	13.7±1.4 14(11;17)	13.8±1.5 14(11;18)	P=0.0544
Estradiol level on the day of transvaginal aspiration, pg/ml	3161.2±1712.4 2868 (213;6426)	2428.2±1305 2202 (354;6125)	1722.8±807.2 1442 (3924216)	P=0.0000 P_{1-2}=0.0003 P_{1-3}=0.0000 P_{2-3}=0.0012

The analysis of the embryological stage revealed that in Group 1, more follicles showed growth than in Groups 2 and 3, and a larger number of oocytes, fresh and frozen embryos were obtained ($P<0.05$) (Table 4). The fertilization rate was high (91.8%–93.4%); there was no difference in the number of embryos of good, satisfactory and low quality in all 3 groups. In Group 1, all embryos corresponded to the day of cultivation; in Groups 2 and 3 — 97.0% and 94.4%, respectively (Table 4).

Table 4.

Characteristics of the embryological stage of oocyte collection cycle

Varizble	Group 1 n=111 (1)	Group 2 n=100 (2)	Group 3 n=89 (3)	Statistics
	M±SD; Me (Q1;Q3)			
Number of aspirated follicles	11.5±4.6 11(2;22)	9.6±2.6 10(5;18)	8.5±2.8 8(4;17)	P=0.0000 P_{1-2}=0.0003 P_{1-3}=0.0000 P_{2-3}=0.0831
Number of oocytes after aspiration	10.1±5.2 10(1;22)	8.6±3.1 8.5(3;18)	7.6±3 7(3;17)	P=0.0001 P_{1-2}=0.0183 P_{1-3}=0.0000 P_{2-3}=0.1987
Number of embryos after fertilization	7.1±4 7(1;19)	6.2±2.4 6(2;13)	5.8±2.3 6(2;14)	P=0.0089 P_{1-2}=0.0860 P_{1-3}=0.0088 P_{2-3}=0.6441
Good quality embryos	45(40.5%)	30(30.0%)	31(34.8%)	P=0.2764
Satisfactory quality embryos	65(58.6%)	67(67.0%)	56(62.9%)	P=0.4480
Low quality embryos	1(0.9%)	3(3.0%)	2(2.3%)	P=0.8142
Embryos corresponded to the day of cultivation	111(100%)	97(97.0%)	84(94.4%)	P=0.1307
Fertilization rate	91.8±11.9	91.8±11.;	93.4±10.5	P=0.5405
Number of frozen embryos	5.1±2.9 5(1;16)	3.2±1.8 3(1;8)	3.1±1.8 3(1 9)	P=0.0000 P_{1-2}=0.0000 P_{1-3}=0.0000 P_{2-3}=0.9509

The analysis of frozen/thawed embryo transfers showed that in Group 2, Day 3 embryos were cryopreserved less frequently, which can be explained by the fact that in Group 2, embryos needed post-thaw cultivation more often to select embryos with good developmental potential (Table 5). Day 4 embryos were most often cryopreserved in Group 1, which is related to a large number of good-quality embryos and no need for their further selection. On Day 5 of embryo cultivation, cryopreservation was performed most often in Group 2, which indicates the worse quality of embryos in this group and the need for their selection during cultivation.

The period of frozen embryo storage in these 3 groups was also different: in Group 1, it was shorter than in Groups 2 and 3, due to the need for additional examination and treatment of endometrial pathology, especially in Group 3 after IVF failure in the induced cycle.

The number of thawed embryos and the percentage of embryos that survived thawing was the same in all groups. The number of remaining frozen embryos in Group 1 was greater than in Groups 2 and 3, which is due to their large number at the time of freezing.

The day to day FET (i.e. without post-thaw cultivation) was conducted only in those patients whose embryos were frozen on Day 5. In Group 2, such cryotransfer was carried out most often — 57.0% (in Groups 2 and 3, 32.4% and 48.3% respectively). In the study groups, the blastocyst (Day 5) stage transfer was performed in 95.5%, 95.0%, and 93.3% cases, respectively. The best pregnancy rates were in Groups 1 and 2 (48.6%–48.0%), and the lowest pregnancy rate was in Group 3 (32.6%).

Table 5.

Characteristics of cryopreserved embryos

Variable	Group 1 n=111 (1)	Group 2 n=100 (2)	Group 3 n=89 (3)	Statistics
	M±SD; Me (Q1;Q3)			
Total number of frozen embryos	5.1±2.9 5(1; 6)	3.2±1.8 3(1;8)	3.1±1.8 3(1; 9)	P=0.0000 P_{1-2}=0.0000 P_{1-3}=0.0000 P_{2-3}=0.9509
Freezing of Day 3 embryos	16(14.5%)	9(9.0%)	16(18.2%)	P=0.1920
Freezing of Day 4 embryos	59(53.6 %)	34(34.0%)	30(34.1%)	P=0.0046
Freezing of Day 5 embryos	36(32.4%)	57(57.0%)	43(48.3%)	P=0,0013
Frozen embryo storage period at the time of thawing (months)	5.8±1.8 2(1;12)	9.4±2.1 5.5(1;32)	9.5±1.7 5(1;46)	P=0.0000 P_{1-2}=0.0000 P_{1-3}=0.0000 P_{2-3}=0.9291
Number of women who have frozen embryos after transfer	57(51.4%)	34(34.0%)	27(30.3%)	P=0.0042
Number of remaining frozen embryos	4.3±2.4 3(1;12)	2.6±1 2(1;6)	3±1.8 3(1;8)	P=0.0000 P_{1-2}=0.0000 P_{1-3}=0.0000 P_{2-3}=0.3006
«Day to day» cryotransfer	36(32.4%)	57(57.0%)	43(48.3%)	P=0.0013
Post-thaw cultivation of embryos	70(63.1%)	38(38.0%)	40(44.9%)	P=0.0008
Transfer of cryopreserved Day 4 embryos (morula)	5(4.5%)	4(4.0%)	6(6.7%)	P=0.6584
Transfer of cryopreserved Day 5 embryos (blastocyst)	106(95.5%)	95(95.0%)	83(93.3%)	P=0.770
Pregnancy rate	54(48.6%)	48(48.0%)	29(32.6%)	P=0.0423

Conclusion

The retrospective analysis of clinical, anamnestic and laboratory parameters in IVF patients with cryopreserved embryos revealed that the patients from the group of transfer cancellation due to OHSS prevention had a higher ovarian reserve, a larger number of eggs and fresh and frozen embryos, and shorter shelf life of frozen embryos. All embryos were of the best quality. During stimulation, lower amounts of gonadotropins were used.

Patients with a thin endometrium and previous IVF failures demonstrated slow growth of follicles, which required a higher course dose of gonadotropins with the addition of LH-containing preparations.

Regardless of the group, in most cases, frozen/thawed embryos were transferred at the blastocyst stage. The pregnancy rate was high in patients at OHSS risk and with a thin endometrium, which tops the figures for the Russian Federation (42.4% in 2018; 41.0% in 2017). Patients with IVF failure (32.6%) had a lower pregnancy rate; this is due to endometrial pathology in the medical history, a smaller number of antral follicles, oocytes, fresh and frozen embryos, and longer shelf life of frozen embryos.

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Conflict of Interests

The authors declare that they have no conflicts of interest.

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ORIGINAL ARTICLE

Reproductive Health

INTERNATIONAL JOURNAL OF BIOMEDICINE

Glutathione-Dependent Mechanisms of Antioxidant Defense in Men with Pathozoospermia after COVID-19 Infection

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Abstract

The article presents results of studying the influence of COVID-19 infections on glutathione metabolism in men with pathozoospermia and COVID-19. In erythrocyte cytolysate, we determined concentration of reduced (GSH) and oxidized (GSSG) glutathione and the GSH/GSSG ratio as the main indication of cell redox-status and an important factor in cell redox-dependent signaling control along with the effect GSH-dependent enzymes. Results of our study allowed us to determine the peculiarity of the influence of COVID-19 infection on thee glutathione metabolic pathway. The understanding of the influence mechanisms of SARS-CoV-2 on metabolic processes in the body of men with pathozoospermia has been expanded. The data obtained give grounds and opportunities for a more reasonable approach to the organization of preventive, curative, and rehabilitative measures for COVID-19 infection.(**International Journal of Biomedicine. 2021;11(4):543-545.**)

Key Words: infertility • men • COVID-19 • glutathione • oxidative stress

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Introduction

The infectious factor accounts for about 15% of the whole structure of reasons for men's infertility.[1] The appearance of new infections with COVID-19 made careful study of its possible consequences necessary. Men and women are equally susceptible to contracting COVID-19, but there is some evidence that men could be at risk for more complications.[2-4] Frequently, COVID-19 is diagnosed in reproductive-age men.[4-6] This fact dictates the importance of a thorough study of the potential impact and possible consequences of this disease on the male reproductive system.

The generation of a certain count of free radicals in cells promotes implementatioon of cell functions.[5,6] The majority of disturbances in reactions of free radical molecules with different peptides, lipids, and signal factors are determined in nosological forms.[7-9] Pathological changes in the glutathione status for a wide range of diseases can play an important role in determining the symptoms and severity of the process. The glutathione system provides a protective effect with the help of three components: antioxidant protection, detoxification, and immunostimulation.[10-12] The sulfhydryl group (SH) is the main tool of glutathione in implementing antioxidant and detoxification effects, and it is used as an electron donor in antioxidant neutralization reactions of more than 3,000 toxic oxidized substrates in the body. The most important role of glutathione as an antioxidant is explained by the high reducing potential of the molecule and the high intracellular concentration. The glutathione system restores peroxides, as well as products of peroxidation of lipids, membrane phospholipids, proteins, and nucleic acids and removes them from the body in the form of non-toxic conjugates.[12,13]

In addition, it regulates the synthesis and recovery of vitamins A, C, and D, and also acts as an immunomodulator, taking part in the activation of natural killer cells (NK cells) and T-lymphocytes.[4,6,14]

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The aim of this research was to study the effect of COVID-19 infection on glutathione metabolism in men with pathozoospermia.

Materials and Methods

The results of the examination of men (n=30; average age of 29.9 ± 5.3 years) with pathozoospermia after COVID-19 infection from infertile married couples (Group 1) were analyzed retrospectively. The comparison group included 38 men (average age of 30.2 ± 3.6 years) with pathozoospermia who did not have COVID-19 infection (Group 2).

Semen was analyzed in accordance with the WHO recommendations[15] (twice with a minimum interval of 2 weeks). Hemolysate prepared from red blood cells was used as the material for biochemical studies. Blood was sampled from the ulnar vein, on an empty stomach from 8 a.m. to 9 a.m., in accordance with the generally accepted procedure—two months after the patient received a negative PCR test for SARS-CoV-2. The concentration of GSH (mmol/L), GSSG (mmol/L) was determined in the erythrocyte cytolysate (Hissin P. Y. et al., 1976). The measurements were carried out on a spectrofluorimeter 02 ABFF-T (Russia).

Statistical analysis was performed using STATISTICA 6.1 software (Stat-Soft Inc., USA).

The study was carried out in compliance with Ethical Principles for Medical Research Involving Human Subjects, Adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, and amended by the 64th WMA General Assembly, Fortaleza, Brazil, October 2013. The study was approved by the Ethics Committee of the Scientific Center for Family Health and Human Reproduction Problems. Written informed consent was obtained from each patient.

Results and Discussion

Currently, in relation to more than 60 illnesses, a pathogenetic association with oxidative stress has been established.[16] Paying to attention the significance of glutathione in forming an antioxidant potential for the whole organism and support functional tone for the immune system in the active phase, we studied the influence of COVID-19 on the metabolic pathway of glutathione in men with pathozoospermia.

As a result of the study, a statistically significant increase in the level of GSH by 8% (P=0.001) and GSSG by 16% (P=0.000) was found in Group 1, compared to Group 2. In turn, the GSH/GSSG ratio in Group 1 was 10% lower than in Group 2 (P=0.01) (Fig.1).

Disturbances in the activity of thiol-dependent ensembles, according to modern concepts, are an indispensable companion of anomalies of spermatogenesis.[8] Specific features of glutathione activity are caused by a character of virus impact on the body when there is a quick need for large amounts of glutathione and its precursors.

The data obtained show that oxidative stress leads to a significant accumulation of GSSG in the body and its release into the blood. A lower level of GSH in Group 2 may be due to increased activity of glutathione peroxidase or a decrease in the activity of glutathione reductase. Glutathione peroxidase forms the first response to oxidative stress and performs the function of a scavenger during the leakage of ROS and the development of chains of uncontrolled processes. The increased content of GSSG in the blood of men of Group 1, in turn, can cause the oxidation and activation of the thiol groups of blood proteins and proteins of the basolateral membranes of tissue cells. In this regard, the process of catabolization and removal of an excessive accumulation of GSSG from the blood circulation is of particular biological importance. The concentrations of GSH and GSSG depend on the body's condition. In many pathological conditions, the GSH/GSSG rate changes towards the growth of oxidized forms, which was revealed in men with pathozoospermia after COVID-19 infection. Oxidative stress leads to a significant accumulation of GSSG in the body and its release into the blood, as evidenced by the data obtained. Numerous publications indicate that maintaining glutathione at a high level provides reliable, non-specific antiviral protection regardless of the type of virus and demonstrates immunomodulatory properties. There is evidence that glutathione inhibits the replication of various types of viruses at different stages of their life cycle, thereby reducing the viral load on the body and preventing a massive release of inflammatory cells in the lungs, including those due to glutathione's own anti-inflammatory properties.[17]

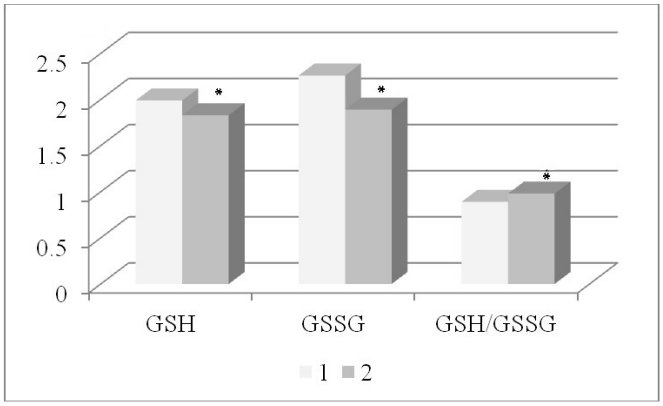

Fig. 1. *The level of GSH, GSSG, and the GSH/GSSG ratio in the study groups. *- P<0.05*

In conclusion, the analysis of the literature data indicates that among all the antioxidants in the body, it is glutathione that provides a stable basis for the normal functioning of the antioxidant system, and disturbances in the thiol-disulfide metabolism can cause serious disorders of various organs and systems. The study of the potential impact of the new coronavirus infection COVID-19 on the reproductive health of men is an extremely relevant topic. Further large-scale studies of various aspects of the pathogenesis of COVID-19 are needed. Determination of the total activity of the enzymes of the thiol-disulfide system is currently acquiring great diagnostic significance, since many proteins localized in various tissues and intracellular compartments have glutathione-S-transferase activity. The results of the conducted studies allowed us to determine the peculiarities

of the impact of a COVID-19 infection on the metabolism of glutathione – the main component of the body's antioxidant system. The data obtained give grounds and opportunities for a more reasonable approach to the organization of preventive, curative, and rehabilitative measures for COVID-19 infection.

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Competing Interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

Pediatrics

INTERNATIONAL JOURNAL OF BIOMEDICINE

The Hygienic Assessment of School Educational Programs

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Abstract

Background: The study presents a methodology for the hygienic assessment of school educational programs from the point of view of safety for the health of students and of effectiveness in improving the quality of education. The aim of the study was a hygienic assessment of educational programs in primary schools.

Methods and Results: The study involved primary school students (n=245) aged between 8 and 9.5 years: 120 children studying under the "Planet of knowledge" program (Group 1) and 125 children studying under the "Primary school of the XXI century" program (Group 2). The hygienic factors are studied using the methodology of assessing the intensity of educational work and the conditions of the organization of the educational process. The obtained data are compared with the main criteria of the state of children's neuropsychological development. We found that the program "Primary school of the XXI century" is characterized by the intensity of educational work (2.7±0.13 points) and approaches to the third degree, according to the criterion of intellectual loads. In contrast, the program "Planet of knowledge" is characterized by a lower intensity of educational work – 2.1±0.08 points (P=0.000) . The indicators of sensory and emotional intensity of educational work under the program "Primary school of the XXI century" were also statistically higher.

Conclusions: The high intensity of educational work does not ensure high rates and levels of intellectual development and mental performance of children. The hygienic assessment of children's educational activities should include a comprehensive hygienic examination of the educational program and the means used in the process of its implementation. An educational program may be allowed to be used in educational organizations only after a hygienic examination of its application in the educational process.(**International Journal of Biomedicine. 2021;11(4):546-550.**)

Key Words: schoolchildren • educational activity • educational programs • hygiene • neuropsychological development

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Introduction

The education system has undergone serious changes over the past decades. The modernization allowed the use of innovative pedagogical technologies, which ideally should have provided an individual approach and improved the quality of education. Despite the measures taken and the increasing educational load, the state of children's health is deteriorating while the quality of education is not improving.[1,2] Many methods have been developed and studies are being conducted to assess the achievements of students and the reasons for the poor quality of education.[3] Therefore, understanding the causes of the deterioration in children's health and the poor quality of education is the primary task of building an education development strategy.

First of all, the search for solutions to educational problems should be sought in accordance with the age characteristics of children (physical, mental, social) and the hygienic conditions of the organization of the educational process, the core of which is pedagogical technology. This means that the hygienic assessment of pedagogical technology is a key link in an organization's choice of such technology.

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Modern researchers have shown that a high level of intensification of education (provided for by the training technology) leads to a decrease in educational results due to a decrease in students' health, such as disorders of the nervous system, manifestations of deviant behavior, mental disorders, hyperactivity and a decrease in motivation for academic work. In combination with an increase in the level of informatization, the devaluation of fundamental disciplines (since informatization prefers the "final result") leads to a decrease in the level of a student's cognitive activity.[1,4-6]

Educational organizations are increasingly using modern, innovative pedagogical technologies and programs aimed at improving the quality of education and meeting the needs of society for the education and socialization of children.

In this regard, the study of the hygienic characteristics of pedagogical technology and educational programs becomes particularly relevant, justifying the use of the study results by educational organizations.

The search for scientific approaches to studying the hygienic characteristics of educational programs allowed us to formulate the purpose of this study. The aim of the study was a hygienic assessment of educational programs in primary schools.

Materials and Methods

Our study was conducted in the primary classes of the children's education center No.47 and of the secondary school No.67 in Irkutsk. Hygienic conditions in educational institutions met the requirements of sanitary rules and did not differ significantly statistically.

The study involved primary school students (n=245) aged between 8 and 9.5 years: 120 children studying under the "Planet of knowledge" program (Group 1) and 125 children studying under the "Primary school of the XXI century" program (Group 2).

The peculiarity of the training system under "Planet of knowledge" is the creation of an integral educational space, the unity of conceptual approaches, and the achievement of high learning results due to the effective combination of regular and extracurricular activities.

The peculiarity of the training system under "Primary school of the XXI century" is the orientation to the individual capabilities and characteristics of the child; the priority of problem-research activities, taking into account the pace of student advancement; the correction of emerging difficulties, the formation of creative thinking and imagination, and high erudition and cultural background.

Groups were formed by a continuous method. The children in the studied groups had no acute and decompensated chronic diseases or congenital pathology; sexual development corresponded to age; the level of physical activity was average – physical culture classes within the school curriculum.

Statistically significant differences in the physical development of the children were not identified

This study is based on the methodology we developed earlier for assessing the intensity of the educational work of schoolchildren.[7] The methodology is based on an assessment of the student's working conditions, as a set of factors of the educational process, and the educational environment in which the student's activities are carried out. The assessment of the intensity of the schoolchildren's work characterizes the educational process, reflects the load mainly on the central nervous system, the sensory organs, and the emotional sphere of the child.[7]

The monotony of loads was assessed by timing observations of schoolchildren during a typical school week. The assessment was carried out throughout the school year. The mode of educational activity was evaluated according to the actual duration of the study time (taking into account the educational process at school, the system of additional education and the preparation of homework). The sensory and emotional loads were assessed in accordance with different levels of complexity of educational activities.[7]

Tension was assessed in points and was based on the average score of all the criteria for the intensity of educational work: Thus, the light degree of educational work corresponded to 1 point, the average degree – 2 points and the heavy degree – 3-4 points.[7]

The assessment was carried out by observing groups of children over time, as well as individual surveys of children, parents, and teachers. At the same time, all types of educational activities were taken into account, including homework and work in circles and sections of additional education.

The obtained indicators were correlated with indicators of intellectual development (according to the Raven test),[8] mental performance (according to V.Ya. Anfimov)[9] and memory.[10]

The indicators of intellectual development were evaluated by means of the Raven test. At first, the assessment was carried out in points, then the percentage of completed tasks was estimated. At the same time, 5 degrees of intellectual development were distinguished: Degree 1 (high intelligence) – more than 95% of test tasks were completed; Degree 2 (above average intelligence) – 75%-94%; Degree 3 (average intelligence) – 25%-74%; Degree 4 (below-average intelligence) – 5%-24%; Degree 5 (defect) – below 5%.[11] The results of the series in the Raven test were evaluated separately in order to identify differentiated indicators of intellectual development: The ability to determine the principle of interrelation in the structure of matrices (A series), the principle of analogy between pairs of figures (series B), the principle of progressive changes in the figures of matrices (C series), the principle of rearrangement of figures (D series), and the principle of decomposition of figures into elements (E series) were distinguished.

The evaluation was carried out by the number of errors made and the number of rows viewed. Each missing line was equated to one error. The productivity coefficient Q was calculated using the formula:

$$Q=c^2/c+d,$$

where c is the number of rows viewed; d is the number of errors (errors were not standardized).[9]

The efficiency was evaluated according to V.Ya. Anfimov's curly tables.[9]

The study was conducted in accordance with ethical principles of the WMA Declaration of Helsinki (1964, ed. 2013). Written informed consent was obtained from the participant's parent/guardian.

Statistical analysis was performed using the Statistica 10 software package (Stat-Soft Inc., USA). Analysis of the distribution of values obtained was performed using the Kolmogorov-Smirnov test. The mean (M) and standard error of the mean (SEM) were deduced. For data with normal distribution, inter-group comparisons were performed using Student's t-test. Differences of continuous variables departing from the normal distribution were tested by the Mann-Whitney U-test. The frequencies of categorical variables were compared using Pearson's chi-squared test. A probability value of $P<0.05$ was considered statistically significant.

Results

The study showed that the intensity of educational work was higher in children studying under the program "Primary school of the XXI century" (Table 1). The intellectual loads under this program amounted to 2.7±0.13 points (Group 2), which was close to the third class of intensity of educational work due to the use of an approach aimed at improving student erudition by mastering a wide range of concepts, developing projects, and solving non-standard tasks. In Group 1, the intellectual loads were determined by the performance of simple tasks that were distributed both for the regular and extracurricular activities and amounted to the second class of the intensity of educational work (2.1±0.08 points).

Table 1.

The intensity of educational work, points

Variable	"Planet of knowledge" (n=120)	"Primary school of the XXI century" (n=125)	*P*-value
Intellectual loads	2.1±0.08	2.7±0.13	0.000
Sensory loads	1.8±0.04	1.6±0.05	0.002
Emotional loads	1.2±0.01	1.4±0.07	0.005
Monotony of loads	1.6±0.05	1.8±0.06	0.011
Operating mode	1.5±0.04	1.8±0.05	0.000

The sensory loads in both groups were determined by the duration of the concentration time, the teaching tools used during the lesson as well as the time spent watching the video terminal monitors. In Group 1, the sensory loads were 1.6±0.05 points and were close to the second class of the intensity of educational work, and in Group 2 – 1.8±0.04 points (P=0.002).

The emotional loads during the work on the program «Primary school of the XXI century» were higher due to the importance of the assessment for the student and individual responsibility for the result, and amounted to 1.2±0.01 points in Group 1, and 1.4±0.07 points in Group 2. In both groups of the study, the indicator of emotional loads corresponded to the first class of intensity of educational work but when compared, it was statistically different (P=0.005).

The obtained data on the intensity of educational work allowed us to correlate the indicators of the intensity of educational work and the results of the neuropsychic development of children studying under various programs (Table 2).

Table 2.

Conditions and learning outcomes (the intellectual loads)

The training program	The level of tension (the intellectual loads), points	The level of intelligence, points
"Planet of knowledge"	2.1±0.08	24.9±0.7
"Primary school of the XXI century"	2.7±0.13	23.1±0.7
P-value	0.000	0.07

The indicators of the Raven test (the percentage of completed tasks) did not increase during training with an increased intellectual load, as one would expect (Table 3).

Table 3.

The indicators of the Raven test, points

Raven's progressive table series	"Planet of knowledge"	"Primary school of the XXI century"	*P*-value
A series	9.9±0.4	9.8±0.4	0.860
B series	7.1±0.5	5.9±0.4	0.062
C series	2.5±0.3	2.4±0.2	0.782
D series	4.3±0.3	4.1±0.3	0.638
E series	1.1±0.2	0.9±0.2	0.480
Total	24.9±0.6	23.1±0.6	0.035

The study showed an inverse relationship between the number of completed tasks and the intensity of intellectual loads. The qualitative analysis of the Raven test results showed that the application of the principle of correlation in the structure of the matrices, the analysis of the structure of the base image and the detection of these features in one of a few

fragments, and the merger of the fragment and its comparison with the surroundings of the main part of the test pattern are equally well formed by this training program.

The principle of identifying the analogies used in the series B scored slightly better in children of Group 1 and was 7.1±0.5 points compared to 5.9±0.4 points in Group 2.

The C series of the Raven test involves identifying the principle of complication of figures; once students have identified this principle, they can pick out the missing figure. There were no statistically significant differences in this indicator.

The D series determines the ability to speculatively rearrange the figures in the matrix, both in horizontal and vertical positions. There were also no statistically significant differences in this series.

In the E series, the missing figures can be found by understanding the principle of analysis and synthesis of figures. This series was the most difficult to complete. Children of Group 2 had slightly lower results: 0.9±0.2 points against 1.1±0.2 points in Group 1.

The absolute indicators (Table 3) of intelligence in the Raven test, while studying according to a standard training program, was statistically significantly higher and had a value of 24.9±0.6 points, compared to 23.1±0.6 points (P=0.035) while studying according to enriched training programs.

The study of the mental performance of younger schoolchildren showed that studying under the program «Primary school of the XXI century» leads to an increase in the number of mistakes (17.5±0.6 in Group 2 versus 13.1±0.4 in Group 1, P=0.000), which may indicate chronic fatigue (Table 4). The productivity of mental labor under this training program is lower than when studying under the program "Planet of knowledge."

Table 4.

The indicators of mental performance

The indicators	"Planet of knowledge"	"Primary school of the XXI century"	*P*-value
The number of rows viewed (c)	14.3±0.3	14.5±0.3	0.638
The number of errors made (d)	13.1±0.4	17.5±0.6	0.000
The productivity of intellectual work (q)	7.5±0.5	6.6±0.4	0.161

Discussion

The study of the hygienic characteristics of school education shows that indicators of the intensity of educational activity can have a significant impact on the state of the nervous system (intellectual development, memory, mental performance). Significant changes in the indicators of the nervous system's functioning are observed when the indicator of the intensity of educational work approaches the third-degree severity. The constant impact of educational stress on the child's body subsequently leads inevitably to health disorders.[1]

Considering the high intensity of educational work, it is necessary to conduct a hygienic assessment of school education programs, which in conditions of school stress can have a serious negative impact on the health of children, without increasing the training and quality of acquired knowledge. Our research has shown that high intensity educational work, which means a higher level of stress, does not necessarily lead to higher academic achievements but leads to "chronic educational stress."

The intensity of educational work is largely determined by pedagogical technologies and programs, which include both the conditions of the educational process and the means of teaching.

Due to the hygienic examination of the educational program, it is possible to predict its impact on the health of children and on the educational effect.

As hygienic criteria for studying the educational program, it is proposed to use the intensity of educational work.

As sensitive criteria, we propose to use the pace of physical development, intellectual development, and mental performance.

Conclusions

1. The high intensity of educational work does not ensure high rates and levels of intellectual development and mental performance of children.

2. The hygienic assessment of children's educational activities should include a comprehensive hygienic examination of the educational program and the means used in the process of its implementation.

3. An educational program may be allowed to be used in educational organizations only after a hygienic examination of its application in the educational process.

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Competing Interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

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Subjective Assessment of Stress and its Relationship with Neuroendocrine Mechanisms of its Development in Obstetricians-Gynecologists against the Background of Professional Burnout

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Abstract

The purpose of this case-control study was to investigate the factors of subjective assessment of stress and their relationship with neuroendocrine mechanisms of its development in obstetricians-gynecologists against the background of professional burnout.

Methods and Results: A total of 96 physicians and nurses from the different clinics specializing in both obstetrics and gynecology were surveyed. The Russian versions of MBI, BDI, SF-12, FFMQ, MAAS, and Coping strategies (the Ways of Coping Checklist) were applied. Blood serum/plasma was tested on the concentration of hormones (DHEA-C and TSH), melatonin, serotonin, and dopamine. Saliva cortisol was also estimated. In the present study, 43.75% of the physicians and nurses showed a high degree of burnout, which was comparable to that among physicians and nurses in other studies. Physicians and nurses with a high degree of burnout had more expressed coping strategies like Confrontive coping, Distancing, Self-controlling, Seeking social support and Escape-avoidance. Also, they have more expressed level of depressive manifestations. We found significant correlations between some factors of subjective assessment of stress (like coping and mindfulness) and neuroendocrine biomarkers. Adaptive coping like Planful problem-solving correlated negatively with the level of melatonin, and subscales of the mindfulness questionnaire were correlated negatively with levels of some biomarkers. Thus, we concluded that coping strategies and mindfulness could theoretically contribute to a decrease in the secretion of several hormones.

Conclusion: Physicians and nurses with a low degree of burnout have a greater level of mindfulness and a lower level of some maladaptive coping strategies – Confrontive coping, Distancing, Escape-avoidance. Our results focus on the predictive role of these factors of subjective assessment of stress, in particular, Confrontive coping and mindfulness, in burnout syndrome. The present data confirm that there are some psychological and physiological aspects related to stress in the medical profession. (**International Journal of Biomedicine. 2021;11(4):551-557.**)

Key Words: obstetricians-gynecologists • stress • professional burnout • biomarkers

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Introduction

Burnout is a state of physical and emotional exhaustion, depersonalization, and a decreased sense of personal accomplishment caused by work-related stress. It is an outcome of chronic depletion of the individual's coping resources resulting from prolonged exposure to stress, particularly work-related stress.[1]

This problem is relevant for representatives of various professions, including medical workers. Its prevalence can be judged, for example, according to Rodrigues et al.,[2] who indicate that burnout is characteristic of workers in general surgery, anesthesiology, obstetrics/gynecology and orthopedics - 40.8%; plastic surgery and pediatrics - 30.0%; otolaryngology and neurology - 15.4%.

The estimates of the prevalence of burnout in ObGyn vary widely but they remain high - 39%,[3] 50%,[4] and up to

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75%.[5] As a consequence of burnout, healthcare professionals may develop symptoms such as anxiety, irritability, mood swings, insomnia, depression, and a sense of failure.[6-8]

Some theoretical bases explain the relationship between stress as a psychological factor and exhaustion. Several studies in the medical area have analyzed the relationship between socio-demographic, occupational, and personality variables and the occurrence of burnout syndrome. The factors that prevent burnout are job satisfaction[3] and self-efficacy.[9] One of the strategies that can prevent burnout is the subjective assessment of stress, which is associated with personality variables such as extraversion, conscientiousness, and openness,[10] coping strategies, and mindfulness.[11] The most commonly used programs are mindfulness programs.[12-14]

Other studies have analyzed the relationship between psychological parameters of burnout symptoms and neuroendocrine mechanisms of stress.[15-20] There are still many possible neuroendocrine mechanisms between burnout and stress that are being discussed. Since burnout is generally the result of a prolonged period of stress, it is often hypothesized that the hypothalamic-pituitary-adrenal (HPA) axis, a part of the neuroendocrine system involved in the regulation of stress reaction, may be disturbed in individuals suffering from burnout. Chronic exposure to stressors can contribute to permanent HPA axis activation. As the major output of the HPA axis is the stress hormone cortisol, cortisol levels are considered to be different among subjects with burnout than among healthy people.[15] Also, chronic stress is associated with hyperstimulation of the hypothalamic-pituitary-thyroid (HPT) axis and related to thyroid function. Besides, the sympathetic-adrenal-medullary axis stimulates adrenal glands to release catecholamine (eg, dopamine). It has been reported that the level of DHEA-S has been associated with burnout.[21] Also, since the level of stress in obstetricians and gynecologists is closely related to night shifts, the level of their burnout may be related to the level of melatonin.

Some studies have shown how different biomarkers predict burnout.[15,12,22] But in general, according to Danhof-Pont et al.,[23] no potential biomarkers for burnout can be found, largely due to the incomparability of different studies (study designs and methods, including the characters of patients, assess biomarkers and control for confounders). There is also a discussion of the context in which factors of subjective assessment of stress, like coping strategies and mindfulness, influence burnout.[11,24-26]

It has not been shown how these factors of subjective assessment of stress are connected with neuroendocrine mechanisms of stress that predict the development of burnout in obstetricians and gynecologists. In turn, the research into these factors and their relationship with stress biomarkers could contribute to the creation of a program to prevent burnout among physicians and nurses employed in obstetrics and gynecology.

Therefore, the purpose of this case-control study was to investigate the factors of subjective assessment of stress and their relationship with neuroendocrine mechanisms of its development in obstetricians-gynecologists against the background of professional burnout.

Materials and Methods

Participants

We invited 181 people to participate in the study, 85 of whom were excluded because they did not meet inclusion criteria (n=37) or declined to participate (n=48). A total of 96 physicians and nurses from the different clinics specializing in both obstetrics and gynecology were surveyed. They were selected personally and in a consecutive manner by a researcher after contact with all of the professionals working on the units.

The inclusion criteria were as follows: fully completed questionnaires and the results of hormonal studies, age over 18 years. All participants were given a document about the objectives and procedures of the study.

Instruments

Symptoms of burnout were measured with the Russian version of the Maslach Burnout Inventory (MBI).[27] This instrument is currently the most commonly used for evaluating burnout in healthcare professionals. The MBI consists of 22 elements. The MBI's three subscales were analyzed separately: Emotional exhaustion (EE), Depersonalization (DP), and Personal accomplishment (PA). Mean values were calculated and subscales were categorized into "low," "moderate," and "high" degrees of burnout using the cut-off values suggested by the Russian adaptation.[28] For the EE subscale, this translates into ≤15, 16–24, and ≥25 points, respectively; for the DP subscale - ≤5, 6–10 and ≥11 points, respectively; and for the PA subscale - ≤30, 37–31, and ≥37 points, respectively. Higher scores on the subscales EE and depersonalization indicate a higher degree of burnout, while a higher score on the subscale PA indicates a lower degree of burnout.

Coping strategies were measured with the Ways of Coping Checklist, Russian adaptation.[29] The Russian version of this psychometric test consists of 50 questions and eight subscales – Confrontive coping, Distancing, Self-controlling, Seeking social support, Accepting responsibility, Escape-avoidance, Planful problem-solving, Positive reappraisal. Each answer was assigned from 0 to 3 points from "never" to "often." Coping strategies where the subject scored the highest score are considered to be leading.

We also used the 21-item Beck Depression Inventory (BDI) and SF-12 questionnaire (Russian version).[29] The 21-item BDI) measures depressive symptoms. This psychometric test consists of 21 questions regarding the subject's recent mood with each answer being assigned from 0 to 3 points. The SF-12 questionnaire is the short form of the SF-36 Health Survey, which measures adequate physical and mental health summary scores.

In addition, we used the Russian version of the Five Facet Mindfulness Questionnaire – FFMQ[30] and Mindful Attention Awareness Scale, MAAS.[31] The first questionnaire consists of 39 statements with a choice of assessment on a 5-point Likert scale from "never or very rarely true" to "very often or almost always true." It includes five factors: Describe (name, ability to turn one's emotions, sensations, thoughts into words), Non-judging of inner experience, Non-reactivity to inner experience, Act with awareness (concentration,

action "not automatically"), Observe (attention to sensations, feelings, thoughts). The second questionnaire contains 15 statements. The gradation of answers from "almost always" to "almost never" is used.

The blood serum, blood plasma, and saliva were used as the biomaterials. Blood serum was tested on the concentration of hormones: DHEA-C and TSH using Alkor Biotest systems (Russia), melatonin using the ELISA Kit for Melatonin (Cloud-Clone Corp., USA), and serotonin using Serotonin ELISA (IBL-International, Germany). Quantitative determination of plasma dopamine was measured using Dopamine ELISA(IBL-International, Germany). Saliva cortisol was estimated using a Cortisol Saliva ELISA(DBC- Diagnostics Biochem Canada Inc.). Measurements were made using ELx808™ Absorption Microplate Reader (BioTek Instruments, Inc., VT, USA).

Procedures

All participants were invited to the study on a voluntary basis. First, before the survey, the purpose of the research was clarified. The participants were instructed on how to fill out the questionnaires and informed that the survey would not have any influence on their work or personal life. Second, socio-demographic information was obtained by a researcher in order to determine whether they met the inclusion criteria. Third, participants signed the written informed consent form and completed the self-administered evaluation and all the above-mentioned questionnaires. Fourth, participants gave saliva and blood. Before any therapy was prescribed, in the fasted state, from 8 a.m. to 9 a.m., after a 15-minute rest, blood was sampled from a median cubital vein, using disposable vacuum blood collection tubes; 4–5 mL of saliva was collected into a clean special test tube (SaliCaps, IBL International GmbH, Hamburg, Germany) without force or inducement and before eating, drinking, or brushing the teeth. Before sampling,, the mouth was simply rinsed out with water. All samples were then stored at 4°C until sent to the laboratory.

Data Analysis

All data have been entered into the REDCap system.[1] Statistical analysis was performed using the IBM SPSS Statistics V23.0. The normality of distribution of continuous variables was tested by one-sample Kolmogorov-Smirnov test. Continuous variables with normal distribution were presented as mean (standard deviation [SD]); non-normal variables were reported as median (interquartile range [IQR]). Means of 2 continuous normally distributed variables were compared by independent samples Student's t test. Mann-Whitney U test was used to compare means of 2 groups of variables not normally distributed. The frequencies of categorical variables were compared using Pearson's chi-squared test or Fisher's exact test, when appropriate. A multiple logistic regression model was made to identify predictive factors (independent variables) of burnout (dependent variables). The results are shown as odds ratios (OR.) with 95% confidence intervals (95% CI) for Exp (B). The fit of the models was judged by the likelihood ratio test statistic. A probability value of $P<0.05$ was considered statistically significant.

The study was carried out in compliance with Ethical Principles for Medical Research Involving Human Subjects, Adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, and amended by the 64th WMA General Assembly, Fortaleza, Brazil, October 2013. The study was approved by the Ethics Committee of the Scientific Center for Family Health and Human Reproduction Problems. Written informed consent was obtained from each participant.

Results

The socio-demographic characteristics of the study sample are presented in Table 1.

Table.1.

Socio-demographic characteristics of the study sample

Variable		Total sample	High burnout (n=42)	Low burnout (n=54)
Age; yrs, mean±SD		40.87±11.51	39.81±11.6	42.15±10.28
Sex, n (%)	Male	10(10.41)	5(11.9)	5(9.3)
	Female	86(89.58)	37(88.1)	49(90.7)
Ethnic group, n (%)	Caucasian	82(85.5)	37(88.1)	45(83.3)
	Asian	14(14.5)	5(11.9)	9(16.7)
	Mixed-race	0(0)	0(0)	0(0)
	Not specified	8(10.39)	8(10.39)	8(10.39)
Professional affiliation, n (%)	Physician	61(63.54)	30(71.43)	31(57.4)
	Nurse	35(36.46)	12(28.57)	23(42.6)
Work experience, n (%)	<1 year	0(0)	0(0)	0(0)
	1-5 years	20(20.83)	12(28.6)	8(14.8)
	6-10 years	13(13.54)	7(16.7)	6(11.1)
	11-20 years	25(26.04)	10(23.8)	15(27.8)
	21-30 years	26(27.08)	8(19.0)	18(33.3)
	31-40 years	9(9.38)	4(9.5)	5(9.3)
	>40 years	3(3.13)	1(2.4)	2(3.7)
	Not specified	0(0)	0(0)	0(0)
Number of working hours per week, Median (Q1, Q3)		40 (39;48.75)	40 (39;51.5)	40 (39;40.75)
Number of night shifts per month, Median (Q1, Q3)		0(0;2)	0(0)	0(0;3.75)
Marital status, n (%)	Unmarried	15(15.63)	6(14.3)	9(16.7)
	Common law marriage	13(13.54)	5(11.9)	8(14.8)
	Separate accommodation with a partner	1(1.04)	1(2.4)	0(0)
	Married	49(51.04)	22(52.4)	27(50.0)
	Divorced	18(18.75)	8(19.0)	10(18.5)
	Not specified	0(0)	0(0)	0(0)
Religiosity, n (%)	I prefer not to answer	13(13.54)	3(7.1)	10 (18.5%)
	Not religious	23(23.96)	11(26.2)	12 (22.2%)
	Religious	60(62.50)	28(66.7)	32 (59.3%)
	Not specified	0(0)	0(0)	0 (0.00%)

A total of 96 physicians and nurses of ObGyn were included in this study. Of these, 89.58% were women and 10.41% men, mostly Caucasian (85.5%), married (51.04%), and religious (62.5%). The average age was 40.87±11.51 years, with an age range of 22–69 years, and clinical work experience mean of 17.4±11.2years. About 30.2% of the physicians and nurses worked night shifts.

First of all, we found that in the sample, the EE subscale score was 25.33±11.22. This value was high for this scale. The DP subscale score was 11.09±7.28, which meant a high level of expression. Finally, the PA subscale score was 38.69±6.69, which meant a high level of expression. Thus, subjects primarily exhibited high levels of EE and DP rather than professional reduction.

There are different criteria for diagnosing burnout.[15,20] We use criteria from Deneva et al.[15] with the amendment for the Russian version of the MBI. A diagnosis of burnout (yes/no) was assigned if respondents presented high levels in at least two subscales (either EE and/or DP, associated or not with low PA) or in three subscales based on the following scores: EE>24, DP>10, and PA<30. According to this, 43.75% of the physicians and nurses indicated a higher degree of burnout and 56.25% had a lower degree of burnout.

We found that there were no significant differences in socio-demographic characteristics between physicians and nurses with a high degree of burnout and those without it (Table 1). No significant differences were found between physicians and nurses employed on night shifts and those not employed on them, or who have different family status and work experience, although these parameters are considered as burnout factors.[32]

In the next step, we found significant differences in psychological characteristics between physicians and nurses with a high degree of burnout and those without it (Table 2). We found statistically significant differences between studied groups: physicians and nurses with a high degree of burnout have more expressed coping strategies like Confrontive coping (*P*=0.006), Distancing (*P*=0.003), Self-controlling (*P*=0.019), Seeking social support (*P*=0.031) and Escape-avoidance (*P*=0.000). Also, they have more expressed level of depressive manifestations (*P*=0.000).

On the contrary, physicians and nurses with a low degree of burnout appreciate the quality of their lives more and have a greater level of mindfulness – in MAAS scale (*P*=0.000) and subscales of FFMQ like Non-judging (*P*=0.038), Non-reactivity (*P*=0.004), and Observe (*P*=0.050).

The correlation analysis (Table 3) between biomarkers and personality variables showed a positive correlation between symptoms of depression (by BDI) and saliva cortisol (r=0.237, *P*=0.05), DHEA-S (r=0.4, *P*=0.01), as well as negative correlations between coping Planful problem-solving and the level of melatonin (r=-0.232, *P*=0.05), subscales of FFMQ like Describe and the level of DHEA-S (r=-0.304, *P*=0.05), Act with awareness and levels of DHEA-S (r=-0.238, *P*=0.05), salvia cortisol (r=-0.207, *P*=0.05) and melatonin (r=-0.247, *P*=0.05).

Multivariate logistic regression analysis (Table 4) was performed to identify predictive factors (independent variables) of burnout (dependent variable). We use all possible predictive factors—psychological (copings, components of mindfulness, depression), demographics (gender, age, religion, marital status, work experience, work hours, and night shifts), and biomarkers (Salivary cortisol, TSH, DHEA-S, Dopamine, Serotonin, Melatonin**).** The logistic regression model was statistically significant (chi-square = 34.506, *P*=0.001). The model explained 59% (Nagelkerke's R^2) of the variance in exhaustion and classified correctly 74.4% of the cases. With the increase of confrontation, depression symptoms, and decrease of mindfulness (MAAS scale), the probability of exhaustion increased.

Table 2.

Differences in psychological characteristics between physicians and nurses with a high degree of burnout and those without it

Scales		M	SD	t	P
Confrontive coping	High burnout (n=42)	9.02	2.909	2.817	.006
	Low Burnout (n=54)	7.39	2.750		
Distancing	High burnout (n=42)	10.26	2.820	3.039	.003
	Low Burnout (n=54)	8.31	3.324		
Self-controlling	High burnout (n=42)	14.33	2.515	2.384	.019
	Low Burnout (n=54)	12.59	4.178		
Seeking social support	High burnout (n=42)	12.07	2.815	2.194	.031
	Low Burnout (n=54)	10.78	2.905		
Accepting responsibility	High burnout (n=42)	7.21	2.170	.964	.338
	Low Burnout (n=54)	6.76	2.387		
Escape-avoidance	High burnout (n=42)	13.14	2.951	4.525	.000
	Low Burnout (n=54)	9.74	4.117		
Planful problem-solving	High burnout (n=42)	13.07	2.840	.351	.726
	Low Burnout (n=54)	12.83	3.612		
Positive reappraisal	High burnout (n=42)	12.95	3.428	-.735	.464
	Low Burnout (n=54)	13.44	3.112		
SF-12	High burnout (n=42)	70.29%	13.31%	-4.385	.000
	Low Burnout (n=54)	80.81%	10.19%		
BDI	High burnout (n=42)	11.74	7.130	4.410	.000
	Low Burnout (n=54)	6.00	5.623		
Describe	High burnout (n=42)	22.69	5.000	.195	.846
	Low Burnout (n=54)	22.47	5.750		
Non-judging	High burnout (n=42)	29.55	5.886	-2.109	.038
	Low Burnout (n=54)	31.85	4.753		
Non-reactivity	High burnout (n=42)	25.81	7.865	-2.931	.004
	Low Burnout (n=54)	29.83	5.487		
Act with awareness	High burnout (n=42)	27.60	5.623	-.661	.510
	Low Burnout (n=54)	28.36	5.565		
Observe	High burnout (n=42)	20.52	3.983	-1.972	.050
	Low Burnout (n=54)	22.28	5.145		
MAAS	High burnout (n=42)	62.10	8.114	-4.137	.000
	Low Burnout (n=54)	69.74	9.542		

Table 3.

Pearson correlation between biomarkers and psychological factors (n=96)

Variable	TSH	DHEA-S	Dopa-min	Seroto-nin	Saliva cortisol	Mela-tonin
Confrontive coping	.046	-.007	.046	.015	.170	.099
Distancing	.106	-.039	.006	,200	.090	-.005
Self-controlling	.103	.114	.015	.038	.079	-.140
Seeking social support	.198	.054	.058	-.004	.028	.100
Accepting responsibility	.136	-.200	-.019	.118	-.053	-.059
Escape-avoidance	.136	.140	.062	.094	.116	.048
Planful problem-solving	.123	-.072	-.019	.190	.050	-.232*
Positive reappraisal	.060	-.096	-.049	.166	-.035	-.204
SF-12	-.086	-.200	-.114	.030	-.158	-.116
BDI	.119	.400**	.125	.103	.237*	.036
Observe	-.027	.024	.040	-.032	-.018	-.121
Describe	.063	-.304*	-.137	-.119	-.092	-.206
Act with awareness	-.101	-,238*	.043	-.001	-.207*	-.247*
Non-judging	-.008	-.012	-.196	-.134	.037	.083
Non-reactivity	.099	-.128	.020	.069	.001	-.161
MAAS	-.094	-.184	-.091	-.160	.001	-.148

*** Correlation is significant at the 0.01 level (two-tailed)*

** Correlation is significant at the 0.05 level (two-tailed)*

Table 4.

Model of multiple logistic regression

Factors	B	S.E.	Wald	df	P	OR	95% CI	
							Lower	Upper
Confrontive coping	.287	.111	6.728	1	.009	1.333	1.073	1.655
MAAS	-.071	.029	5.866	1	.015	.931	.879	.986
BDI	.128	.043	8.817	1	.003	1.136	1.044	1.236

Discussion

The present study about burnout and its relationship with psychological (first of all coping strategies and mindfulness) and socio-demographic factors among physicians and nurses employed in ObGyn is the first large-scale survey in the Siberia region. In the present data, 43.75% of the physicians and nurses showed a high degree of burnout, which was comparable to that among physicians and nurses in other studies.[2-4]

In this study, a higher percentage of physicians and nurses exhibited high scores of EE and DP. These results could be connected with the place of employment and the nature of the job: 24-h work, nightshifts, emergency conditions, COVID-19 pandemic. But we could not find any significant differences between the results of physicians and nurses with a high degree of burnout and those without it in work experience, night shifts, and other factors, which usually are predictors of burnout. We attribute this to the fact that other studies have not taken into account psychological factors, such as coping strategies and mindfulness, which can help (or not) to cope with burnout. Conceivably, these factors allow physicians and nurses to maintain a high level of PA, as shown in our study.

As we mentioned above, there are different opinions about the role of coping and mindfulness in reducing burnout. In one study,[11] it was concluded that coping skills may not mitigate physician EE in some situations; in another the authors concluded that coping is a significant factor in preventing burnout. In this study, we found that not all ways of coping seem to be able to cope with burnout.[24,26]

According to the results of logistic regression and comparison of physicians and nurses with a high degree of burnout and those without it, Confrontive coping and, supposedly, coping strategies like Distancing and Escape-avoidance do not discourage but encourage burnout.

Various studies describe maladaptive coping strategies, including Escape-avoidance and similar behavior.[33-35] Susan Folkman and Richard S. Lazarus pointed out that in different situations Confrontive coping and Distancing could be both adaptive and maladaptive.[36] Supposedly, for physicians and nurses employed in ObGyn these coping strategies are maladaptive.

Although some studies show that mindfulness does not help burnout,[24] many others have shown that developing mindfulness prevents burnout.[7,12,13] This disagreement is also shown in our model.

We found significant correlations between some factors of subjective assessment of stress (like coping and mindfulness) and neuroendocrine biomarkers. Although other studies have attempted to connect psychological factors and biomarkers,[15] usually those factors were depression and anxiety. We found that adaptive coping like Planful problem-solving correlated negatively with the level of melatonin, and subscales of the mindfulness questionnaire were correlated negatively with levels of some biomarkers. Thus, we concluded that coping strategies and mindfulness could theoretically contribute to a decrease in the secretion of several hormones. But this assumption requires further confirmation.

We plan to use our results and the regression model to create a program to help physicians and nurses in obstetrics and gynecology reduce burnout.

In conclusion, our results indicate that there are differences in some personality and psychological factors between physicians and nurses in obstetrics and gynecology with a high degree of burnout and those without it. Physicians and nurses with a low degree of burnout have a greater level of mindfulness and a lower level of some maladaptive coping strategies – Confrontive coping, Distancing, Escape-avoidance. Our results focus on the predictive role of these factors of subjective assessment of stress, in particular, Confrontive coping and mindfulness, in burnout syndrome. The present data confirm that there are some psychological and physiological aspects related to stress in the medical

profession. Indeed, it may be relevant for further research to implement prevention programs aimed at reducing the negative aspects of professional distress and preventing burnout.

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Competing Interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

Epidemiology/Population Health

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Prospective Risk Factors of Toxoplasmosis Seropositivity in Pregnant Women: The Fundamental Role of Community Healthcare Education

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Abstract

Background: The present study conducted a survey on awareness of toxoplasmosis infection, with an emphasis on risk factors, and an assessment of toxoplasmosis prevalence in pregnant women in Al-Kharj province of KSA.

Methods and Results: A cross-sectional study with a qualitative approach was carried out from August 2018 to February 2019. The study recruited all pregnant women who attended maternity and children's outpatient clinics. The non-probability sampling approach was used to select 345 blood samples from study subjects. *T. gondii*-specific IgG and IgM antibodies were identified using ELISA. ach participant enrolled in the study was provided with a validated questionnaire to fill out by an assistant of the laboratory technician or an antenatal care nurse. In addition to socio-demographic data, simple closed-ended questions about established risk factors for *T. gondii* exposure were included in the questionnaire items, and answers were listed in a three-point Likert scale (agree, disagree, I am not sure). The overall prevalence of T. gondii-specific antibodies among study subjects was 12.75%; 29(8.40%) women were positive for IgG only, 9(2.6%) - for IgM, and 6(1.7%) - for both IgG and IgM antibodies. About 41.4% of participants were in the first trimester of pregnancy; among them 31(9%) were positive for T. gondii antibodies. 82.8% of pregnant women had chronic infection in the first trimester, while 44.4% of those women also had an acute infection. The number of respondents to the questionnaire was 345 participants with a response rate of about 100%. It is important to note that 81.5% of women were unaware that toxoplasmosis is dangerous, and two-thirds of them didn't know the dangerous complications for the fetus and newborn.

Conclusion: The current study concludes that there is a low prevalence of toxoplasmosis among Saudi pregnant women in Al-Kharj province. A general program must be implemented to increase population awareness, especially among the at-risk populations. (**International Journal of Biomedicine. 2021;11(4):558-563.**)

Key Words: *Toxoplasma gondii* • risk factors • IgG • IgM • pregnancy

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Introduction

Toxoplasmosis is a parasitic infection caused by the intracellular protozoan *Toxoplasma gondii*.[(1)] *T. gondii* oocysts can infect people through the environment, including in contaminated foods, water, or soil. Pregnant women are at high risk of infection. Acute infections with *T. gondii* during pregnancy and their potentially tragic outcomes for the fetus and newborn continue to occur worldwide.[(2,3)] Our understanding of the biological life cycle and clinical significance of *T. gondii* has grown throughout the last four decades. *T. gondii* was identified more than a century ago, and it was first identified as a pathogen responsible for congenital infection, although its clinical manifestation and the significance of reactivations of infectious agents in people with compromised immune systems were identified subsequently, specifically in the area of organ transplantation and HIV infection. Current observations about

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the relationships between host cell and parasite, as well as parasite virulence, have added to our knowledge of infection pathophysiology.[4]

Most immunocompetent individuals who contract the parasite do not develop symptoms, or might experience nonspecific flulike symptoms.[5-8] However, when *T. gondii* infection is acquired in pregnancy, the parasite can be transmitted across the placenta to the fetus, resulting in congenital toxoplasmosis, which can have grave consequences.[9] Infection during pregnancy can cause severe disease in the fetus (hydrocephalus, intra-cerebral calcification, retinochoroiditis, and mental retardation).[10]

Initial maternal serological screening relies on detecting IgM and IgG antibodies using an enzyme-linked immunosorbent assay (ELISA). The presence of elevated levels of *T. gondii*-specific IgG antibodies indicates infection has occurred at some point but does not distinguish between an infection acquired recently and one acquired in the distant past. The presence of a high *T. gondii*-specific IgM titer combined with a high IgG titer probably indicates an acute infection within the previous 3 months. A low-to-medium IgM titer and a high IgG titer might indicate an acute infection 3-6 months previously, but IgM antibodies have been detected as long as 18 months after initial infection.[11-15]

The onset of acute toxoplasmosis in pregnant women may pose a risk to their growing fetuses. The timely diagnosis of infection in managing the disease and preventing its harmful consequences on the fetus is very important.[16] Systematic serological screening for *T. gondii*-specific IgG and IgM antibodies in all pregnant women as early in gestation as feasible (ideally during the first trimester) and in seronegative women each month or trimester thereafter would be optimal. A positive Toxoplasma IgM test is often considered a marker of an acute infection. However, IgM can persist from several months to years after an acute infection, thus making the distinction between an acute and a chronic infection challenging.[16-19]

Ultrasonography and PCR with amniotic fluid are being used predominantly in the prenatal diagnosis of congenital toxoplasmosis.[20,21] Pregnancy, direct contact with a cat, soil contact, consumption of undercooked meat, and drinking unpasteurized milk, as well as sources of drinking water, are considered as significant risk factors for acquiring toxoplasmosis.[10,21] Numerous pregnant women, nevertheless, are unaware of such risk factors. The present study conducted a survey on awareness of toxoplasmosis infection, with an emphasis on risk factors, and an assessment of toxoplasmosis prevalence in pregnant women in Al-Kharj province of KSA.

Materials and Methods

A cross-sectional study with a qualitative approach was carried out from August 2018 to February 2019. The study recruited all pregnant women who attended maternity and children's outpatient clinics. The study was performed in Al-Kharj province, with a population of 332,243, in 2017.

The non-probability sampling approach was used to select 345 blood samples from study subjects. Samples were centrifuged to separate serum and preserved at 20°C for serological examination for one week. *T. gondii*-specific IgG and IgM antibodies were identified using ELISA, a commercial kit (Biokit-Bioelisa Toxo IgG/Italy, and Organon-Toxonostika IgM II Mikro ELISA kit), and the manufacturer's guidelines.[22]

Each participant enrolled in the study was provided with a validated questionnaire to fill out by an assistant of the laboratory technician or an antenatal care nurse. In addition to socio-demographic data, simple closed-ended questions about established risk factors for T. gondii exposure were included in the questionnaire items, and answers were listed in a three-point Likert scale (agree, disagree, I am not sure).

Statistical analysis was performed using the IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.). Baseline characteristics were summarized as frequencies and percentages for categorical variables. Group comparisons were performed using chi-square tests with Yates correction. A probability value of $P<0.05$ was considered statistically significant.

The study was approved by the Ethics Committee of the department of Medical Laboratory Sciences, Al-Neelain University. Written informed consent was obtained from each research participant.

Results

A total of 345 blood samples were collected by laboratory staff at Al-Kharj maternity and children's hospital. All pregnant women were screened for toxoplasmosis using ELISA to identify *T. gondii*-specific IgG and IgM antibodies. The overall prevalence of *T. gondii*-specific antibodies among study subjects was 12.75%; 29(8.40%) women were positive for IgG only, 9(2.6%) - for IgM, and 6(1.7%) - for both IgG and IgM antibodies (Tables 1).

Table 1.

T. gondii-specific IgG and IgM antibodies in study blood samples

Antibodies	Positive n (%)	Negative n (%)
IgG(+), IgM(-)	29 (8.4)	301 (87.25)
IgG(-), IgM(+)	9 (2.6)	
IgG(+), IgM(+)	6 (1.7)	
Total	44 (12.75)	

The majority of participants (57.7%) were in the age group of 25-34 years, and among them 25(7.2%) were positive for *T. gondii* antibodies (Table 2). About 41.4% of participants were in the first trimester of pregnancy; among them 31(9%) were positive for *T. gondii* antibodies. The study showed that gestational age, abortion, as well as cat ownership, had a significantly positive correlation with seropositivity of toxoplasmosis. Table 3 and Figure 1 display the frequency of *T. gondii*-specific antibodies related to gestational age: 82.8% of pregnant women that had chronic infection were in the first trimester, while 44.4% of those women also had an acute infection.

Table 2.

Clinical and demographic characteristics of the study population

Pattern	Total n(%)	Positive for *T. gondii* antibodies [P]	Negative for *T. gondii* antibodies [N]	*P* (P-N)
Age group, yrs				
15 – 24	86(24.9)	12(3.5)	74(21.4)	0.913
25 – 34	199(57.7)	25(7.2)	174(50)	
35 45	60(17.4)	7(2.1)	53(10.1)	
Total	345(100)	44(12.8)	301(87.2)	
Education				
Primary	113(32.8)	14(4.1)	99(28.7)	0.981
Secondary	121(34.8)	16(4.6)	105(30.4)	
Tertiary	111(32.2)	14(4.1)	97(28.1)	
Gestational age				
First trimester	143(41.4)	31(9)	112(32.5)	0.000
Second Trimester	125(36.2)	9(2.6)	116(33.6)	
Third trimester	77(22.3)	4(1.2)	73(21.2)	
History of abortion				
Yes	59(17.1)	21(6.1)	38 (11)	0.000
No	286(82.9)	23(6.7)	263(76.2)	
Risk factors				
Cat ownership				
Yes	159 46.1)	39(11.3)	120(34.8)	0.000
No	186(53.9)	5(1.4)	181(52.4)	
Blood transfusion				
Yes	28(8.1)	4(1.2)	24(7.0)	0.800
No	317(91.9)	40(11.6)	277(80.2)	
Direct soil contact				
Yes	224(65)	19(5.5)	205(50.4)	0.001
No	121(35)	25(7.2)	96(27.8)	
Consumption of undercooked meat				
Yes	287(83.2)	24(6.9)	263 (76.2%)	0.000
No	58(16.8)	20(5.8)	38 (11%)	

Table 3.

The frequency of T. gondii-specific antibodies related to gestational age

Parameters	IgG(+)/ IgM(-) n(%)	IgG(+)/IgM(+) n(%)	IgG(-)/IgM(+) n(%)	*P*
First trimester (n=113)	24(82.8)	3 (50%)	4 (44.4%)	0.000
Second trimester (n=121)	2(6.9)	3 (50%)	4 (44.4%)	0.880
Third trimester (n=111)	3(10.3)	0	1 (11.1%)	0.454
Total	29(100)	6 (100%)	9 (100%)	

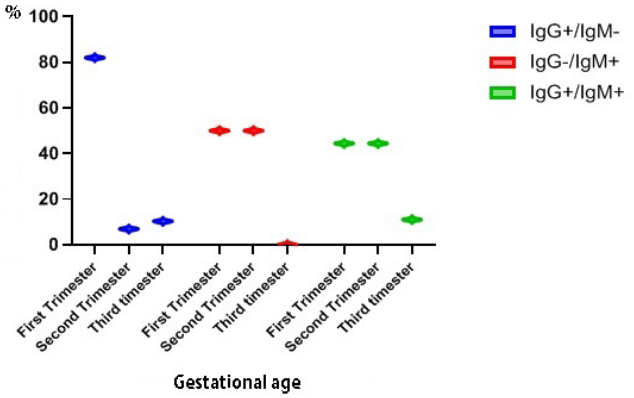

Fig. 1. *The frequency of T. gondii-specific antibodies related to gestational age*

Table 4 summarized the level of population awareness about toxoplasmosis. The number of respondents to the questionnaire was 345 participants with a response rate of about 100%. When we asked whether participants had heard of *T. gondii*, 50.4% of respondents answered, "agree." Almost half (49.6%) of women stated that toxoplasmosis is not a dangerous infection. However, only 35% agreed that *T. gondii* is transmitted through the eating of poorly washed vegetables and undercooked meat, and 73.6% agreed that cats are the main host for transmitting infection. Two-thirds of subjects didn't know that blood transfusion may be a source of infection, and only 22.9% agreed that toxoplasmosis is symptomless.

Table 4

The level of population awareness about toxoplasmosis

Questions	Agree n(%)	Disagree n(%)	Not sure n(%)
Do you know toxoplasmosis infection	174(50.4)	161(46.6)	10(2.9)
Toxoplasmosis is dangerous	64(18.6)	171(49.6)	110(31.9)
It is an infectious disease	76(22)	23(6.7)	246(71.3)
It is transmitted by improbably washed vegetables	123(35.7)	98(28.4)	124(35.9)
It is transmitted by eating undercooked meat	124(35.9)	32(9.3)	189(54.8)
Cats are a major cause of transmission of disease	254(73.6)	6(1.5)	85(24.6)
It can be transmitted through blood transfusion	110(31.9)	165(47.8)	70(20.3)
Direct contact with soil may be a source of infection	98(28.4)	119(43.5)	128(37.1)
There are no apparent symptoms in healthy people	79(22.9)	162(47)	104(30.1)
It can cause abortion or stillbirth of a child	131(38)	102(29.6)	112(32.5)

Discussion

Toxoplasmosis is a widespread, preventable-but-fatal, devastating infection that primarily affects pregnant women due to their weakened immune systems, and its prevalence varies globally. Early diagnosis in the first months of pregnancy, as well as knowledge of the disease's nature and modes of transmission, will significantly contribute to reducing the frequency of toxoplasmosis and thus the potential risks. Our results showed that the overall prevalence of anti-*T. gondii* antibodies in serum of pregnant women was 12.8%, which was similar to data reported by Sarah et al., who revealed a prevalence rate of about 8.57% in the city of Hail.[23] However, our seroprevalence was much higher than that (1.4%), documented by Mohajab et al. in Jeddah.[24] At the same time, our outcomes were less than the previous studies conducted by Alghamdi et al.,[25] who revealed 32.5% IgG seroprevalence in Riyadh, Elsafi et al.[26] in Dhahran - 28.5%, and Aqeely et al.[27] in Jazan - 24.1%.

In total, our results were more than the 7.9 % measured for the overall population of China in a survey carried out by China's Ministry of Health, and lower than initially reported in Thailand (22%),[28] Taiwan (31.06%),[29] and Tanzania (40.2%).[30] These data imply that the parasitic infection is endemic in the region of study. Such discrepancy could be related to various environmental factors and variation of climates between regions, such as air humidity and increased temperature, that control and favor dissemination and infectivity of *T. gondii* oocyst, as well as other considerations, such as different study populations, design of the study, ethnic groups, diagnostic techniques, sample size, and the lifestyle of recruited study participants.

Interestingly, chronic toxoplasmosis infection was found in 8.4% of pregnant women; besides this, anti-*T.gondii* IgG and IgM antibodies were observed in 1.7% of cases, which is difficult to interpret because IgG avidity was not conducted in the present study, and specific IgM antibodies can persist for several months or even years post-primary infection. We found acute toxoplasmosis in 2.6% of study participants. A total of 31(9%) pregnant women were positive for anti-*T.gondii* antibodies in the first trimester, and 4(44.4%) of them were IgM positive. This is a major concern because the effects of toxoplasmosis infection on the fetus are more severe during the first trimester of pregnancy, and there is a possibility of vertical transmission and therefore congenital infection.[31] The same outcomes were reported by Aqeely et al.[27]

Regarding possible demographic and clinical data, we did not find any statistically significant differences between seropositive subjects according to age and education levels. However, a positive relationship was revealed between abortion and the presence of *T. gondii*-specific antibodies.

We found a strong relationship between *T. gondii* seropositivity and cat ownership, as well as direct contact with soil, and no association with blood transfusion and eating undercooked meat. This is in agreement with different studies implying that cats were indeed a potential risk.[32,33] Other studies, on the other hand, found no correlation between *T. gondii* infection and the existence of cats in the home.[34,35]

It is obvious that the infected women get the infections from the cat, since sporozoites really are not detected on cat fur and are regularly buried in soil with cat feces, and soil contact is ubiquitous and hard to avoid. Other risk factors such as blood transfusion and eating undercooked meat play a critical role in the transmission of toxoplasmosis.

However, we did not find significant correlations of those factors with toxoplasmosis, which may be attributed to the strict precautionary measures and protocol followed in Saudi blood banks for safe blood transfusions.[36]

The preventive measures mostly depend on the women's knowledge about toxoplasmosis, its transmission, and its origin. Regarding the knowledge of toxoplasmosis among the Saudi population, about 50.4% of the study subjects said that they know about toxoplasmosis. However, it seems like poor or fake knowledge, because two-thirds of respondents give wrong answers, and there is a lack of knowledge concerning disease symptoms, complications, and prevention. These findings are similar to a study carried out by Obaid et al.[37] in Iraq and disagree with many studies in which awareness of toxoplasmosis was much lower. Low knowledge of toxoplasmosis [30] was also reported in Asian countries, such as Malaysia, the Philippines, and Thailand.In Egypt,[38] it was found that only 9.9% of the studied sample had a good knowledge of toxoplasmosis. Similar results were reported in Tanzania and in Ethiopia, where only 5% and 5.7%, respectively, of pregnant women had known about the disease.[39,40] This rate difference may be due to the different cultural or socio-demographic factors in each country.[41] Interestingly, an association between toxoplasmosis and cat ownership was noted by 73.6% of those surveyed, which may explain the low prevalence of toxoplasmosis among Saudi pregnant women in Al-Kharj province. It is important to note that 81.5% of women were unaware that toxoplasmosis is dangerous, and two-thirds of them didn't know the dangerous complications for the fetus and newborn. With this information, provider- and patient-centered strategies, such as monitoring, surveillance, and awareness programs to instruct pregnant women about *T. gondii,* can be established.

Our results cannot be generalized, since the data were collected from one healthcare facility and a low sample size. Furthermore, the seroconversion window period could not be tested because only one blood sample from each woman was evaluated, without any effort to authenticate such infection using IgG avidity tests or polymerase chain reaction.

Conclusion

The current study concludes that there is a low prevalence of toxoplasmosis among Saudi pregnant women in Al-Kharj province. We need more studies with large samples size to perfectly determine the exact prevalence and incidence rate of toxoplasmosis. A general program must be implemented to increase population awareness, especially among the at-risk populations.

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Competing Interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

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Socio-Demographic Factors and Epidemiological Characteristics of HIV-Positive Pregnant Women with High Risk of Vertical Transmission of the Immunodeficiency Virus

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Abstract

The purpose of this study was to determine the features of the course of pregnancy, delivery, and the postpartum period in HIV-positive women with a high risk of HIV vertical transmission.

***Methods and Results*:** A retrospective, longitudinal cohort study of mother-child pairs for the period from 2017 to 2019 was conducted in the Irkutsk City Perinatal Center (level III). The clinical observation group included HIV-positive women (n=213) and their newborn children with a high risk of perinatal immunodeficiency virus transmission (n=214). The findings of the conducted study demonstrated that most HIV-seropositive women with a high risk of HIV vertical transmission had an aggravated social history, a high prevalence of pelvic inflammatory diseases, and a high incidence of opportunistic and AIDS-defining conditions. Evaluation of PMTCT preventive complex showed that the target parameters in women with a high risk of HIV transmission were not reached: the first stage was performed for 49.3% of pregnant women with good ART adherence, the second stage – for 97.1% of obstetric patients, the third stage – in 100% of HIV perinatally exposed children. HIV RNA was detected in 3.7% of children, which evidences their antenatal infection.

***Conclusion*:** Development of efficient communication with HIV-positive women aimed at preservation of their health and decrease of logistic barriers to access to medical care.(**International Journal of Biomedicine. 2021;11(4):564-569.**)

Key Words: HIV infection • pregnancy outcomes • vertical transmission • retrospective study

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Abbreviations

ART, antiretroviral therapy; **HIV**, human immunodeficiency virus; **PMTCT**, prevention of mother-to-child transmission; **VL**, viral load.

Introduction

The infection caused by human the immunodeficiency virus (HIV) continues to be a priority medical and social problem and seriously threatens the health of women and children. Significant progress in strategies aimed at preventing mother-to-child transmission (PMTCT) of HIV was achieved over the past decades; these strategies allow an HIV-positive woman to realize her reproductive potential.

However, the final elimination of HIV infection in the pediatric population directly depends on parental HIV status, which actualizes the study of the nature of disease distribution and necessitates taking coordinated tactical decisions. HIV infection continues to be registered in all the constituents of the Russian Federation, and the number of constituent regions with a high prevalence of the disease is continuously growing (more than 0.5 % of the overall population number): from 22 in 2014 to 38 in 2020. One of the territories is the Irkutsk region,

where 1,951.5 HIV-infected people have been registered per 100,000 of the population since 2020. The high prevalence of HIV in the region is also among fertile women.[1,2]

Despite the trend to decrease the incidence of HIV vertical transmission, we should not forget about the abnormal reproductive health of an HIV-positive woman, which can not only complicate the course of pregnancy and delivery, but also affect the state of a newborn.[1] It should be noted that an HIV-positive woman often has comorbid and severe forms of the disease which significantly increases the incidence of unfavorable perinatal outcomes.[2-4] Positive epidemiological anamnesis and decreased access of HIV-positive fertile women to specialty care also increase the probability of mother-to-child transmission of HIV because the target parameters of three-stage chemoprophylaxis of vertical transmission of HIV infection (during pregnancy, delivery, and to a newborn) are still unachieved.

Therefore, further in-depth study to understand the features of perinatal outcomes in HIV-infected women who have maximum risks of mother-to-child transmission of HIV and who are living in a territory with a high prevalence. It is a necessary condition to successfully realize a regional, adopted system of activities to control the HIV infection epidemic and completely eliminate mother-to-child transmission of the disease.

The purpose of this study was to determine the features of the course of pregnancy, delivery, and the postpartum period in HIV-positive women with a high risk of HIV vertical transmission.

Materials and Methods

A retrospective, longitudinal cohort study of mother-child pairs for the period from 2017 to 2019 was conducted in the Irkutsk City Perinatal Center (level III). The clinical observation group included HIV-positive women (n=213) and their newborn children with a high risk of perinatal immunodeficiency virus transmission (n=214).

The inclusion criteria were: no maternal chemoprophylaxis during pregnancy within at least four previous weeks; no chemoprophylaxis during pregnancy and/or delivery; insufficient pre-delivery prophylaxis (VL>50 copies/ml prior to delivery); positive maternal rapid HIV test at delivery; the increased risk of infection during pregnancy (epidemiological indications, i.e. injecting a drug within the previous 12 weeks or sexual contact with an HIV-positive partner).

Since birth, all the HIV perinatally exposed children received ART with three-drug products (zidovudine, nevirapine, lamivudine), formula-fed and tested for HIV nucleic acids by molecular and genetic techniques within the first 48 hours of life.

Several maternal factors affecting the state of a child after delivery were evaluated. External (social) maternal factors include low social status, the absence of a family and/or a partner, regular place of work, high/professional education; substance use during the current pregnancy. Internal (medicinal) maternal factors include HIV-positive status; coinfections (hepatitis C and/or B virus, herpes virus infections, fungal infections); HIV-VL at pregnancy; compliance with ART at pregnancy (as part of PMTCT of HIV as well as treatment of maternal HIV infection prior to pregnancy). Medical records of each HIV-positive woman and her child were reviewed; clinical observation and examination of children within the specified group for their first 28 days of lifee, and their microbiological and virological monitoring were performed.

Statistical analysis was performed using the Statistica 6.1 software package (Stat-Soft Inc., USA). For descriptive analysis, results are presented as median (Me) and interquartile range (IQR; 25th to 75th percentiles). The frequencies of categorical variables were compared using Pearson's chi-squared test. The Wilcoxon signed-rank test was used to compare the differences between the two dependent groups (for non-parametric data). Odds ratios (ORs) and their 95% confidence intervals (CIs) were calculated A probability value of $P<0.05$ was considered statistically significant.

Results

The study findings showed that the median and quartiles of the age of pregnant women with a high risk of HIV vertical transmission were 30(27–34 years). Table 1 demonstrates a roughly equal ratio of female patients in the age categories from 21 to 30 years of age (46%) and from 31 to 40 years of age (45%). The majority of HIV-positive pregnant women were Russians (97.6%), permanently residing in the city (71.8%), and had secondary (38%) or lower secondary (30.9%) education. Interestingly, 74.2% of HIV-positive women were found not to be active in the labor force before they became pregnant. By social position, 1.8% of the unemployed HIV-seropositive pregnant women were students, 98.2% - housewives; 63.3% of HIV-positive women were not in a registered marriage. The partners of one-third of pregnant women (31.45%) were also HIV-seropositive, and the percentage of women with the annulled parental rights was 7%.

The study findings showed that 80% of women had a history of substance use. Smoking cigarettes and substance use were found to be most common among HIV-infected women (56.8% and 13.6% cases, respectively); combined substance use was observed often. Most women with a risk of HIV vertical transmission continued to inject drugs.

The evaluation of the obstetric and gynecological history of HIV-positive women with a high risk of HIV vertical transmission showed that pregnancy was planned by only one-third of female patients (29.1%). The median and quartiles of pregnancy ranking were found to be 3(2–5), and the first pregnancy was recorded only in 10.3% of women. Social deprivation was also reflected at the antenatal phase of monitoring HIV-positive pregnant women. The median and the quartiles of pregnancy registration date were shown to be 13(9–20). About 78.8% of HIV-positive women were registered at the female health department. However, only half of them were registered prior to Week 12 of gestation (54.7%), and irregular perinatal care was found in 60.7% of cases.

Table 1.

Social and demographic characteristics of pregnant women with a high risk of perinatal HIV transmission (n=213)

Parameter	Abs.	% [95% CI]
Age		
< 20 years	17	7.98 [4.8–12.6]
21–30 years	98	46 [39.2–52.9]
31–40 years	96	45 [38.3–52]
> 40 years	3	1.4 [0.37–4.4]
Ethnicity		
Russians	208	97.65 [94.3–99.13]
Place of residence		
City	153	71.8 [65.2–77.66]
Education		
Lower secondary	66	30.99 [24.9–37.7]
Secondary	81	38 [31.5–44.9]
Secondary special	44	20.66 [15.5–26.8]
High	22	10.33 [6.7–15.4]
Primary school	2	0.9 [0.16–3.7]
Other		
Employment	55	25.8 [20.1–32.3]
Marriage not registered	135	63.3 [56.4–69.7]
HIV-positive partner	67	31.4 [25.3–38.2]
Deprivation of paternal rights	15	7 [4,1–11,5]
Substances use		
Nicotine	121	56.8 [49.8–63.5]
Alcohol	21	9,8 [6.3–14.8]
Parenteral drugs	29	13,6 [9.4–19.1]

The obstetric history of many HIV-seropositive pregnant women was aggravated: 15% had a premature birth, 17.3% – miscarriages, and 48.3% – induced abortions. Many were diagnosed with chronic inflammatory diseases of reproductive system organs: salpingitis (1.8%), oophoritis (0.4%), vaginitis (2.8%), cervicitis (8.4%), and colpitis (17.8%).

Table 2 shows the structure of somatic pathology in HIV-positive women. The leading nosologies were urinary system inflammatory diseases (13.6%) and endocrinopathies (5.6%). Reproductive system tumors at different sites were not uncommon (3.2%).

Interestingly, various abnormalities in the course of pregnancy were observed in most women with a high risk of HIV transmission. Change in the amount of amniotic fluid was observed in HIV-positive women equally as often – hydramnios (1.4%) and oligoamnions (1.4%); 4.6% of patients were diagnosed with preeclampsia of various severities, 11.7% had threatened miscarriage, 26.7% – placental insufficiency, 3.7% – gestational diabetes. Anemia prevailed (87.7%) among hematologic disorders in HIV-seropositive pregnant women; thrombocytopenia (1.8%) and bleeding abnormality as hypercoagulation (0.4%) were also observed.

According to our data, many women with a high risk of HIV vertical transmission at pregnancy and delivery had inflammatory diseases: gestational pyelonephritis (2.8%), chorioamnionitis (2.8%), cryptogenic hepatitis (0.4%), community-acquired pneumonia (0.4%), acute enteric infection, and ARVI (4.5%). One (0.4%) HIV-seropositive woman was diagnosed with sepsis. The evaluation of verified infectious pathology showed that hepatitis C (49.2%), syphilis (23.9%), mycotic infection (31.9%) as well as pulmonary tuberculosis (8.4%) were the dominant nosologies.

Table 2.

Somatic pathology in pregnant women with a high risk of perinatal HIV transmission (n=213)

Somatic pathology	Abs.	% [95% CI]
Respiratory tract diseases	6	2.8 [1.1–6.3]
Cardiovascular system diseases	2	0.9 [0.16–3.7]
Genitourinary system diseases	29	13.6 [9.4–19.1]
Gastrointestinal tract diseases	2	0.9 [0.16–3.7]
Neuropsychiatric diseases	3	1.4 [0.37–4.4]
Endocrinopathies	12	5.6 [3–9.8]
Reproductive system neoplasms	7	3.2 [1.4–6.9]
Inflammatory diseases of reproductive system	67	31.4 [25.3–38.2]

The evaluation of the course of the intranatal period established that most pregnant women with a high risk of HIV vertical transmission were waiting for the second labor (83%). About 1.4% of women had outpatient labor; the others were hospitalized for labor; 36.1% of HIV-positive women had premature labor, 17.3% – <12-hour period between rupture and delivery, 4.2% – abnormal labor, 1.4% – a fetal presentation, 1.4% – umbilical cord prolapse. One HIV-seropositive woman had ingrowth of placenta to the uterine wall, 3.2% – a complete detachment of a normally situated placenta, and 1.8% – hemorrhage at the fourth stage of labor with major blood loss. Some women had invasive procedures at delivery by urgent obstetric indications: 0.9% underwent amniotomy, 2.3% – episiotomy. One patient underwent a hysterectomy during the postpartum period due to major bleeding.

Careful examination of epidemiological anamnesis is performed for making a decision on the management of female patients admitted to an obstetric institution with a confirmed diagnosis of HIV infection or without perinatal care. Evaluation of HIV status of women with a high risk of vertical transmission of immunodeficiency virus established that most patients were diagnosed before the current pregnancy (70.4%). One-third of patients became aware of their HIV-seropositive status when they sought medical attention during the current pregnancy(29.5%). Median and quartiles of HIV infection duration were 3(1-7) years. The main route of transmission was heterosexual contacts (61.5%) and the parenteral route(12.6%). The HIV transmission route was unknown in 25.3% of cases. The study of HIV status showed that most women were diagnosed with subclinical Stage 3 (36.1%) and secondary diseases Stages 4A, 4B, 4C(40.3%).

Of mothers with HIV infection, 10.8% were found to be continuously monitored in the regional center for prevention

and control of AIDS and received ART prior to pregnancy. Only 68.5% of patients with a current pregnancy visited an infectious disease specialist, and the monitoring was regular only in 69.8% of women in this cohort. Chemoprophylaxis regimens of HIV infection treatment (first stage of PMTCT) were administered to all the women at pregnancy when they visited an infectious disease specialist as per the protocols. However, their compliance with the treatment was not high; only 49.3% of HIV-positive women adhered to the medical prescriptions.

Pregnant women observed in the regional center for prevention and control of AIDS showed a 4.7-fold decrease in the VL at control examination on Weeks 34-36 of gestation, as compared to the first visit of an infectious disease specialist (Z=3.4; PW=0,0006). A statistically significant decrease of HIV-positive women with VL of 100,000 copies/ml and more ($\chi 2$=17,7; df=1; P<0,001) (Table 3) was observed.

Table 3.

Dynamics of the HIV viral load of the examined women with a high risk of HIV vertical transmission, observed at the Regional Center on Prevention and Control of AIDS (n=146)

Viral load, cop/ml	Observation stages				*P-value*
	First visit during pregnancy		Visit at Weeks 34–36 of gestation		
	Abs	% [95% CI]	Abs	% [95% CI]	
< 400	32	21.9[15.6–29.6]	37	25.3[18.6–33.3]	0.49
400–10000	49	33.5[26–41.9]	43	29.4[22.3–37.6]	0.44
> 10001	61	41.7[33.7–50.2]	47	18.4[12.7–25.9]*	< 0.0001

**Statistically significant differences between the groups by χ^2 criterion.*

About 49% of pregnant women with adequate compliance with ART received chemoprophylaxis in the antenatal period, which was shown in the estimated adequacy of PMTCT preventive measures complex. The second stage of PMTCT was performed in 97.1% of obstetric patients with a high risk of HIV vertical transmission. No chemoprophylaxis of this stage in 2.8% of women was related to outpatient delivery or to admission in the active pushing phase. One hundred percent of newborns received ART and switched to formula feeding in the postnatal period.

The study of clinical and anamnestic features of HIV perinatally exposed newborns (n=214) found that more often children were born by cesarean section (55.6%). The median body weight of these newborns was 2,630g (IQR: 1980g–3050g), median body height at birth was 46 cm (IQR: 28cm–55 cm), and median gestation age was 37 weeks(IQR: 34–38 weeks). More than one-third of children were prematurely born (35.9%), 15.5% – with extremely low body weight, 22% – very low body weight; 27.5% of newborns were small for gestation date; 3.7% had HIV RNA, which evidences their antenatal infection. The diagnosis was confirmed after examination of a second blood sample of a child taken on Day 2 after a positive result was received. All children were directed to a specialist on HIV infection for diagnosis verification, in-depth examination, and treatment administration.

Discussion

Socio-demographic factors of pregnant women with a high risk of HIV vertical transmission found in the study reflect regional and all-Russian trends. The influence of the educational level of women and their partners on the prevalence of HIV infection was previously shown by various authors. It was found that every additional year of education in an educational institution statistically significantly decreased the risk of HIV infection by 7%.[5] It was also observed that better-educated women are capable of better processing the information on the infection risks, rules of safe behavior, and the need for preventive measures that mitigate the probability of HIV transmission and infection.[6] Many authors previously noted a low behavioral and social status of pregnant HIV-positive women.[7-10]

The study showed that most women continued to inject drugs during pregnancy. This fact reflects regional features of the course of the epidemic process. It should be noted that the substances are an additional risk factor of the course of pregnancy in HIV-positive women and significantly affect the fetoplacental complex and a newborn's status.[11] Smoking cigarettes during pregnancy is a proven risk factor for unfavorable outcomes in HIV-positive pregnant women, which is related to intrauterine fetal hypoxia, architectural distortion of placental vessels, inflammation, immune status abnormality, and increased risk of miscarriage.[12-14] It should be noted that there is a causal relationship between HIV infection and the use of alcohol or drugs because such consumers are prone to risky behavior, which increases the risk of immunodeficiency virus infection. In addition, the response to HIV infection may be considered by a woman as a traumatic event that involves cognitive and behavioral as well as emotional aspects of mental health and needs psychosocial support.[15]

Our results showed a high prevalence of reproductive system diseases in women with a high risk of HIV vertical transmission. It should be stressed that a high prevalence of pelvic inflammatory diseases not only increases the probability of reproductive losses but also increases the risk of sexually transmitted diseases as well as activation of latent infections.[2,16,17]

The prevalence of kidney diseases in HIV-positive patients observed in our study was also noted by other authors. There is a probable relation between the pathogenetic role of HIV infection and the development of nephropathy.[8] Literature data for the last years also show that not infrequently severe cervical injuries are recorded.[18] High frequency of carriage of human papillomaviruses of oncogenic types in HIV-seropositive women as well as statistically significantly increased prevalence of cervical intraepithelial dysplasia were observed in the territory of Irkutsk city.[19]

As noted above, perinatal outcomes mainly depend on HIV infection comorbidity. The analysis of the findings again draws attention to the high prevalence of opportunistic

infections and AIDS-defining conditions, which, on the one hand, may aggravate the immunosuppression and increase the risk of mother-to-child transmission of other pathogens, and on the other, HIV infection is considered an important determinant of hepatitis C virus vertical transmission from mother to child. Perinatal transmission of hepatitis C was demonstrated to be met in immunocompetent individuals (<1% of cases), but it increases up to 20% in HIV-positive women with progressive immunodeficiency. The risk of perinatal transmission is supposed to be mitigated by the administration of ART during pregnancy and is less than 3% with operative delivery.[20] It should be also stated that unbalanced oxidant-antioxidant status is observed with HIV infection, which enables the sustained chronic inflammation and development of inadequate immune response.[21]

Our observations again emphasize the significance when patients comply with not only the therapy but also medical monitoring, which is one of the key parameters of health care delivery to HIV-positive patients. As is known, the leading factor affecting the probability of HIV transmission from mother to fetus or child is the blood concentration of the virus of a woman – VL during pregnancy and delivery. Despite the trend to decrease VL, the findings of our study show that most women with a high risk of HIV vertical transmission did not reach undetectable levels. The probability of mother-to-child transmission is known to correlate with the VL and duration of antiretroviral therapy, meanwhile, about 75% of HIV transmission occurs during delivery or during the last few weeks of pregnancy. HIV vertical transmission prior to the third trimester of pregnancy is observed in about 10% of cases, during breastfeeding – in 10%-15%.[22] If the VL in a woman is below the detection limit, the probability of vertical transmission is extremely low. On the other hand, absence or insufficient HIV suppression significantly reduces the risk of vertical transmission.

A vertical route of transmission was also observed in 3.7% of newborns. It was found that not one of the mothers of newborns with perinatal HIV transmission received ART during pregnancy. Most women were not registered at the female health department (62.5%). A common use of substances during pregnancy was observed. HIV denialism was found in one woman, which interfered with antenatal and intranatal stages of PMTCT. The second stage of PMTCT was not implemented in 37.5% of HIV-positive women. The third stage of PMTCT at the postnatal stage (boosted ART regimen) was initiated in all newborns. However, the absence of a complete complex of preventive measures of PMTCT threatened the health of newborns and increased the probability of HIV vertical transmission, in spite of boosted ART drug products administered to all children of PMTCT postnatal stage.

Conclusion

The findings of the conducted study demonstrated that most HIV-seropositive women with a high risk of HIV vertical transmission had an aggravated social history, 80% used various substances, 74.2% were not active in the labor force, most (60.7%) had no regular antenatal care during pregnancy. A high prevalence of pelvic inflammatory diseases(31%), nephropathies(13.6%), and endocrinopathies (5.6%) was observed. High incidence of opportunistic and AIDS-defining conditions was recorded with hepatitis C(49.2%), syphilis(23.9%), and fungal infection (31.9%) prevailing. Abnormality of the intranatal period was observed in more than half of the women (65.7%). The main routes of HIV transmission were heterosexual (61.5%) and parenteral(12.6%). Most women were diagnosed with HIV before their current pregnancy (70.5%). Attention is paid to the low compliance of HIV-positive pregnant women with medical supervision and the use of ART. A 4.7-fold progressive decrease in VL was found in HIV-positive women who received specialty care ($Z=3,4$; $pW=0.0006$). The number of pregnant women with a VL of 100,000 copies/ml and higher was also decreased ($\chi 2=17.7$; $df=1$; $p<0.001$). However, an undetectable level was not reached due to ART use and low compliance of HIV-positive pregnant women with medical supervision, despite the availability of qualitative special medical care and the presence of efficient chemoprophylaxis regimens at pregnancy. Evaluation of PMTCT preventive complex showed that the target parameters in women with a high risk of HIV transmission were not reached: the first stage was performed for 49.3% of pregnant women with good ART adherence, the second stage – for 97.1% of obstetric patients, the third stage – in 100% of HIV perinatally exposed children. HIV RNA was detected in 3.7% of children, which evidences their antenatal infection.

Hence, the findings of our study show that each mother of a newborn in the high-risk group had anamnesis factors of pregnancy and delivery abnormalities, and a newborn had prerequisites for infection. This necessitates the enhancement of activities on medical and social support of women of fertile age from risk groups, on the provision of measures of psychological support with the transformation of their behavioral settings. Development of efficient communication with HIV-positive women aimed at preservation of their health and decrease of logistic barriers to access to medical care.

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Competing Interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

Population Genetics

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Association of the *IRF6* rs2235371 and rs861019 Polymorphisms with Non-Syndromic Cleft Lip with or without Cleft Palate in the Yakut Population

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Abstract

Background: Non-syndromic cleft lip with or without cleft palate (NSCL/P) is one of the most common birth defects. NSCL/P can be broadly divided into cleft lip only (CLO), cleft palate only (CPO), and cleft lip with cleft palate (CLP) based on clinical presentation. The aim of this study was to investigate the relationship between the *IRF6* gene polymorphisms and NSCL/P in the Yakut population.

***Methods and Results*:** In 23 OFC patients and 58 unrelated control subjects from the Yakut population, we tested two SNPs (rs2235371 and rs861019) with a minor allele frequency of more than 5% in the candidate gene *IRF6.* We found that the SNP marker rs861019 showed significant differences in allele frequencies (OR=2.07, 95% CI: 1.01–4.23, *P*=0.04) between the NSCL/P patients and the comparison group. Analysis of allele frequencies for rs861019 SNP in subgroups showed that there was a difference in the frequency between CLP and control (OR=5.00, 95% CI: 1.61-15.53, *P*=0.11); however, this result was not significant.

Genotype analysis showed significant differences in patients from the CLP subgroup in comparison with controls for homozygous (AA compared with GG) (OR=9.00, 95% CI: 1.03–78.58, *P*=0.03), heterozygous (GA compared with GG) (OR=5.50, 95% CI: 1.05-28.75, *P*=0.04,), recessive (GG compared with GA + AA) (OR=6.67, 95% CI: 1.61-27.58; RR=4.78, 95% CI: 1.42–16.10, *P*=0.008,) and co-dominant (GG compared with GA, compared with AA) (*P*=0.02) inheritance models.

Diplotype analysis showed that the NSCL/P group was more likely to have the [CC]-[GG] diplotype than the comparison group. This diplotype carries the risk GG genotype (rs861019) (30.4%) and does not carry the risk T allele(rs2235371). In the CLP subgroup, two diplotypes ([CT]-[GG] and [CC]-[GG]) were found more often than in the comparison group. Both diplotypes carry the risk GG genotype(rs861019; 33.3%). In the CPO subgroup, the [CT]-[GG] diplotype was more common. In the CLO subgroup, only two diplotypes ([CC]-[GA] and [CC]-[GG]) were found, both of which were more common than in the comparison group (75% and 25%).. It is likely that these results for the CLO and CPO subgroups were influenced by the small size of both samples. Unlike the NSCL/P and CLP groups, in these samples, diplotypes with the homozygous genotype GG (rs861019) without the homozygous genotype TT (rs2235371) were more common. Diplotypes with a homozygous genotype of the TT risk allele were not found in the studied groups except for the comparison group, where the [TT]-[AA] diplotype was represented by a low frequency (0.17%).

Conclusion: The present study provides strong statistical support (for the first time to our knowledge) that genetic variants of the *IRF6* rs861019 SNP are associated with NSCL/P in Yakuts.(**International Journal of Biomedicine. 2021;11(4):570-575.**)

Key Words: orofacial cleft • IRF6 gene • rs2235371 • rs861019 • haplotype • diplotype • Yakuts

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Abbreviations

CLP, cleft lip with cleft palate; **CLO**, cleft lip only; **CPO**, cleft palate only; **IRF6**, interferon regulatory factor 6; **LD**, linkage disequilibrium**; NSCL/P**, non-syndromic cleft lip with or without cleft palate; **OFC**, orofacial cleft; **SNP***,* single nucleotide polymorphism.

Introduction

Non-syndromic cleft lip with or without cleft palate (NSCL/P) can be broadly divided into cleft lip only (CLO), cleft palate only (CPO), and cleft lip with cleft palate (CLP) based on clinical presentation.[1] NSCL/P is one of the most common birth defects that carries a serious physical and financial burden to affected patients and their families.[2] The overall prevalence of orofacial cleft (OFC) abnormalities is estimated at about 1 in 700 live births, which is almost half of all craniofacial anomalies.[3,4] According to the World Health Organization, the prevalence of OFC at birth varies worldwide, from 0.34–2.29 per 1000 live births for CLP and 0.13–2.53 per 1000 live births for CPO.[5] The frequency of CLP and CPO can vary greatly from study to study. Prevalence was found to vary by origin, with the highest incidence rates in the Asian population (0.82–4.04 per 1000 live births), intermediate rates among Caucasians (0.9–2.69 per 1000 live births), and the lowest rates among the African population (0.18–1.67 per 1000 live births).[1,6,7] It was also found that prevalence varied even more by subgroup; for example, one study reported lower OFC rates among residents of the Far East than among Filipinos.[8] The frequency of CLP in Russia is 1:700-1:1000, while in Yakutia (mixed population) it is 1:548 (1.82 per 1000 newborns), and an unfavorable tendency of their increase was noted over the period from 2000-2016.[9,10]

NSCL/P is a complex malformation that is influenced by both genetic and environmental factors. In early pregnancy, maternal exposure to tobacco smoking, alcohol use, malnutrition, drugs, viral infections, and environmental pollution increases the risk of having children with NSCL/P.[11] Approximately 20 genes are involved in the etiology of NSCL/P, such as *IRF6, MSX1, TGF-beta, MTHFR*, and *FOXE1*.[12,13] Among these genes, *IRF6*, located on the long arm of chromosome 1 (1q32.3-q41), encodes a member of the interferon regulatory transcription factor family.[13] Interferon regulatory factor 6 (IRF6) belongs to a family of nine transcription factors that share a highly conserved helix-turn-helix DNA-binding domain and a less conserved protein-binding domain. Genetic variations in the *IRF6* gene were first identified in the etiology of autosomal dominant van der Wood syndrome, which included cleft lip and/or cleft palate and lower lip pits. In addition, the role of the *IRF6* gene has also been studied in animal models of NSCL/P. Ingraham et al.[14] reported that the *IRF6* knockdown mice have abnormal skin, limb, and craniofacial development. Richardson et al.[15] demonstrated that IRF-6 acts as a key determinant of the keratinocyte proliferation-differentiation switch. Subsequently, these authors further demonstrated that IRF6 plays an important role in the formation and maintenance of the oral peridemis, the spatio-temporal regulation of which is important to ensure adequate palatal adhesion. In 2004, Zucchero et al.[13] investigated 36 SNPs in the *IRF6* gene with 8003 individual subjects originating from 10 populations, including Asian, European, and American. One particular SNP (rs2235371) was the first marker in the *IRF6* gene that was significantly associated with NSCL/P in Asians and South Americans. Subsequently, similar studies in different populations provided additional evidence that the *IRF6* rs2235371 SNP is significantly associated with NSCL/P. Although the exact functions of the *IRF6 gene* remain unknown, these findings indicate that the *IRF6* gene plays a critical role in NSCL/P. Wang et al.[16] performed the first meta-analysis investigating the relationship between the *IRF6* rs2235371 SNP and the risk of NSCL/P. These authors performed subgroup analyses stratified by ethnicity (including Caucasians, Asians, and mixed) and NSCL/P types (CLP and CPO), but did not analyze CLO due to the lack of available data. The results showed that genes sensitive to NSCL/P can differ in different ethnic groups and types of NSCL/P.[16] The relationship between the common *IRF6* rs2235371 variant and NSCL/P has been widely studied in the world, but the results of these studies are contradictory.

These results are especially controversial in studies with a mixed population, for example, in Brazil, where the results varied depending on the geographic region and the studied ethnicity.[17] Therefore, in order to better understand the association between the *IRF6* gene and NSCLP, other polymorphisms are also studied, for example, rs861019.[18,19]

The Yakuts, the largest ethnic group of indigenous people of Siberia in Russia with a special life environment, race, customs, and socio-economic status, live mainly in Yakutia (Siberia). The number of Yakuts is about 500,000 people, and their CLP level exceeds the national level.

However, no studies have been done to examine candidate genes associated with NSCL/P among the Yakuts, and we do not know of a single study that has been published in English. Taking into account the genetic heterogeneity of NSCL/P in different populations and the negative tendency of an increase in the incidence of NSCL/P in newborns in Yakutia,[10] we conducted a case-control study to find out whether the *IRF6* polymorphism affects susceptibility to NSCL/P in the Yakuts. This work is a continuation of the previously published work.[20]

Materials and Methods

The experimental part of the work on the genotyping of the *IRF6* SNPs (rs2235371, rs861019) was performed in the Department of Molecular Genetics at the Yakutsk Scientific Center for Complex Medical Problems (YSC CMP). For the study, we used DNA samples from the collection of biomaterials of the YSC CMP using the Unique scientific equipment "Genome of Yakutia" (registration no. USU_507512). The sample of the examined persons consisted of 23 children (9 girls and 14 boys) of the Yakut ethnicity with congenital OFCs. According to the indications, a cytogenetic examination was performed to exclude chromosomal pathology in this group of children. The comparison group (control) included 58 healthy Yakut volunteers (35 women and 23 men) who had no history of relatives with congenital

OFCs. The average age of the patients and the volunteers was 12.09±2.35 years and 40.52±0.19 years, respectively.

Genomic DNA samples were isolated from the peripheral blood leukocytes using a commercial DNA kit, Excel biotech (Yakutsk, Russia). The study of the *IRF6* SNPs (rs2235371, rs861019) was performed by PCR and RFLP analysis.

Primer sequences, conditions for amplification, restriction pattern, restriction enzyme, and the lengths of the restoration fragments are presented in Table 1.

Table 1.

The primers and restriction enzymes used for detection of IRF6 SNPs using PCR-RFLP methods

SNP	Primers	AT, °C	RE	RFL, bp
rs2235371	F: 5'-ATCAGTCCT CTGTCCATGACG-3'	61	MboI	CC: 310 bp, CT: 310+222+88 bp TT: 222+88 bp
	R: 5'-GCATGAGTC ACAGGGATGAAC-3'			
IRF6 rs861019	F: 5′-ATGACACCA CCATGATGAGGGA-3′	61	TfiI	GG: 350 bp, GA: 350+212+138 bp AA: 212+138 bp
	R: 5′-CTAGCCATG CAAAGCTTGTCTC-3′			

AT, annealing temperature; RE, restriction enzyme; RFL - restriction fragment length; bp, base pair

Genotypes were determined by analyzing the sizes of the resulting fragments by gel electrophoresis on a 4% agarose gel with ethidium bromide in a standard Tris-acetate buffer at 120V for 1 hour. Restriction products were visualized using a UV gel documentation system (Vilber Lourmat, France).

The study was approved by the Ethics Committee of the YSC CMP. Written informed consent was obtained from each research participant (or the participant's parent/guardian).

Statistical analysis was performed using statistical software package SPSS version 17.0 (SPSS Inc, Chicago, IL). Differences in the allele and genotype distribution between the groups were assessed by $\chi 2$- test or Fisher's exact test, when appropriate. The odds ratio (OR), relative risk (RR) and the corresponding 95% CI were calculated to estimate the strength of the association.

The following genetic models and test were analyzed: allelic model: D compared with d (d - the minor allele); genotypic tests - homozygous model (DD compared with dd), heterozygous model (Dd compared with dd); recessive model (dd compared with Dd + DD); dominant model (dd+Dd compared with DD); over-dominant model (DD+dd compared with Dd); co-dominant model (DD compared with Dd, compared with dd). The Haploview 4.2. software (Broad Institute, Cambridge, MA, USA) was use to analyze the Linkage disequilibrium between variants. A probability value of $P<0.05$ was considered statistically significant.

Results and Discussion

Among OFC patients, the largest number of children (n=12, 6 boys and 6 girls) was diagnosed with CLP. CLO and CPO were diagnosed in 4 children (all boys) and 7 children (4 boys and 3 girls), respectively.

In 23 OFC patients and 58 unrelated control subjects from the Yakut population, we tested two SNPs (rs2235371 and rs861019) with a minor allele frequency of more than 5% in the candidate gene *IRF6* (Tables 2 and 3). There were no deviations from HWE for any of the genotyped markers in both groups (Table 2).

Statistical analysis showed that polymorphic variants of the *IRF6* gene may be associated with an increased risk of NSCL/P in the Yakut population. Of the two studied SNPs, we found that the SNP marker rs861019 showed significant differences in allele frequencies (OR=2.07, 95% CI 1.01–4.23, P=0.04) between the NSCL/P patients and the comparison group. Analysis of allele frequencies for rs861019 SNP in subgroups showed that there was a difference in the frequency between CLP and control (OR=5.00, 95% CI: 1.61-15.53, *P*=0.11); however, this result was not significant. Genotype analysis showed significant differences in patients from the CLP subgroup in comparison with controls for homozygous (AA compared with GG) (OR=9.00, 95% CI: 1.03–78.58, *P*=0.03), heterozygous (GA compared with GG) (OR=5.50, 95% CI: 1.05-28.75, *P*=0.04), recessive (GG compared with GA + AA) (OR=6.67, 95% CI: 1.61-27.58; RR=4.78, 95% CI: 1.42–16.10, *P*=0.008) and co-dominant (GG compared with GA, compared with AA) (*P*=0.02) inheritance models.

To show the genetic relationship between the tested SNPs, paired LD analysis and haplotype estimation were performed for the studied SNPs. Haplotypes were constructed using the data on the genotypes we obtained in this work. LD patterns for combinations of 2 SNPs are shown in Table 4 and Figure 1 There was very weak LD between two SNPs of the *IRF6* gene (Table 3 and Fig. 1) in almost all groups, except for the CPO subgroup, where a weak LD was observed (D '= 1 with LOD = 2). The frequency distribution of the *IRF6* gene haplotypes for two SNPs (rs2235371 and rs861019) is shown in Table 3.

There are four possible haplotypes (CG, CA, TA, and TG) for the rs2235371 and rs861019 SNPs. All four haplotypes were found in the NSCL/P group and ComG. The fact that the TG and TA haplotypes were not found in the CPO and CPO subgroups probably indicates the small size of these subgroups. The frequency of 2-marker combined haplotypes did not show significant differences from the comparison group.

The distribution of diplotype frequencies for two SNPs (rs2235371 and rs861019) of the *IRF6* gene, based on all detected variants, is presented in Table 4. Seven diplotypes out of nine possible variants were found. The NSCL/P group was more likely to have the [CC]-[GG] diplotype than the comparison group. This diplotype carries the risk GG genotype (rs861019) (30.4%) and does not carry the risk T allele(rs2235371). In the CLP subgroup, two diplotypes ([CT]-[GG] and [CC]-[GG]) were found more often than in the comparison group. Both diplotypes carry the risk GG genotype(rs861019; 33.3%). In the CPO subgroup, the [CT]-[GG] diplotype was more common. In the CLO subgroup, only two diplotypes ([CC]-[GA] and [CC]-[GG]) were found, both of which were more common than in the comparison group (75% and 25%).

Table 2.

Association of the IRF6 rs2235371 and rs861019 SNPs with the development of NSCL/P, CLP, CLO, CPO

NSCL/P and sub-groups	SNP	Alleles	MAF/ARA	Sample size (case/ control)	Case genotypes	Control genotypes	OR and RR Allelic model (95 % CI) *P*	OR and RR Homozygous model (95 % CI) *P*	OR and RR Heterozygous model (95 % CI) *P*	OR and RR Recessive model (95 % CI) *P*	OR and RR Dominant model (95 % CI) *P*	OR and RR Over-dominant model (95 % CI) *P*	CDM model *P*
NSCLP	rs2235371	C/T	T	23/58	13/10/0	26/31/1	0.70 (0.31-1.57) 0.77 (0.42-1.41) 0.38	0	0	0	0.62 (0.24-1.65) 0.78 (0.39-1.51) 0.34	0.67 (0.25-1.77) 0.75 (0.37-1.51) 0.42	0.55
CLP				12/58	6/6/0	26/31/1	0.84 (0.31-2.30) 0.86 (0.37-2.01) 0.81	0	0	0	0.81 (0.23-2.82) 0.84 (0.30-2.36) 0.76	0.87(0.25-3.02) 0.89 (0.32-2.50) 1	0.87
CLO				4/58	4/0/0	26/31/1	0 0 0.11	0	0	0	0 0 0.05	0 0 0.11	0.10
CPO				7/58	3/4/0	26/31/1	1.01 (0.29-3.4) 1.01 (0.34-3.01) 1.00	0	0	0	1.08 (0.22-5.28) 1.07 (0.26-4.42) 1	1.16 (0.24-5.66) 1.14 (0.28-4.71) 1	0.93
NSCLP	rs861019	A/G	G	23/58	3/9/11	18/22/18	2.07 (1.01-4.23) 1.69 (0.99-2.89) 0.04	3.67 (0.87-15.38) 2.65 (0.84-8.36) 0.11	1.49 (0.51-4.39) 1.31 (0.63-2.69) 0.46	2.04 (0.76-5.48) 1.64 (0.83-3.25) 0.16	3.00 (0.79-11.40) 2.33 (0.77-7.06) 0.16	1.05 (0.39-2.83) 1.04 (0.51-2.10) 1	0.19
CLP				12/58	1/2/9	18/22/18	5.00 (1.61-15.53) 2.47 (0.83-7.36) 0.11	9.00 (1.03-78.58) 6.33 (0.87-45.91) 0.03	5.50 (1.05-28.75) 4.00 (0.96-16.72) 0.04	6.67 (1.61-27.58) 4.78 (1.42-16.10) 0.008	4.95 (0.59-41.30) 4.10 (0.57-29.63) 0.16	0.33 (0.07-1.63) 0.38 (0.09-1.61) 0.02	0.02
CLO				4/58	0/3/1	18/22/18	1.67 (0.38-7.30) 1.61 (0.40-6.46) 0.72	Infinity -	0.41 (0.04-4.26) 0.44 (0.05-3.89) 0.62	0.74 (0.07-7.62) 0.75 (0.08-6.79) 1	Infinity 0.31	4.91 (0.48-50.18) 4.44 (0.49-40.30) 0.29	0.28
CPO				7/58	2/4/1	18/22/18	0.75 (0.24-2.30) 0.77 (0.28-2.10) 0.78	0.50 (0.04-6.02) 0.53 (0.05-5.34) 1	0.31 (0.03-2.98) 0.34 (0.04-2.82) 0.38	0.37 (0.04-3.31) 0.40 (0.05-3.13) 0.66	1.12 (0.20-6.36) 1.11 (0.23-5.25) 1	2.18 (0.45-10.68) 2.00 (0.49-8.21) 0.42	0.56

The order of genotypes: DD/Dd/dd (d is the minor allele); Allelic model: D compared with d; Homozygote model: DD compared with dd; Heterozygote model: Dd compared with dd; Recessive model: dd compared with Dd+DD; Dominant model: dd+Dd compared with DD; Over-dominant model: DD+dd compared with Dd; Co-dominant model: DD compared with Dd, compared with dd; P: HWE P-value. ARA, associated risk allele; CDM, Co-dominant model

Table 3.

The frequency distribution of IRF6 gene haplotypes for two SNPs (rs2235371 and rs861019) of the IRF6 gene in Yakuts with NSCL/P and OFC subgroups

NSCL/P and subgroups	Haplotype frequency				Linkage disequilibrium (LD)		
	T-G	C-G	T-A	C-A	D'	LOD	r^2
NSCLP (n=23)	0.123	0.377	0.161	0.339	0.115	0.02	0.008
Control (n=58)	0.130	0.544	0.088	0.238	0.134	0.08	0.007
CLP (n=12)	0.178	0.656	0.072	0.094	0.247	0.12	0.036
CPO (n=7)	0	0.625	0	0.375	1	0.77	0.533
CLO (n=4)	0.286	0.143	0	0.571	0	0	0

D', the coefficient of LD; r^2, an alternative to D' is the correlation coefficient between pairs of loci, usually expressed as its square.

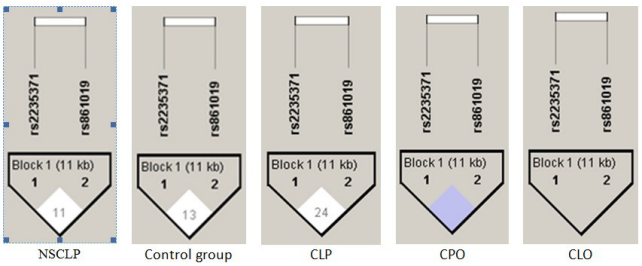

Fig. 1. LD between the IRF6 (rs2235371, rs861019) SNPs
The color scheme shows the strength of adhesion between SNPs: white squares – a poor link (D'<1, LOD<2); grey squares – a strong kink (D'=1 with LOD=2); kb, kilobase.

It is likely that these results for the CLO and CPO subgroups were influenced by the small size of both samples. Unlike the NSCL/P and CLP groups, in these samples, diplotypes with the homozygous genotype GG (rs861019) without the homozygous genotype TT (rs2235371) were more common. Diplotypes with a homozygous genotype of the TT risk allele were not found in the studied groups except for the comparison group, where the [TT]-[AA] diplotype was represented by a low frequency (0.17%).

Table 4.

Distribution of diplotypes for two SNPs (rs2235371 and rs861019) of the IRF6 gene in NSCLP patients and the comparison group

№	Genotype / SNP		Diplotype	Diplotype frequency				
	rs2235371	rs861019		NSCL/P (n=23)	Control (n=58)	CLP (n=12)	CPO (n=7)	CLO (n=4)
1	TT	GG	[TT]-[GG]	0.000	0.000	0.000	0.000	0.000
2	CT	GG	[CT]-[GG]	0.174	0.155	0.333	0.077	0.000
3	TT	GA	[TT]-[GA]	0.000	0.000	0.000	0.000	0.000
4	CT	GA	[CT]-[GG]	0.217	0.224	0.111	0.308	0.000
5	TT	AA	[TT]-[AA]	0.000	0.017	0.000	0.000	0.000
6	CC	AA	[CC]-[AA]	0.087	0.138	0.056	0.154	0.000
7	CC	GA	[CC]-[GA]	0.174	0.155	0.111	0.231	0.750
8	CC	GG	[CC]-[GG]	0.304	0.155	0.333	0.077	0.250
9	CT	AA	[CT]-[AA]	0.043	0.155	0.056	0.154	0.000

IRF6 belongs to a family of nine transcription factors that play a key role in the formation and maintenance of the oral peridemis and palatine shelves. Knockdown of the *IRF6* gene in mice showed an abnormal multilayer development of the epidermis, skin, limbs, and craniofacial regions, and gene expression analysis showed that the primary defect was in the proliferation-differentiation switch of keratinocytes. Since Zucchero et al.[13] found a significant association between the *IRF6* gene and non-syndromic clefts in several populations from Asia, Europe, and North and South America, a number of replication studies have been conducted with different populations and ethnic groups. Notably, one SNP (rs2235371) that changed the conserved amino acid valine to isoleucine at codon 274 in the SMIR-binding domain was significantly associated with cleft lip and palate, especially in Asians and South Americans. We assessed 2 SNPs of the *IRF6* gene in 81 subjects and found evidence of an association between these SNPs and NSCL/P in the Yakut population, further supporting previous findings that the *IRF6* gene is involved in the pathogenesis of NSCL/P. Patients with NSCL/P had a significantly higher frequency of the G allele than the comparison group, with a relatively high odds ratio (OR=2.07, 95% CI: 1.01-4.23, *P*=0.04). This indicates that patients with the rs861019 G allele are predisposed to a 2.07-fold increased risk of developing this anomaly. As far as we know, the relationship between the *IRF6* common variant rs861019 and non-syndromic cleft mouth has been extensively studied around the world. However, to date, the results remain controversial. Inconsistent results may arise due to different ethnic origins, differences in environment, anthropological diversity, different research methods, and complex genetic etiology of the disease. It is generally accepted that any SNP can have only a moderate effect, and combined variants within a gene can provide a more complete assessment in association studies. When LD between markers is weak, haplotype and diplotype analyses have advantages over SNP alone. The results of our diplotype analysis are consistent with the results of the genotypic analysis for rs861019, that is, that the G allele is a risk factor for NSCL/P. We also suspect that patients carrying the [CC]-[GG] diplotype are at a higher risk of developing NSCL/P. Based on these results, we hypothesized that diplotype analysis might be useful to assess the relationship between haplotypes and NSCL/P. Patients with more risk alleles are more likely to develop NSCL/P than patients with fewer or no alleles.

One of the main limitations of this study is the relatively small sample size; some of the minor genotypes are number fewer than five. Therefore, our study was probably not powerful enough to detect a mild to moderate association between SNPs and clefts. The most widely studied marker rs2235371 in the *IRF6* gene, which showed a significant association with an increased risk of NSCL/P in Europeans and Asians, in our study did not show an association with NSCL/P.

Conclusion

In the present case-control study, there were no significant associations between the *IRF6* rs2235371 SNP and NSCL/P. The present study provides strong statistical support (for the first time to our knowledge) that genetic variants of the *IRF6* rs861019 SNP are associated with NSCL/P in Yakuts. Despite the discovery of an association between the rs861019 SNP and the risk of NSCL/P in our patients, more studies are required in similar populations, but with larger sample sizes, to further explore potential associations with different NSCL/P sub-phenotypes and to determine the structure of *IRF6* genetic variants in populations, for example, in Yakutia.

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Competing Interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

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MTHFR and MDR1 Gene Polymorphisms in Yakut Patients with Non-Syndromic Orofacial Clefts

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Abstract

Background: Non-syndromic malformations of the face, jaws, and teeth are quite frequent, and, often, serious diseases, representing one of the complex problems of maxillofacial surgery and surgical dentistry. The aim of our study was to investigate the relationship between the *MDR1* and *MTHFR* gene polymorphisms and non-syndromic cleft lip with or without cleft palate (NSCL/P) in the Yakut population in the Republic of Sakha (Yakutia).

Methods and Results: The sample of examined persons consisted of 60 children with NSCL/P. The NSCL/P group was divided into the CLP (cleft lip with cleft palate) subgroup (n=31), CLO (cleft lip only) subgroup (n=14), and CPO (cleft palate only) subgroup (n=15). The comparison group (control) included 174 healthy volunteers who did not have relatives with OFCs. The study of the *MDR1* rs1045642 SNP and the *MTHFR* rs1801133 SNP was performed by PCR and RFLP analysis.

Analysis of the frequency distribution of alleles and genotypes depending on the severity of clefts showed that the carriage of the TT homozygous genotype of the *MDR1* rs1045642 SNP was associated with significant risk for the development of NSCL/P (OR=2.52, 95% CI: 1.19-5.32, *P*=0.02). Analysis of the recessive model (TT vs CC + TC) also found a significant risk of NSCL/P with the TT genotype carriage (OR=2.20, 95% CI: 1.06-4.57, *P*=0.04). Analysis of the over-dominant model (TC vs TT + CC) showed that the heterozygous TC genotype had a protective effect (OR=0.41; 95% CI: 0.22-0.77, *P*=0.01) on the development of NSCL/P. Subgroup analysis according to NSCL/P subtypes (CLO, CPO and CLP) showed that the *MDR1* rs1045642 SNP was significantly associated with a high risk of CPO in three genetic models: heterozygous [(TT vs TC): OR=5.03; 95% CI: 1.55-16.32; *P*=0.01], recessive [(TT vs CC + TC): OR=3.96; 95% CI: 1.32-11.95; *P*=0.02], and over-dominant [(TC vs TT + CC): OR=0.23; 95% CI: 0.08-0.66; *P*=0.01].

Conclusion: A study of two SNPs in the *MDR1*and *MTHFR* genes revealed a statistically significant increased risk for NSCL/P in carriers of the TT genotype of the *MDR1* rs1045642 SNP. (**International Journal of Biomedicine. 2021;11(4):576-580.**)

Key Words: orofacial cleft • MDR1 gene • MTHFR gene • single nucleotide polymorphism

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Abbreviations

CLP, cleft lip with cleft palate; **CLO**, cleft lip only; **CPO**, cleft palate only; **IRF6**, interferon regulatory factor 6; **LD**, linkage disequilibrium; **MTHFR**, methylenetetra-hydrofolate reductase; **MDR1**, multidrug resistance 1; **NSCL/P**, non-syndromic cleft lip with or without cleft palate; **OFC**, orofacial cleft; **SNP**, single nucleotide polymorphism.

Introduction

Non-syndromic malformations of the face, jaws, and teeth are quite frequent, and, often, serious diseases, representing one of the complex problems of maxillofacial surgery and surgical dentistry. According to Ushnitsky et al., in the structure of clefts, heredity accounts for 7.55±2.18%. Moreover, every eighth cleft lip and (or) palate is part of multiple congenital malformations.[1]

The organ primordia of the maxillofacial system develop in the first trimester of pregnancy from the first and second branchial arches, as well as the frontal protrusion of the cerebral region. From the branchial arches, tissues of the maxillofacial and submandibular regions are formed, with the exception of the central part of the midface zone, which develops from the frontal protrusion of the cerebral region. The development of the fetal lips ends at about 5-6 weeks, and the palate closes at about 10 weeks of gestation.[2] Disorders of the fusion of the secondary palate can be caused by an already existing cleft in the primary palate or can occur separately with a normally developed primary palate.

To clarify the etiology of congenital facial clefts, it is necessary to study how genetic susceptibility factors interact with environmental factors. In recent years, studies of genetic predisposition in the development of non-syndromic cleft lip with or without cleft palate (NSCL/P) have become widespread. Reliable associations of the *MDR1* and *MTHFR* gene variants with an increased risk for NSCL/P have been found in different populations.[3-5]

The *MTHFR* gene, encoding the synthesis of the MTHFR enzyme, is located on chromosome 1p36.3. MTHFR plays a key role in folic acid metabolism. The value of folate in the prevention of neural tube defects (NTDs) is well established,[6,7] and recent studies showing hypomethylation of neural tissue in cases of NTD support this observation.[8-11]

The rs1801133 SNP (also known as 677C>T) is localized in exon 4 of the *MTHFR* gene and is formed by the transition from cytosine (C) to thymine (T). The 222nd genetic code of the *MTHFR* gene changes from GCC to GTC, which leads to the replacement of alanine (Ala) with valine (Val) in the *MTHFR* polypeptide. Animal studies have shown that reducing the formation of methionine from homocysteine plays a key role in the development of neural tube defects. A number of studies investigated the relationship between the polymorphisms of the *MTHFR* gene and OFCs, but with mixed results.[3,5,10]

Functional SNPs in the *MDR1* gene can affect the expression and activity of transport proteins located on the apical and basolateral surfaces of syncytiotrophoblast and placental capillary endothelial cells. These proteins are able to remove toxins or drugs from the environment that enter the mother's body, into the mother's bloodstream, and can lead to an altered response of the fetus on xenobiotics and a subsequent increase in the risk of complex genetic disorders or birth defects.(4) Pels et al. suggest that the rs1045642 SNP (also known as 3435C> T) has an adaptive significance, or is linked to other polymorphic sites that have an adaptive significance.[12]

The aim of our study was to investigate the relationship between the *MDR1* and *MTHFR* gene polymorphisms and NSCL/P in the Yakut population in the Republic of Sakha (Yakutia).

Materials and Methods

The experimental part of the work on the genotyping of the *MDR1* rs1045642 SNP and the *MTHFR* rs1801133 SNP was carried out in the Department of Molecular Genetics at YSC CMP. For the study, we used DNA samples from the collection of biomaterials of the YSC CMP (Project "The Genome of Yakutia"; No. USE_507512).

The sample of examined persons consisted of 60 children (29 girls and 31 boys) with NSCL/P. The NSCL/P group was divided into the CLP subgroup (n=31), CLO subgroup (n=14), and CPO subgroup (n=15). According to indications, a cytogenetic examination was performed to exclude chromosomal pathology in this group of children. The comparison group (control) included 174 healthy volunteers (128 women and 46 men) who did not have relatives with OFCs.

The study was approved by the Ethics Committee of the Yakut Science Center of Complex Medical Problems (YSC CMP). Written informed consent was obtained from each research participant (or the participant's parent/guardian).

Genomic DNA samples were isolated from the peripheral blood leukocytes using a commercial kit for DNA isolation Excell biotech (Yakutsk, Russia). The study of the *MDR1* rs1045642 SNP and the *MTHFR* rs1801133 SNP was performed by PCR and RFLP analysis. Primer sequences, conditions for amplification, restriction pattern, restriction enzymes, and the lengths of the restoration fragments are presented in Table 1.

Table 1.

The primers and restriction enzymes used for detection of MDR1 and MTHFR SNPs using PCR-RFLP method

Gene / RefSNP ID	Primers	AT	RE	RFL
MDR1 rs1045642	F: 5′-TTGATGGCA AAGAAATAAAGC-3′	54˚C	DpnI	CC: 130,76 bp CT: 206,130,76 bp TT: 206 bp
	R: 5′-CTTACATTA GGCAGTGACTCG-3′			
MTHFR rs1801133	F: 5'-TGGGGTCAG AAGCATATCAGTCA -3'	62˚C	TaqI	CC: 497 bp TC: 497,271,226 bp TT: 271,226 bp
	R: 5'-CTGGGAAGA ACTCAGCGAAC-3'			

AT, annealing temperature; RE, restriction enzyme; RFL - restriction fragment length; bp, base pair

Genotypes were determined by analyzing the sizes of the resulting fragments by gel electrophoresis on a 4% agarose gel with ethidium bromide in a standard Tris-acetate buffer at 120V for 1 hour. Restriction products were visualized using a UV gel documentation system (Vilber Lourmat, France).

Statistical analysis was performed using Microsoft Excel 2010. Differences in the allele and genotype distribution between the groups were assessed by χ2- test with Yates correction or Fisher's exact test, when appropriate. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. The following genetic models and test were analyzed: allelic model: D compared with d (d - the minor allele); genotypic tests – homozygous model (DD compared with dd), heterozygous model (Dd compared with dd); recessive model (dd compared with Dd + DD); dominant model (dd+Dd compared with DD); over-dominant model (DD+dd compared with Dd); co-dominant model (DD compared with Dd, compared with dd). A probability value of $P<0.05$ was considered statistically significant.

Results and Discussion

Among 60 OFC patients, CLP was found in 31(51.7%) cases, CLO in 14(23.3%) cases, and CPO in 15(25%) cases. As known, the prevalence of OFC varies according to gender and the nature of the cleft. For example, Mossey et al.[13] found a predominance of CLP in men, with a sex ratio (M/F) of 1.81(95% CI:1.75–1.86) and the opposite ratio for CPO – 0.93(95% CI:0.89–0.96); however, one Danish study failed to find a significant predominance of women with cleft palate.[14] In our study, among CLP cases, boys predominated slightly (51.7%). Analysis of the gender distribution found that CLO was more common in boys (57.2%) and CPO in girls (66.7%). Our study is comparable to the results obtained by Tafazzoli and Shahryari. [15]

NSCL/P has a multifactorial etiology that includes both genetic and environmental factors, and several genes have been associated with this malformation.[16]

More than 40% of OFC children had a family history of the disease. Since the mechanism of occurrence of each type of cleft is different, we compared the genetic variants of the *MDR1* rs1045642 SNP and the *MTHFR* rs1801133 SNP in patients with CLP, CLO, and CPO. Our analysis showed (Table 2) no

Table 2.
Results of allelic and genotype analysis

Gene	Group	CC	CT	TT	C	T	χ2	*P*-value	Genotypes		Alleles	
									HWE	*P*-value	HWE	*P*-value
MTHFR rs1801133	CLP	69.4	30.6	0	0.847	0.153	1.171	0.279				
	M	73.7	26.3	0	0.868	0.132	0.436	0.509	NA	NA	0.40	0.841
	F	64.7	35.3	0	0.824	0.176	0.781	0.377				
	CLO	50	50	0	0.750	0.250	0.889	0.346				
	M	50	50	0	0.750	0.250	0.667	0.414	NA	NA	0.444	0.505
	F	50	50	0	0.750	0.250	0.222	0.637				
	CPO	68.8	31.3	0	0.844	0.156	0.549	0.459				
	M	50	50	0	0.750	0.250	0.667	0.414	NA	NA	0.395	0.530
	F	80	20	0	0.900	0.100	0.123	0.725				
	NSCL/P	66.7	33.3	0	0.833	0.167	2.400	0.121				
	M	64.5	35.5	0	0.823	0.177	1.442	0.230	NA	NA	0.07	0.935
	F	69.0	31.0	0	0.845	0.155	0.978	0.323				
	Control	70.1	28.2	1.7	0.842	0.158	0.588	0.443				
	M	78.3	19.6	2.2	0.880	0.120	0.230	0.632	2.306	0.316	1.026	0.311
	F	67.2	31.3	1.6	0.828	0.172	1.224	0.269				
Gene	Group	CC	CT	TT	C	T	χ2	*P*-value	Genotypes		Alleles	
									HWE	*P*-value	HWE	*P*-value
MDR1 rs1045642	CLP	11.1	63.9	25.0	0.431	0.569	3.303	0.690				
	M	5.3	68.4	26.3	0.395	0.605	3.544	0.60	1.396	0.498	0.169	0.681
	F	17.6	58.8	23.5	0.471	0.529	0.554	0.457				
	CLO	37.5	62.5	0	0.688	0.313	1.653	0.199				
	M	50	50	0	0.750	0.250	0.667	0.414	NA	NA	0.97	0.755
	F	0	100	0	0.500	0.500	2.000	0.157				
	CPO	18.8	43.8	37.5	0.406	0.594	0.139	0.710				
	M	16.7	16.7	66.7	0.250	0.750	1.852	0.174	3.810	0.149	1.045	0.307
	F	20	60	20	0.500	0.500	0.400	0.527				
	NSCL/P	16.7	58.3	25.0	0.458	0.542	1.834	0.176				
	M	16.1	54.8	29.0	0.435	0.565	0.412	0.521	0.563	0.755	0.113	0.737
	F	17.2	62.1	20.7	0.483	0.517	1.710	0.191				
	Control	9.7	77.1	13.1	0.483	0.517	51.917	0				
	M	10.9	73.9	15.2	0.478	0.522	10.645	0.01	0.344	0.842	0	0.983
	F	9.4	78.1	12.5	0.484	0.516	40.720	0				

F, female; M, male. χ2- test with Yates correction

significant differences in the allele and genotype frequencies between all subgroups. We also did not find differences in the frequency distribution of alleles and genotypes between boys and girls. As a result, we compared these subgroups with the comparison group regardless of gender.

We found no significant association between the carriage of the unfavorable allele T of the *MTHFR* rs1801133 SNP and OFC in all subgroups (Table 3).

Analysis of the frequency distribution of alleles and genotypes depending on the severity of clefts showed that

Table 3.

Association of the MDR1 (rs1045642) and MTHFR (rs1801133) polymorphisms with the development of NSCL/P, CLP, CLO, CPO

MTHFR (rs1801133)				
NSCL/P and subgroup	NSCLP	CLP	CLO	CPO
MAF / Associated risk alleles	T			
Control genotypes	122/49/3			
Sample size (case/control)	60/174	36/174	8/174	16/174
Case genotypes CC/TC/TT	40/20/0	25/11/0	4/4/0	11/5/0
Allelic model (T vs C) (95% CI) *P*-value	OR = 1.06 (0.61-1.86) 0.82	OR = 0.96 (0.47-1.94) 0.91	OR = 1.78 (0.55-5.71) 0.36	OR = 0.99 (0.36-2.67) 0.98
Dominant model (TC + TT vs CC) (95% CI) *P*-value	OR = 1.17 (0.63-2.20) 0.62	OR = 1.03 (0.47-2.25) 0.94	OR = 2.35 (0.56-9.74) 0.25	OR = 1.07 (0.35-3.22) 0.91
Reverse heterozygous model (TC vs CC) (95% CI) *P*-value	OR = 1.24 (0.66-2.34) 0.50	OR = 1.10 (0.50-2.40) 0.82	OR = 2.41 (0.60-10.35) 0.22	OR = 1.13 (0.37-3.43) 0.83
Over-dominant model (TC vs TT + CC) (95% CI) *P*-value	OR = 1.28 (0.68-2.40) 0.45	OR = 1.12 (0.51-2.45) 0.77	OR = 2.55 (0.61-10.60) 0.20	OR = 1.16 (0.38-3.51) 0.79
MDR1 (rs1045642)				
MAF / Associated risk alleles	T			
Control genotypes	17/135/23			
Sample size (case/control)	60/175	36/174	8/174	16/174
Case genotypes CC/TC/TT	10/35/15	4/23/9	3/5/0	3/7/6
Allelic model (C vs T) (95% CI) *P*-value	OR = 1.10 (0.73-1.67) 0.64	OR = 1.23 (0.74-2.06) 0.42	OR = 0.42 (0.14-1.25) 0.11	OR = 1.36 (0.65-2.85) 0.40
Homozygous model (TT vs CC) (95% CI) *P*-value	OR = 1.11 (0.40-3.06) 0.84	OR = 1.66 (0.44-6.31) 0.45	NA	OR = 1.48 (0.32-6.77) 0.61
Heterozygous model (TT vs TC) (95% CI) *P*-value	OR = 2.52 (1.19-5.32) 0.02	OR = 2.30 (0.94-5.58) 0.08	NA	OR = 5.03 (1.55-16.32) 0.01
Reverse heterozygous model (TC vs CC) (95% CI) *P*-value	OR = 0.44 (0.19-1.05) 0.07	OR = 0.72 (0.22-2.35) 0.60	OR = 0.21 (0.05-0.96) 0.06	OR = 0.29 (0.07-1.24) 0.12
Recessive model (TT vs CC + TC) (95% CI) *P*-value	OR = 2.20 (1.06-4.57) 0.04	OR = 2.20 (0.92-5.27) 0.09	NA	OR = 3.96 (1.32-11.95) 0.02
Dominant model (TC + TT vs CC) (95% CI) *P*-value	OR = 0.54 (0.23-1.25) 0.16	OR = 1.16 (0.37-3.68) 0.80	OR = 0.18 (0.04-0.82) 0.04	OR = 0.47 (0.12-1.80) 0.30
Over-dominant model (TC vs TT + CC) (95% CI) *P*-value	OR = 0.41 (0.22-0.77) 0.01	OR = 0.52 (0.24-1.13) 0.11	OR = 0.49 (0.11-2.16) 0.36	OR = 0.23 (0.08-0.66) 0.01

MAF, minor allele frequency

the carriage of the TT homozygous genotype of the *MDR1* rs1045642 SNP was associated with significant risk for the development of NSCL/P (OR=2.52, 95% CI: 1.19-5.32, *P*=0.02).

Analysis of the recessive model also found a significant risk of NSCL/P with the TT genotype carriage (OR=2.20, 95% CI: 1.06-4.57, *P*=0.04).

Analysis of the over-dominant model (TC vs TT + CC) showed that the heterozygous TC genotype had a protective effect (OR=0.41, 95% CI: 0.22-0.77, *P*=0.01) on the development of NSCL/P.

Subgroup analysis according to NSCL/P subtypes (CLO, CPO and CLP) showed that the *MDR1* rs1045642 SNP was significantly associated with a high risk of CPO in three genetic models (Table 3): heterozygous [(TT vs TC): OR=5.03, 95% CI: 1.55-16.32, *P*=0.01], recessive [(TT vs CC + TC): OR=3.96, 95% CI: 1.32-11.95, *P*=0.02], and over-dominant [(TC vs TT + CC): OR=0.23, 95% CI: 0.08-0.66, *P*=0.01].

Conclusion

The present case-control study of two SNPs in the *MDR1*and *MTHFR* genes revealed a statistically significant increased risk for NSCL/P in carriers of the TT genotype of the *MDR1* rs1045642 SNP.

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Competing Interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

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The Effectiveness of Local Application of Melatonin in the Original Dermal Film in Experimental Thermal Trauma

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Abstract

Background: The development and pathogenetic substantiation of the new agents used for local therapy of thermal trauma (TT) is an urgent problem in medicine. Melatonin (MT) is an endogenous factor of homeostasis regulation with pleiotropic potential. The aim of our study was to assess the morphology, expression of matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor (VEGF), indicators of repair, oxidative destruction of lipids in the skin lesion focus in the dynamics of experimental TT under the conditions of using the original dermal film (DF) with MT.

***Methods and Results*:** The experiment was performed on 104 male Wistar rats weighing 200-240 g. For modeling TT II degree according to ICD-10 a relative area of 3.5% of the body surface, an interscapular region isolated from the surrounding tissues, was immersed in distilled water at 98-99 °C for 12 sec. DF based on sodium carboxymethylcellulose with an area of 12 cm2 with MT at a concentration of 5 mg/g was applied daily for five days. The wound area and epithelialization rate were calculated. The content of MMP-9 and VEGF in the burn wound was assessed by an immunohistochemical method. In the homogenate of the burn wound, the content of LPO products was assessed. Morphological and biochemical studies were performed on Days 5, 10 and 20 after TT induction.

With experimental TT from Day 5 to Day 20, the absolute area of the burn wound decreases by 35%, the rate of epithelialization increases, the number of neutrophils in the focus of thermal damage decreases, while the representation of lymphocytes, histiocytes, and fibroblasts increases; the expression of MMP-9 and VEGF increases; predominantly secondary and final LPO products in the heptane phase accumulate, the final products of LPO in the isopropanol phase of the lipid extract. The use of MT in the composition of DF daily for 5 days with experimental TT leads to a decrease in the area of the wound defect (by 46% of the original area on Day 20), an increase in the rate of its epithelialization, an increase in the content of lymphocytes and fibroblasts in the burn wound on Days 5, 10 and 20 of TT, a decrease in the representation of neutrophils and macrophages on Days 5 and 10, as well as an increase in VEGF expression on Days 5 and 10, MMP-9 - on Day 5 and a decrease in MMP-9 expression on Days 10 and 20 of TT. In addition, the use of MT in the composition of DF leads to a decrease in the content of predominantly secondary and end products of LPO in the heptane and isopropanol phases of the burn wound on Days 10 and 20 of TT. Correlation analysis revealed that a decrease in the burn surface area under a local application of MT occurs with an increase in the content of VEGF in the wound area and a decrease in the content of MMP-9 and secondary and final LPO products in the heptane phase and the isopropanol phase. On Day 20, there were direct moderate correlations between the absolute burn surface area, on one hand, and secondary and final LPO products, on the other, in the heptane phase ($R=0.51$, $R=0,68$; $P<0.05$) and the isopropanol phase ($R=0.44$, $R=0.46$; $P<0.05$), respectively.

Conclusion: The results obtained expand the existing understanding of the role of changes in the expression of MMP-9 and VEGF in the pathogenesis of TT. We believe that the repair-stimulating effect of MT in the DF, which we established during TT at the preclinical stage, is associated with the LPO-limiting effect of MT and a change in the expression of MMP-9 and VEGF in the burn wound and is a prerequisite for further study of the mechanism of action and the effectiveness of MT application in clinical conditions in TT. (**International Journal of Biomedicine. 2021;11(4):581-589.**)

Key Words: MMP-9 • VEGF • lipid peroxidation • thermal trauma • melatonin

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Abbreviations

BSA, burn surface area; **CD**, conjugated dienes; **CT**, conjugated trienes; **DF**, dermal films; **FRO**, free-radical oxidation; **GPO**, glutathione peroxidase; **HPh,** heptane phase; **IPh**, isopropanol phase; **KD**, ketodienes; **LPO**, lipid peroxidation; **MT**, melatonin; **MMP**, matrix metalloproteinase; **MDA**, malondialdehyde; **SOD**, superoxide dismutase; **SB**, Schiff bases; **TT**, thermal trauma; **VEGF**, vascular endothelial growth factor.

Introduction

A wide range of therapeutic approaches is used for local therapy of small-area thermal trauma (TT). In particular, early surgical necrectomy, which restrains excessive inflammation and infections in the burn wound, and enzymatic debridement of the burn wound.[1] Modern wound dressings are woven or non-woven materials, including hydrogel, gel dressings on a textile basis, hydrocolloid dressings, polyurethane sponge dressings, and calcium alginate dressings, There are wound dressings, on absorbent fabric and non-woven bases, impregnated with solutions of drugs: hemostatic agents, antiseptics, wound healing agents, antioxidants, regenerants of various natures, anti-inflammatory drugs, hemostatic agents, etc. Despite the variety of dressings, the issue of developing drugs for TT local therapy remains topical. To close the burn wound, nylon dressings with collagen, hydrolyzed collagen, or carbon nanotubes can be used, as well as dermal films (DF) based on carboxymethyl cellulose containing various biologically active substances.[2]

Adrenaline, thrombin, fibrin, recombinant tissue factor, etc. are used as active components of wound dressings.[3] Hydrogels based on polyvinyl alcohol, chitosan, alginate or polyethylene glycol are widely used when conjugated with zinc oxide, phlorotannins, hyaluronic acid polymyxin B, VEGF, which form a barrier against pathogens and a hydrated environment for wound healing.[4] Fibrin gel is used in patients with TT as a hemostatic and graft fixator and skin substitute.[5] Hydrogels based on hyaluronic acid improve skin regeneration and reduce the area of scar tissue by increasing VEGF secretion, reducing the expression of TGF-β1.[6] The skin is the largest organ with intensive FRO processes; oxidative stress during TT is recorded not only in the lesion focus, but also in distant tissues, and therefore antioxidants in TT have shown effectiveness in the coagulation zone (necrosis), limiting cell death in the ischemic zone.[7] In this regard, it is of interest to study the LPO products in the TT focus as markers of the effectiveness of FRO and antioxidant use.

When searching for new therapeutic approaches to TT, special attention is paid to homeostasis regulators of endogenous origin.[8-10] A potential, but theoretically justified, interest in the discussion of promising therapeutic agents for TT is associated with melatonin (MT). MT is one of the most ancient molecules in the evolutionary sense, having been present in living organisms for about 2-3 billion years. It is found in animals, plants, fungi, and prokaryotes, where it originally served as an antioxidant. MT biosynthesis occurs from tryptophan, mainly in the pineal gland.[11] MT is also synthesized by retinal cells, enterochromaffin cells of the gastrointestinal tract, monocytes, lymphocytes, dendritic and mast cells.[12] Currently, MT is considered as an endogenous factor with multitrophic effects in various cells, including antioxidant, pro- and anti-inflammatory, immunomodulatory, antiapoptogenic, regulating cell proliferation and differentiation, and anti-aging.[13] Mammalian skin has its own melatoninergic system involved in maintaining homeostasis and integrity due to MT synthesis and the presence of specific receptors. Skin cells synthesize MT; its metabolites are found in keratinocytes, melanocytes, and dermal fibroblasts, as well as in melanoma cells.[14] The MT1 receptor is found in keratinocytes and fibroblasts of the skin and hair follicle cells; the MT2 receptor is found mainly in the eccrine glands, blood vessels of the skin, and melanocytes.[15] The RORα receptor has been identified in epidermal keratinocytes, fibroblasts, and melanocytes. When the skin is damaged, MT accumulates in the epidermis, protecting mitochondria and ensuring the synthesis of ATP.(15) MT regulates the proliferation and differentiation of epidermal cells, the development of hair follicles, the expression of keratin, and involutin in the epidermis. Information was obtained on the radioprotective effect of MT after exposure to X-ray and gamma radiation on the skin in vivo and in cell culture.[16] Considering the above, it is of interest to study the effectiveness of MT local application in the skin TT.

The aim of our study was to assess the morphology, expression of MMP-9 and VEGF, indicators of repair, oxidative destruction of lipids in the skin lesion focus in the dynamics of experimental TT under the conditions of using the original DF with MT.

Methods

The experiment was performed on 104 male Wistar rats weighing 200-240 g. The animals were randomly divided into 4 groups: Group 1 (n=14) included intact control, Group 2 (n=30) – animals with TT and an aseptic dressing applied to the BSA (TT+ASP); Group 3 (n=30) – animals with TT and the DF-matrix and aseptic dressing imposition on the BSA (TT+ASP+DF), and Group 4 (n=30) – animals with TT in conditions of DF/MT and aseptic dressing application on the BSA (TT+ASP+DF/MT). The aseptic dressing was changed daily up to 20 days after TT. Since the most common causes of TT are hot liquid and flame, and in two-thirds of patients the area of the burn is less than 10% of the body surface,[17] for modeling TT II degree according to ICD-10 a relative area of 3.5% of the body surface, an interscapular region isolated from the surrounding tissues, was immersed in distilled water at 98-99 °C for 12 sec. From the area of the skin in the interscapular region intended for the burn, the hair was clipped and shaved, washed with warm saline and wiped dry. To create TT, a device made of heat-resistant plastic with a hole diameter of 38 mm was used, into which the animal was placed (Fig.1). The prepared area of the skin interscapular region was aligned with the hole of the device, the animal was fixed. The depth of the burn was verified by morphological methods. For anesthesia,

the drug Zoletil-100 (tiletamine, zolazepam) (Virbac Sante Animale; France) was used at a dose of 20 mg/kg. DF with an area of 12 cm^2 in Groups 3 and 4 was applied immediately after TT, fixing with an aseptic bandage; The TT was dressed daily for 5 days. In preliminary studies, a DF composition was developed based on sodium carboxymethylcellulose (poly-1,4-β-O-carboxymethyl-D-pyranosyl-D-sodium glycopyranose), MT was included at a concentration of 5 mg/g, and it was evaluated by pharmacological and technological parameters: organoleptic indicators (appearance, color, transparency, elasticity, presence of impurities and microcracks), adhesive ability, mechanical tensile strength, and thickness.[18] Group 3 used DF, similar in area and properties, but not containing MT.

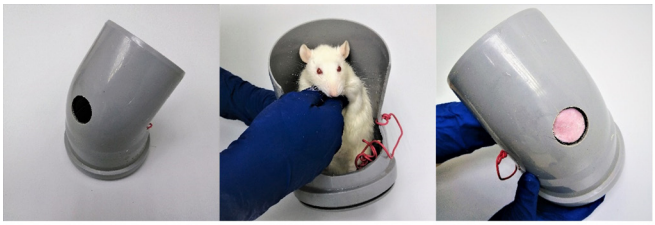


Fig.1. *Device for simulation of rat's TT.*

A Nikon Coolpix S2800 camera (China) and a Microsoft Office Visio software package were used to calculate the wound area on 24h, Days 5 and 10 after TT by digital planimetry. The epithelialization rate (VS) was calculated by the formula: VS = S - Sn/t, where S is the initial area of the wound before treatment (hereinafter, the area at the previous measurement); Sn is the area in the subsequent measurement; t is the number of days between measurements.

The area of the wound (BSA) in subsequent measurements was determined in percent, taking as 100% the area before treatment, the result was expressed in percent/day. A skin flap was excised along the border of the wound with the capture of an area of intact skin for morphological and immunohistochemical studies. The morphology of the lesion focus was examined on a DMRXA microscope (Leika, Germany) using the ImageScope M computer program (Germany) at ×50, ×200, and ×400 magnifications. From paraffin blocks, histological sections with a thickness of 5-7 microns were prepared, which were stained with H&E (Biovitrum, Russia). The depth of skin damage, the reaction of the vascular bed, the presence and composition of the cellular infiltrate, the timing of the appearance of granulation tissue in the wound, and the timing of wound epithelialization were assessed. The following morphometric parameters per mm^2 were determined: the number of fibroblasts, neutrophils, histiocytes, and lymphocytes. The content of MMP-9 and VEGF in the burn wound was assessed by an immunohistochemical method using specific polyclonal antibodies to rat MMP-9 (host - rabbit, cat. No. PAA553Ra01 "Cloud-Clone Corp.", China) and polyclonal antibodies to rat VEGF (host - rabbit, cat. No. PAA143Ra01 "Cloud-Clone Corp.", China) and a polymer detection system Rabbit HRP/DAB Detection IHC Detection Kit ("Abcam," Latvia) for detecting antigen-bound rabbit immunoglobulins in tissue sections. Diaminobenzidine was used as a substrate/chromogen for visualization of the polymer complex in the system. We determined the relative area of MMP-9 - positively and VEGF - positively stained structures and determined the integral index of the content of MMP-9 and VEGF as the product of the relative area of the stained structures on the intensity of the color in points. The result was expressed in arbitrary units per mm^2 (unit/mm^2). In the homogenate of the burn wound, the content of LPO products was assessed. To prepare a 10% homogenate, the burn wound was excised, 40mg was washed in chilled buffer, dried, ground and homogenized at 2-4 °C in 0.4 ml (1:10) of chilled 0.1M phosphate buffer (pH=7.4). LPO products were determined on a spectrophotometer SF-56 ("LOMO – Spectrum," St. Petersburg).[19] The optical density of heptane and isopropanol extracts was measured at 220nm (isolated double bonds), 232 nm (CD), 278 nm (KD and CT), 400 nm (SB). The relative content of LPO products was expressed in units of oxidation indices (u.o.i.): E_{232} / E_{220} (CD), E_{278} / E_{220} (KD and CT), and E_{400} / E_{220} (SB). Morphological and biochemical studies were performed on Days 5, 10 and 20 after TT induction.

Statistical analysis was performed using statistical software package SPSS version 19.0 (Armonk, NY: IBM Corp.). The results are presented as median (Me) and and interquartile range (IQR [Q1;Q3]). A non-parametric Kruskal-Wallis test was used for comparisons of median values among groups, followed by post-hoc testing using un-paired Mann-Whitney U tests. Differences were considered statistically significant at $P<0.01$, taking into account the Bonferroni correction. Spearman's rank correlation coefficient was calculated to measure the strength and direction of the relationship between two variables.

The experiments were performed in accordance with the norms for the humane treatment of animals and approved by the Ethics Committee of the South Ural State Medical University.

Results and Discussion

When evaluating the parameters of burn wound repair, it was found that in the dynamics of TT on Day 10 of observation, compared with Day 5, the absolute area of the wound defect decreased, which led to an increase in the rate of wound epithelialization and the proportion of decrease in its area (Table 1). On Day 20 of TT, the absolute area of the burn decreased, in comparison with Days 5 and 10; the relative area of the burn decreased compared with Day 5 of observation, which was accompanied by an increase in the rate of wound epithelialization and the proportion of a decrease in its area, compared to Days 5 and 10. The area of the wound defect from Day 5 to Day 20 of experimental TT decreased by 35% along the median.

On Days 5 and 10, histological examination of the skin determined typical signs of the skin TT: necrosis of the epidermis and dermis layers to the papillary layer, thickening of the connective tissue fibers of the dermis, hyperchromia of the nuclei of the cells of the interstitial tissue (Fig. 2A, 3A). On Day 5, in the perifocal zones in all layers of the skin, we found paretic venous and capillary plethora, small diapedetic hemorrhages, erythro- and leukostasis, sludge of erythrocytes; by Day 10, edema of the interstitial tissue led to the accumulation of exudate and the formation of blood clots in the large vessels lumen (Fig. 2A, 3A).

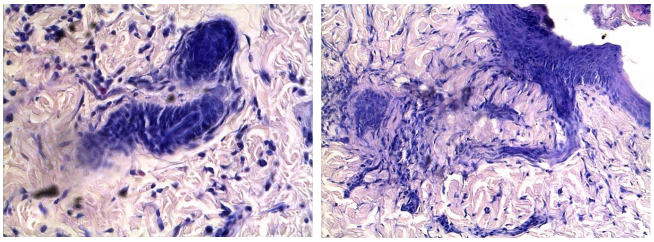
Table 1.

Influence of MTin the composition of DF on the indicators of wound repair in TT (Me [Q25; Q75])

Parameters	Group 2 TT + ASP			Group 3 TT+ASP+DF			Group 4 TT+ASP+MT/DF		
	Day 5 (n=16)	Day 10 (n=20)	Day 20 (n=20)	Day 5 (n=16)	Day 10 (n=20)	Day 20 (n=20)	Day 5 (n=16)	Day 10 (n=16)	Day 20 (n=12)
Burn square, cm^2	11.66 (11.50;11.94)	9.48 (9.28;9.93)*	7.59 (7.23;7.84)**	11.59 (11.00;11.99)	9.40 (9.22;9.81)	7.29 (7.01;7.52)	10.33 (10.17;10.56)#	8.34 (8.19;8.51)#	5.54 (5.24;5.88)#
Percent area, %	3.34 (3.25;3.39)	3.17 (3.10;3.29)	2.99 (2.94;3.12)*	3.31 (3.22;3.42)	3.16 (3.10;3.28)	2.81 (2.74;3.09)	3.36 (3.23;3.42)	3.02 (2.91;3.13)#	1.98 (1.87;2.23)#
Epithelization speed, %/day	0.89 (0.86;0.89)	1.90 (1.88;1.95)*	2.26 (2.14;2.55)**	0.91 (0.85;0.92)	2.01 (1.93;2.05)	3.06 (2.73;3.15)	1.33 (1.29;1.35)#	6.57 (5.92;6.93)#	14.30 (13.38;15.17)#
Wound square decreasing, %	2.61 (2.59;2.64)	3.68 (3.53;4.23)*	11.49 (11.43;11.64)**	2.60 (2.58;2.64)	3.71 (3.63;4.31)	13.58 (12.93;14.01)	9.80 (9.64;10.08)#	16.10 (14.62;17.73)#	19.98 (19.30;20.38)#

** - significant (P<0.01) differences with Group 2 on Day 5, ** - with Group 2 on Day 10; # with Group 3.*

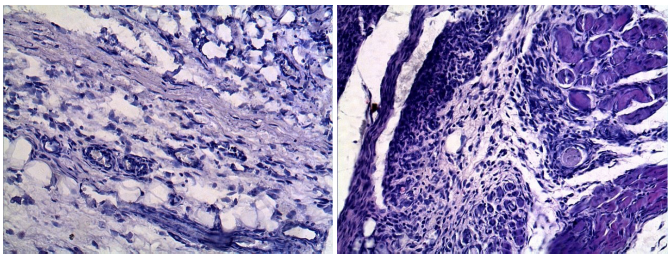
Cellular infiltration consisted of neutrophils, lymphocytes, and to a lesser extent, macrophages and plasma cells. By Day 10, multiple, diffusely scattered foci of macrophage infiltration were visible. In the middle layers of the dermis there were areas where fibroblasts proliferated, forming bundles and strands, and epithelization was weak, with hyperplasia of cells of the basal layer of the preserved epidermis and proliferation under the scab (Fig. 3A). On Day 20 of TT, an immature scar was determined under the burn scab in the form of compactly packed, dense, little-wrinkled fibers with multiple foci of neutrophilic-lymphocytic and macrophage infiltration (Fig. 4A). Proliferating fibroblasts formed cords and bundles parallel to the skin surface. Newly formed vessels were few in number with endothelial lining and differentiated walls.



A B

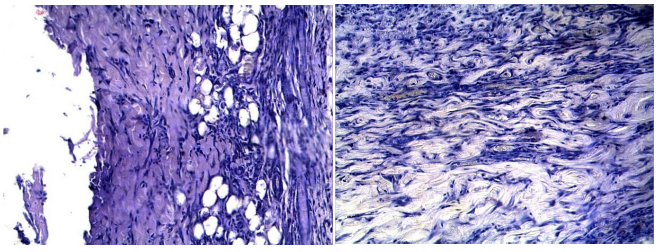
Fig. 2. *Morphological changes in the dermis of the perifocal zone on Day 5 of TT. H&E, magnification × 400. A - Group 2 (TT+ASP): stretching and reorientation of hair follicle cell nuclei; B - Group 4 (TT+ASP+DF/MT): proliferation of cells of the sebaceous glands and hair follicles, the initial phenomena of neoangiogenesis.*

A morphometric assessment of the cellular composition of the infiltrate in the TT focus was carried out (Table 2). In the dynamics of TT, the number of neutrophils on Day 10 did not differ from the values on Day 5; on Day 20, it was significantly lower than on Days 5 and 10. The number of lymphocytes on Day 10 was significantly lower than on Day 5 of TT, and on Day 20, it was significantly higher than on Days 5 and 10. The number of histiocytes and fibroblasts on Day 10 was significantly higher than on Day 5 of TT, and on Day 20 – significantly higher than on Days 5 and 10. On Days 5, 10, and 20 of TT, VEGF expression significantly increased in the burn wound; on Days 10 and 20 of TT, the expression of MMP-9 significantly increased also (Table 3). In the dynamics of the experiment, the expression of VEGF on Day 10 was higher than on Day 5, on Day 20 – lower than on Day 10; the expression of MMP-9 on Days 10 and 20 was higher than on Day 5 of TT.



A B

Fig. 3. *Morphological changes in the perifocal zone in the hypodermis on Day 10 of TT. H&E, magnification × 200. A - Group 2 (TT+ASP). Focal neutrophilic-lymphocytic infiltration and accumulations of macrophages; B - Group 4 (TT+ASP+DF/MT). Perifocal zone: in the hypodermis, the proliferation of immature granulation tissue with a large number of cellular elements and the initial phenomena of fibrillogenesis.*



A B

Fig. 4. *Morphological changes in scar tissue on Day 20 of TT. H&E, magnification × 200. A - Group 2 (TT+ASP). Densely packed connective tissue fibers of immature scar tissue; B - Group 4 (TT+ASP+DF/MT). Full-blooded newly formed vessels and small foci of macrophage and round-cell infiltration.*

In the wound, the content of KD/CT and SB in the HPh and IPh of the extract increased on Day 5 of TT (Table 4). On Days 10 and 20, in the HPh of the burn wound, the content of KD/CT and SB increased, in the IPh – only SB. In the dynamics of TT, the SB content in the HPh and IPhs was less on Day 10 than on Day 5, and more on Day 20 ($P<0.01$) than on Day10.

Table 2.

Influence of MT in the composition of DF on morphometric parameters in the focus of skin damage during TT (Me [Q25; Q75])

Parameters	Group 2 TT + ASP			Group 3 TT+ ASP+DF			Group 4 TT+ ASP+MT/DF		
	Day 5 (n=43)	Day 10 (n=26)	Day 20 (n=26)	Day 5 (n=43)	Day 10 (n=26)	Day 20 (n=26)	Day 5 (n=25)	Day 10 (n=25)	Day 20 (n=25)
Neutrophil, unit/mm^2	1184.7 (1080.0;1320.0)	1117.7 (1020.0;1240.0)	120.0 (100.0-160.0)$^{*\,**}$	1132.6 (1055.0;1302.0)	1069.7 (1001.0;1126.0)	117.0 (104.0;128.0)	384.0 (340.0;420.0)$^{\#}$	373.9 (220.0; 20.0)$^{\#}$	110.5 (60.0;160.0)
Lympho-cytes, unit/mm^2	282.3 (220.0;340.0)	131.5 (100.0;160.0)*	326.9 (260.0;380.0)$^{*\,**}$	277.3 (226.0;324.0)	135.1 (103.0;157.0)	321.4 (257.0;371.0)	1187.8 (1040.0;1360.0)$^{\#}$	312.8 (240.0;360.0)$^{\#}$	336.8 (240.0;380.0)
Fibro-blasts, unit/mm^2	140.9 (100.0;160.0)	113.1 (80.0;140.0)	840.8 (700.0;940.0)$^{*\,**}$	144.2 (109.0;162.0)	119.4 (85.0;146.0)	849.7 (752.0;931.0)	175.2 (140.0;220.0)$^{\#}$	335.2 (300.0;380.0)$^{\#}$	973.6 (840.0;1140.0)$^{\#}$
Macro-phages, unit/mm^2	366.9 (320.0;420.0)	569.2 (440.0;640.0)*	667.7 (600.0;740.0)$^{*\,**}$	341.8 (310.0;405.0)	572.9 (467.0;630.0)	612.7 (545.0;668.0)	164.8 (120.0;200.0)$^{\#}$	290.4 (220.0;360.0)$^{\#}$	420.8 (340.0;480.0)$^{\#}$

** - significant (P <0.01) differences with Group 2 on Day 5; ** - with Group 2 on Day 10; # with Group 3.*

Table 3.

Influence of MT in the composition of DF on immunohistochemical parameters in the burn wound (Me [Q25; Q75])

Parameters	Group 1 (control) (n=14)	Group 2 TT + ASP			Group 3 TT+ASP+DF			Group 4 TT+ASP+MT/DF		
		Day 5 (n=7)	Day 10 (n=7)	Day 20 (n=7)	Day 5 (n=7)	Day 10 (n=7)	Day 20 (n=7)	Day 5 (n=7)	Day 10 (n=7)	Day 20 (n=7)
VEGF, unit/mm^2	3.30 (2.90;3.50)	25.05 (23.35;28.30)*	35.50 (33.40;38.10)$^{*\,\#}$	25.80 (22.30;27.90)$^{*\,\#\#}$	25.40 (22.20;29.30)*	32.50 (31.50;34.20)*	26.90 (25.80;28.30)*	33.00 (30.20;34.90)$^{*\,\&}$	45.60 (41.70;49.20)$^{*\,\&}$	27.10 (22.50;29.10)*
MMP-9, unit/mm^2	2.50 (2.10;2.90)	2.85 (2.45;3.05)	11.90 (10.80;14.10)$^{*\,\#}$	12.40 (12.20;12.90)$^{*\,\#}$	3.30 (2.20;4.40)	12.10 (10.30;12.90)*	12.90 (12.70;13.50)*	14.20 (11.30;18.10)$^{*\,\&}$	3.40 (1.10;4.20)$^{\&}$	3.30 (1.40;4.80)$^{\&}$

** - significant (P<0.01) differences with Group 1, # - with Group 2 on Day 5, ## - with Group 2 on Day 10, & – with Group 3.*

The use of MT in the composition of DF during TT led to a statistically significant decrease in the area of the burn wound in absolute values on Days 5, 10, and 20, in relative values on Days 10 and 20 of TT (Table 1). On Days 5, 10, and 20 of observation, the rate of wound epithelialization and the relative decrease in the area of the wound increased. On Day 5 of TT, the absolute area of the BSA decreased by 12.2%; the maximum changes were recorded on Day 20, when the absolute area of the wound defect decreased by 31.6%, and the epithelialization rate increased 4.7 times relative to Group 3. Thus in Group 4, from Day 5 to Day 20 of TT, the absolute area of the BSA decreased by 46%. Morphological examination of the burn wound on Day 5 revealed that along with signs of necrosis of the epidermis and dermis to the papillary layer (changes in the connective tissue characteristic of TT) there was a decrease in the infiltration of the focus by neutrophils and macrophages, the predominance of lymphocytes, young spindle-shaped fibroblasts, a large number of immature newly formed vessels in the form of acellular gaps, and cells of the preserved hair follicles and sebaceous glands participated in epithelialization (Fig.2B). On Day 10, immature granulation tissue with fibroblasts forming bundles and cords, vessels with differentiated walls and endothelial lining were determined in the hypodermis (Fig.3B). In the epidermal layer, at the border of the necrosis focus and intact skin, the proliferation of cells of the basal layer was visible. On Day 20, a completely epithelized young connective tissue scar with fibroblasts, small foci of macrophage, round cell infiltration, and newly formed vessels with differentiated walls were determined (Fig.4B). Young scar tissue was completely epithelialized, with distinct stratification and proliferating cells of the sebaceous glands and hair follicles. Compared to animals of Group 3, in Group 4 the number of neutrophils and histiocytes significantly decreased in the focus of the BSA on Days 5 and 10 of TT, the number of lymphocytes and fibroblasts increased; on Day 20 of TT, the content of histiocytes in the focus of damage decreased, and the number of fibroblasts increased (Table 2).

When evaluating the expression of VEGF and MMP-9 in a burn wound under the conditions of local application of MT, it was found that on Day 5 of the experiment, the expression in the BSA of MMP-9 and VEGF significantly increased; on Day 10, the expression of MMP-9 decreased, VEGF increased; on Day 20 of TT, the expression of MMP-9 decreased, VEGF did not change significantly (Table 3). The expression of VEGF on Days 5, 10, and 20, and MMP-9 on Day 5 was significantly higher than in intact animals; the expression of MMP-9 on Days 10 and 20 did not differ from the values in the group of intact animals.

In Group 4, on Day 5 of TT, in the IPh of the lipid extract of the burn wound, the amount of KD/CT and SB decreased (Table 4). On Day 10 of TT, in the HPh, the amount of KD/CT decreased, in the IPh - SB decreased also. On Day 20, a decrease in the content of KD/CT, as well as SB, was recorded in the HPh and IPh of the lipid extract of the burn wound. The LPO product content in a burn wound in animals of Group 3 significantly differed from that of intact animals on Days 5, 10, and 20 in relation to KD/CT in the HPh of the lipid extract, in relation to SB in the HPh and IPh of the lipid extract.

In Group 4, an inverse, significant, weak relationship was found between the absolute BSA and VEGF expression on Days 5 and 10 of the experiment, and a direct moderate relationship with the expression of MMP-9 on Days 10 and 20 of TT (Table 5). The absolute BSA had a direct weak correlation with the content of KD/CT in the HPh, and direct moderate correlations with the content of KD/CT and SB in the IPh on Day 5. On Day 10, we found direct moderate correlations between the absolute BSA and the KD/CT content in HPh and SB in the IPh. On Day 20, there were direct moderate correlations between the absolute BSA, on one hand, and KD/CT and SB, on the other, in the HPs and IPh.

So, with TT, well-known morphological patterns and dynamics of the change in the cellular composition during the inflammatory process were recorded: neutrophilic infiltration was most pronounced on Days 5 and 10 and it decreased on Day 20.[20] The maximum representation of lymphocytes and histiocytes, which create the necessary conditions for switching vascular-exudative reactions to proliferative ones, was observed on Day 20 of TT. At the same time, the maximum representation of fibroblasts participating in the synthesis of the extracellular matrix of the connective tissue was recorded. It is necessary to note the uniformity of the depth of skin damage when using this experimental model of TT with hot water. An increase in the expression of VEGF and MMP-9 in a burn wound is important in TT repair. MMP-9 is involved in the destruction of the extracellular matrix (especially type IV and V collagens), an increase in vascular permeability, chemotaxis of neutrophils, and activation and inactivation of autocoids. It also prepares a springboard for reparative reactions and successful epithelialization, and is inhibited by a tissue inhibitor of proteinases, α-1-antichymotrypsin. MMP-9 reflects the representation and activity of predominantly neutrophils and macrophages in the TT focus.[21] VEGF is involved not only in the regulation of angiogenesis, but also in the synthesis of collagen and other components of connective tissue, scar formation due to direct and/or indirect activation of fibroblasts, endotheliocytes, neutrophils, macrophages, and mast cells.[22]

The reparation in the TT focus is influenced by the redox status of the burn wound.[23] According to other researchers, after TT, the content of LPO products in the plasma, such as MDA, increases and persists for up to 30 days, CD increases slightly on the first day, and then decreases; the levels of reduced glutathione, the activity of SOD, catalase, and total antioxidant capacity of serum decreased.[24]

When simulating TT in mice, rats, and pigs, in serum and in a burn wound, the activity of SOD, catalase, GPO, and the total antioxidant activity of serum decrease; the levels of MDA and carbonyl derivatives of proteins increase.[25] An increase in the LPO product content in the skin TT focus is a result of the activation of FRO under conditions of excessive generation of free radicals and/or a decrease in the activity of antioxidant defense system. Inducers of FRO in TT are NADFH oxidase

Table 4.

Influence of MT in the composition of DF on the content of LPO products in the burn wound homogenate (Me [Q25; Q75])

Parameters (u.o.i.)	Group 1 (control) (n=20)	Group 2 TT + ASP			Group 3 TT+ASP+DF			Group 4 TT+ASP+MT/DF		
		Day 5 (n=21)	Day 10 (n=32)	Day 20 (n=25)	Day 5 (n=21)	Day 10 (n=32)	Day 20 (n=25)	Day 5 (n=25)	Day 10 (n=28)	Day 20 (n=17)
CD (HPh)	0.920 (0.863;0.975)	0.889 (0.834;0.966)	0.891 (0.836;0.944)	0.927 (0.873;0.951)	0.914 (0.844;0.969)	0.911 (0.852;0.947)	0.921 (0.869;0.948)	0.866 (0.808;0.908)	0.889 (0.834;0.942)	0.909 (0.861;0.921)
KD и CT (HPh)	0.049 (0.013;0.088)	0.123 (0.112;0.141)*	0.115 (0.101;0.141)*	0.126 (0.092;0.155)*	0.119 (0.110;0.138)*	0.117 (0.098;0.142)*	0.116 (0.093;0.145)*	0.109 (0.103;0.161)*	0.095 (0.058;0.131)*&	0.086 (0.085;0.094)*&
SB (HPh)	0 (0;0.011)	0.018 (0.013;0.031)*	0.009 (0.003;0.018)*#	0.025 (0.015;0.056)*##	0.016 (0.011;0.029)*	0.008 (0.003;0.016)*	0.022 (0.012;0.041)*	0.019 (0.012;0.028)*	0.004 (0.003;0.017)*	0.005 (0.004;0.006)*&
CD (IPh)	0.601 (0.596;0.622)	0.594 (0.570;0.732)	0.580 (0.568;0.614)	0.613 (0.590;0.647)	0.589 (0.562;0.729)	0.582 (0.564;0.620)	0.608 (0.585;0.631)	0.587 (0.579; 0.613)	0.600 (0.584;0.625)	0.609 (0.556;0.617)
KD и CT (IPh)	0.217 (0.209;0.228)	0.259 (0.200;0.313)*	0.210 (0.169;0.264)	0.224 (0.211;0.263)	0.248 (0.200;0.311)*	0.208 (0.175;0.244)	0.218 (0.207;0.244)	0.214 (0.183;0.219)&	0.195 (0.165;0.239)	0.185 (0.171;0.191)&
SB (IPh)	0 (0; 0.011)	0.030 (0.015;0.04)*	0.007 (0.004;0.026)*#	0.034 (0.016;0.039)*##	0.028 (0.012;0.041)*	0.008 (0.004;0.021)*	0.031 (0.024;0.032)*	0.019 (0.016;0.025)*&	0.004 (0.002;0.009)*&	0.006 (0.005;0.008)*&

** - significant (P<0.01) differences with Group 1, # - with Group 2 on Day 5, ## - with Group 2 on Day 10, & - with Group 3.*

and myeloperoxidase of neutrophils, monocytes/macrophages, endotheliocyte xanthine oxidase, monocyte/macrophage NO synthase, and mitochondrial complex I.[26,27] A decrease in the activity of antioxidant defense components during TT is due to their consumption to inactivate excess free radicals, a decrease in the level of zinc and copper in the body (SOD components), and selenium deficiency (GPO component).[28]

Table 5.

Correlation coefficient ® between the absolute BSA and the expression of VEGF and MMP-9 and content of LPO products in the lesion focus during TT in conditions of using DF with MT

Parameters	Day 5 (n=7)	Day 10 (n=7)	Day 20 (n=7)
VEGF, unit/mm^2	– 0.47*	– 0.44*	0.17
MMP-9, unit/mm^2	– 0.26	0.51*	0.57*
CD (HPh), u.o.i.	0.21	0.18	0.19
KD and CT (HPh), u.o.i.	0.34*	0.52*	0.51*
SB (HPh), u.o.i.	0.17	0.27	0.68*
CD (IPh), u.o.i.	0.17	0.15	0.24
KD and CT (IPh), u.o.i.	0.68*	0.21	0.44*
SB (IPh), u.o.i.	0.53*	0.51*	0.46*

*-*P<0.05*

The MT effect in the original DF that accelerates the healing of a burn wound, which we discovered, is due to its pleiotropic action. First of all, we believe that the LPO-limiting effect of MT reduces the area of secondary damage during TT, the activation and attraction of neutrophils, monocytes, and lymphocytes to the damage focus. Local application of MT in the composition of DF reduces the infiltration of the lesion focus by neutrophils and histiocytes, and increases the content of lymphocytes and fibroblasts, which helps to reduce the severity of alterative and vascular-exudative reactions in the TT focus with the participation of neutrophils and histiocytes, as well as the early onset of repair with the participation of fibroblasts. The use of MT during TT leads to a decrease in the content of LPO end products in the HPh, and in the primary, secondary, and end products in the IPh of the wound extract. The LPO-limiting effect of MT in a burn wound is associated with a reduction in its area, according to correlation analysis. The pronounced decrease in the level of primary LPO products in the IPh reflects the MT-dependent limitation of the early stages of LPO and shielding of phospholipids due to the predominant oxidation of proteins. The antioxidant effect of MT coming from DF into the burn wound through passive diffusion, as well as with the use of glucose and oligopeptide transporters, may be due, first, to the direct absorption of reactive oxygen species (ROS); and, second, to an increase in the synthesis of glutathione; the activity of SOD, catalase, GPO, and hemoxidase-1; and a decrease in the activity of NOS.[14] Finally, the antioxidant effect of MT is realized by maintaining the potential of the mitochondrial membrane and increasing oxidative phosphorylation, production of ATP, not ROS.[29]

Modulation of the inflammatory process reactions under the influence of MT may be associated with a decrease in the severity of alteration and vascular-exudative reactions, the production of cytokines, and other autocoids, which will lead to changes not only in the TT focus, but also in the acute phase response in general. Previously, we found that the use of MT in the composition of DF during experimental TT limits the death of lymphocytes in the bloodstream.[30] The direct influence of MT on the production and activity of the factors involved in repair cannot be ruled out. Using correlation analysis, we have demonstrated an association between the VEGF and MMP-9 expression in a burn wound and the BSA under conditions of using DF with MT in TT.

MT in vitro on a culture of brain microvessel endothelial cells reduces the permeability of activated IL-1β cells by inhibiting MMP-9.(31) MT binds MMP-9 excess in COVID-19 - mediated immune response and oral squamous cell carcinoma.[32]

MT reduces the MMP-9 overexpression and activity by regulating the signaling pathways NOTCH3/NF-κB, TLR4/NF-κB.[33] Information on the effect of MT on VEGF synthesis and expression is ambiguous: MT inhibits VEGF synthesis in human neuroblastoma cells and prostate cancer by blocking stabilization of the STAT3 and HIF-1α complex.[34] Recent data indicate that MT has a regulatory effect on angiogenesis, depending on the dose (concentration in tissues) and the initial state of the tissue. During bone repair, skin healing, ulcerative defects of the gastric mucosa, and myocardial ischemia, the use of MT enhances the angiogenic potential of mesenchymal stem cells due to the synthesis of VEGF along the Erk1/2 pathway and stimulation of the synthesis of platelet growth factor.[34,35]

Conclusion

With experimental TT from Day 5 to Day 20, the absolute area of the burn wound decreases by 35%, the rate of epithelialization increases, the number of neutrophils in the focus of thermal damage decreases, while the representation of lymphocytes, histiocytes, and fibroblasts increases; the expression of MMP-9 and VEGF increases; predominantly secondary and final LPO products in the heptane phase accumulate, the final products of LPO in the IPh of the lipid extract. The use of MT in the composition of DF daily for 5 days with experimental TT leads to a decrease in the area of the wound defect (by 46% of the original area on Day 20), an increase in the rate of its epithelialization, an increase in the content of lymphocytes and fibroblasts in the burn wound on Days 5, 10 and 20 of TT, a decrease in the representation of neutrophils and macrophages on Days 5 and 10, as well as an increase in VEGF expression on Days 5 and 10, MMP-9 - on Day 5 and a decrease in MMP-9 expression on Days 10 and 20 of TT. In addition, the use of MT in the composition of DF leads to a decrease in the content of predominantly secondary and end products of LPO in the HPh and IPh of the burn wound on Days 10 and 20 of TT. Correlation analysis revealed that a decrease in the BSA under a local application of MT occurs with an increase in the content of VEGF in the wound area and a decrease in the content of MMP-9 and secondary and final LPO products in the HPh and IPh. The results obtained expand the

existing understanding of the role of changes in the expression of MMP-9 and VEGF in the pathogenesis of TT, and serve as a prerequisite for further research studies. We believe that the repair-stimulating effect of MT in the DF, which we established during TT at the preclinical stage, is associated with LPO-limiting effect of MT and a change in the expression of MMP-9 and VEGF in the burn wound and is a prerequisite for further study of the mechanism of action and the effectiveness of MT application in clinical conditions in TT.

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Conflict of Interests

The authors declare that they have no conflicts of interest.

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CASE REPORT

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Consulting a Patient on Hormonal Contraception: A Clinical Case of Vulvar Vestibulitis

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Abstract

This article presents a clinical case of a 23-year-old female who developed vulvodynia and dyspareunia while taking combined oral contraceptives (OCs). The case study shows that physicians should not recommend any combination of OCs over another to reduce weight gain, headache, breast tenderness, breakthrough bleeding, sexual dysfunction, dyspareunia, and decreased libido. Hormonal contraception counseling should be based on known, evidence-based recommendations and not be limited to the unnecessary substitution of one drug for another.(**International Journal of Biomedicine. 2021;11(4):590-593.**)

Key Words: vestibulodynia • vulvar vestibulitis • dyspareunia • hormonal oral contraceptives • pain

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Introduction

Modern hormonal contraception is generally an affordable, reversible, and highly effective family planning method. The frequency of sexual activity and sexual pleasure are positively correlated with satisfaction with contraception. OCs were not thought to improve sexual function in addition to reducing anxiety about the risk of unintended pregnancy. Few good quality studies report the effects of OC on female sexuality. The possible sexual side effects of OC are unknown to many patients and questionable to many physicians. Questions about the establishment of a causal relationship between the use of OC and sexual health continue to be relevant in clinical practice.[1]

Vulvar vestibulitis syndrome (VVS) is believed to be the leading cause of dyspareunia in premenopausal and postmenopausal women. Current epidemiological estimates suggest a prevalence in women of about 10%. Gynecologists who cannot find an organic basis for vulvar pain often advise their patients to see a mental health professional.[2]

On the other hand, mental health professionals can make various assumptions about the nature of pain, ranging from the peculiarities of sexual technique to the somatization of the problem. Women become increasingly frustrated with an endless series of referrals and little pain relief.

Case Presentation

A 23-year-old woman, a law student, reported that she had consulted a large number of gynecologists without any positive effect on her recurrent dyspareunia. The woman, having started sexual activity at the age of 21, immediately turned to the gynecologist for the selection of OC. Considering her complaints of severe acne on the face, chest, and back, the doctor recommended OC (Cyproteronum [2.0 mg] + Aethinyloestradiolum [0.035 mg]). During the next year of taking this OC, the patient noted an improvement in the quality of the skin, which consisted in the almost complete disappearance of acne on all parts of the body and face, she also noted good tolerance of the contraceptive drug, stable weight, and regular menstruation after 28 days for 4-5 days which has become less painful. A year later, the patient again turned to a gynecologist for a scheduled preventive examination. The gynecologist recommended that she replace the current OC with another (Drospirenonum [3 mg] + Aethinyloestradiolum

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[0.002 mg] + Calcii levomefolas [0.451 mg]), arguing that the previous OC contains a high dose of hormones and is not suitable for someone of a young age. The patient started taking the recommended drug. Bloody spotting, headache, and pain during intercourse appeared within the next 3 months of taking the new OC. The patient again turned to the gynecologist with these complaints. The doctor's recommendation was as follows: replace the second OC with a third OC (Drospirenonum [3 mg] + Aethinyloestradiolum [0.003 mg] + Calcii levomefolas [0.451 mg]). In the next 2 months, the intermenstrual flow stopped, but the pain or discomfort during intercourse did not disappear. At this time, the patient notes that the insertion of the tampon has become much more inconvenient and painful than usual, and she abandoned tampons and switched to sanitary napkins. The patient experienced severe burning and cutting pain during penetration with each intercourse, although she noted arousal and readiness for sex. Despite this pain, she continued sexual intercourse, after which a burning sensation persisted during urination for the next 2 days. Then the patient turned to different gynecologists, who each time replaced her OC with another. Thus, within one year, 7 substitutions of OC were recommended to her. In parallel, the doctors periodically prescribed treatment for yeast infections with fluconazole preparations. Last time, the patient again turned to the gynecologist with previous complaints about the inability to live a sexual life due to painful sensations during intercourse, the lack of effectiveness of substitutions for OC, and the use of antifungal treatment. After a gynecological examination, the doctor announced to the patient that she did not have any objective abnormalities in the vagina and recommended that she consult a psychiatrist. The patient informed us that she had not had intercourse for several months due to severe pain during the penetration attempt. This pain often lasted for several hours after intercourse and intensified with urination. The pain did not occur if there was no pressure on the vulva, for example, intercourse, gynecological examination, or tampon insertion. The patient became afraid of pain and reported avoiding opportunities for relationships and sex; she also reported a marked decrease in sexual desire.

According to her somatic history, the patient was in excellent health and did not take any medication. She also provided me with an extensive set of test results and doctors' reports indicating that she did not have an infection, human papillomavirus, vulvovaginal disorder or disease, or any chronic disease or abnormal hormonal parameters. Gynecological examination with mirrors and colposcopy did not reveal any significant abnormalities. However, upon careful examination of the vulva and the vestibular zone of the entrance to the vagina, a shiny erythematous edematous mucosa attracted attention, and touching the vulvar mucosa with a gloved finger and a cotton swab caused the patient unbearable pain. Further, the patient was recommended to abolish hormonal contraception and at the same time to use Estriolum cream 1mg on the vulva 1 time per day for 3 weeks. At the end of this therapy, the patient reported that her pain had significantly decreased. After 2 months of observation, the patient decided to resume sexual activity, while she notes that the symptoms of vulvodynia practically do not bother her. The couple in this situation was asked to use a barrier method of protection.

Discussion

There are several scientific studies that have shown the relationship of OC with the development of dyspareunia, not only due to vaginal dryness, but also to the development of vestibulitis.[3-7] The likelihood of superficial pain during intercourse appears to be higher when OCs are first used at a young age, and increases with duration of use.[4-6] A study of Israeli women taking OC showed that 51% of them use low doses of estrogen (≤20 mcg) and 49% use higher doses of estrogen (30–35 mcg). Of the 132 women in the study, 86 (65%) used OC: 68 (79%) used low doses of estrogen (P<0.002) compared to the general population), while only 18 (21%) used higher doses of estrogen (P<0.002 compared to the general population). The authors conclude that significantly more patients with SVS use low doses of estrogen than those who use high doses of estrogen.[5]

In gynecology, the traditional diagnostic strategy for chronic dyspareunia is to look for organic causes and, in their absence, suggest a psychogenic etiology. In fact, there are no reliable organic diagnostic markers of VVS, and a routine gynecological examination does not imply a thorough examination of the vestibule of the vulva.[8] Interestingly, psychiatric nosologies are similar to gynecological ones and also define dyspareunia in terms of an organic/psychogenic dichotomy. Again, this position ignores the localization of pain. As a result, dyspareunia without an organic cause is determined by the activity that it interferes with, that is, sexual intercourse.

There are the following criteria for VVS: 1) severe pain on vestibular touch or attempt at vaginal entry; 2) sensitivity to pressure localized on the eve of the vulva; 3) vestibular erythema of varying degrees.[8] The diagnosis is usually based on the woman's report of pain during penile penetration and is confirmed by palpation of a cotton swab on the vestibule of the vulva. Approximately 90% of women ultimately diagnosed with VVS describe their pain with adjectives such as "burning" or "cutting pain."[9] Patients also usually describe their pain starting from the moment the penis enters the vagina. This pain can be reproduced by a gynecologist palpating the vestibule of the vulva with a cotton swab. This palpation is usually perceived as light pressure, but is extremely painful for women with VVS. Visual or colposcopic examinations of the vulva are not helpful in diagnosing VVS. On the other hand, there are numerous urogenital infections and dermatological conditions, the symptoms of which coincide with those of VVS.[10-13] There is also evidence indicating that VVS is difficult to distinguish from vaginismus.[14,15] Finally, there are a number of chronic pain syndromes of the vulva or genitourinary system, called essential or dysesthetic vulvodynia, in patients who experience pain during intercourse and a positive cotton swab test. Usually, these patients are easily distinguished from women with VVS in that their pain is not limited to external stimulation, but occurs spontaneously over long periods of time and often on a daily basis.[16] There is a very long list of proposed etiological factors and mechanisms for the development of VVS; however, this list is not accompanied by evidence-based randomized trials.[14] There are currently several weakly evidence-based findings:

1. SVS appears to be associated with the use of OCs containing a low dose of estrogen.[17]

2. There may be local vestibular changes, reflecting increased inflammation or increased nerve innervation.[18]

3. There is evidence of a possible genetic predisposition to VVS associated with the gene for interleukin-1 receptor antagonist (IL-1Ra), which is involved in inflammatory processes.[19]

4. Hypertonicity of the pelvic floor muscles is associated with urogenital pain.[20]

The American College of Obstetricians and Gynecologists proposed an algorithm for the treatment of VVS, starting with conservative treatment and gradually moving to more invasive interventions. Interestingly, there is no randomized controlled evidence to support any of the proposed interventions.[21] In fact, there have been three randomized controlled trials that investigated medical interventions using cromolyn cream and fluconazole (showing ineffectiveness),[22,23] and topical estrogen and testosterone with good clinical results.[24-26] There are also two randomized controlled trials that have documented the effectiveness of non-medical approaches, such as cognitive behavioral therapy and biofeedback/pelvic floor physiotherapy and vestibulectomy.[27]

A recent review by Coelho et al. emphasized that OCs were associated with dyspareunia, not only due to possible vaginal dryness, but also due to the risk of vestibulitis.[1] Several studies indicate a link between OC use and vestibular pain.[3-7] The likelihood of superficial pain during intercourse appears to be higher when OCs are first used at a young age, and increases with duration of use.[4-6] Other authors also reported that the use of OC containing 20mg of ethinyl estradiol was a risk factor for the development of vestibulodynia.[6,28] Most international clinical guidelines believe that "Physicians should not recommend any combination of OC over another to reduce weight gain, headache, breast tenderness, breakthrough bleeding, mood disorders, acne, nausea, and decreased libido. There are no significant differences between the compositions."[29] Long-term adverse symptoms are often alleviated by changes in OC; however, none of the OCs have been proven to be superior in terms of side effects.(30) With a decrease in libido, it is necessary to reassure the patient that this problem will disappear over time, otherwise, consider increasing the dose of estrogen if the current dose is low.[30,31] The adverse effects of OCs usually decrease to acceptance levels with continued use of the same drug. Physician reassurances that symptoms are likely to resolve within three to five months are often the only treatment needed.[32]

Conclusion

Dyspareunia due to VVS is a fairly commonly misdiagnosed problem. The previously mentioned categorical classification systems capture the complex relationships between genital pain, sexual interference, fear of penetration, muscle tension, and emotional stress. Failure to assess and adequately address this problem is of great importance for the quality of life of women and their partners. It might be better to think of the problem as a chronic pain disorder rather than sexual dysfunction, as this focuses clinical and research attention on a central symptom of pain. Studies of adverse sexual effects in women using OCs are controversial and require further research, but advice on oral hormonal contraception should be based on known evidence-based recommendations and not be limited to the unreasonable substitution of one OC for another.

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CASE REPORT

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A Novel Disease-Causing ASPA Gene Mutation (c.432+1 G>C) in an Iranian Patient with Canavan Disease: A Case Report*

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Abstract

Canavan disease is an autosomal recessive genetic disease and rare fatal childhood neurological disorder caused by mutations in the *ASPA* gene, which resulted in a catalytic deficiency of the *ASPA* enzyme that catalyzes the hydrolysis of N-acetylaspartic acid into aspartate and acetate. Herein, we report an Iranian patient diagnosed with Canavan disease with a novel splice-site mutation in the *ASPA* gene (NM_000049.4; c.432+1 G>C). This report is based on a homozygous c.432+1 G>C mutation in the *ASPA* gene identified from an Iranian patient. As a result, a novel homozygous pathogenic mutation on *ASPA* is the cause of disease in the patient.(**International Journal of Biomedicine. 2021;11(4):594-597.**)

Key Words: Canavan disease • novel mutation • ASPA gene • aspartoacylase • N-acetylaspartic acid

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Abbreviations

NAA, N-acetylaspartic acid; **MRS**, magnetic resonance spectroscopy; **MRI,** magnetic resonance imaging; **gDNA**, genomic DNA; **WES,** whole-exome sequencing; **ACMG**, American College of Medical Genetics and Genomics.

Introduction

Canavan disease is an autosomal-recessive leukodystrophy and fatal neurological disease which is characterized by developmental delay, neurologic deterioration with severe intellectual disability, and early death.(1) The underlying cause of this disease is the deficiency in the enzyme aspartoacylase, which leads to high levels of N-acetylaspartic acid (NAA) in the urine, brain, and body fluids. The *ASPA* gene mutations are responsible for this deficiency (RefSeq NM_000049.4).(2) ASPA is a catabolic enzyme that is primarily in oligodendrocytes in the central nervous system.(3) ASPA catalyzes the hydrolysis of NAA to generate aspartate and acetate, it is a homodimer and essential in the synthesis of myelin. Patients who are deficient in the ASPA enzyme activity have an abnormal elevation of NAA in the brain. This can be identified by applying magnetic resonance spectroscopy (MRS) even before increasing its concentration in the urine, which is suitable for the early diagnosis of Canavan disease.(4) Clinical symptoms are not manifested at the time of birth; however, the clinical triad of hypotonia, macrocephaly, and head lag often in association with macrocephaly and

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seizures are initial diagnostic manifestations for Canavan disease in early childhood.[5] Neuroimaging presents brain white matter signal abnormalities, and, at later time-points, ventricular enlargement.[6,7] Patients with Canavan disease in the first months of life have dysmyelination, intramyelinic edema, and characteristic spongiform degeneration of the white matter of the brain with impairment of psychomotor development, which is specified by cognitive delay, ataxia, and irritability. In atypical cases, disease onset is postponed until some years after birth when some aspartoacylase enzymatic activity remains.[8,9] Other symptoms involve difficulties in sucking and visual tracking, progressive macrocephaly, and preserved social interactions. Disease progression is marked by atrophy of the optic nerve, spastic tetraparesis, intellectual disability, seizures, and early death. Magnetic resonance imaging (MRI) is routinely used for the diagnosis of the characteristic spongy degeneration of the white matter, which typically shows signal abnormalities of the white matter and the basal ganglia. Up to now, the Human Gene Mutation Database has presented 83 mutations in the *ASPA* gene (www.hgmd.cf.ac.uk). Novel mutations in some genes can be inherited in an autosomal dominant or autosomal recessive inheritance.[10,11] Herein, we present a novel mutation in the ASPA gene in one patient in an Iranian family with severe Canavan disease.

Case presentation

Our patient was the second born male child (Fig. 1A; IV:2) of consanguineous parents with no family history of any genetic condition or mental retardation. The parents have two children. The father and mother are first cousins and showed no signs or symptoms of Canavan disease. The 6-year-old girl is healthy and the second child is a boy with 2 years old and has symptoms of Canavan disease. The mother started to notice a delay in acquiring developmental milestones by the age of 4 months as he has the symptoms of hypotonia. At 19 months of age, he had severe developmental delay, a lack of neck support, frontal bossing, and macrocephaly. MRI of the brain revealed a diffuse lesion of the white matter affecting the U-fibers. The diagnosis of Canavan disease was confirmed by the findings of a very high concentration of NAA in urine. The parents of the patient provided written informed consent in accordance with the Helsinki Declaration.

A salting-out method for genomic DNA (gDNA) extraction was done. gDNA sample from peripheral blood lymphocyte of the patient was examined by whole-exome sequencing (WES) technique (Macrogen, Seoul, South Korea) to identify the mutations associated with this disease. A novel homozygous ASPA c.432+1 G>C mutation in exon2/intron2 boundary region (NM_000049.4) was found. Finally, to confirm the presence of the *ASPA* variant, a direct Sanger sequencing method for the patient and his parents were done. So, the specific sets of primers were designed to amplify the mutated sites in the genome by the PCR method. After amplification of *ASPA* sequences, we sequenced the PCR products directly on the automated genetic analyzer (ABI 3130XL; USA) and the results are represented in Figure 1B. This finding has not been reported in the other Canavan patients.

Furthermore, the splice-site mutation (c.432+1 G>C) was classified as pathogenic according to the American College of Medical Genetics and Genomics (ACMG) guidelines. MutationTaster (bioinformatics program) predicted that this mutation is disease-causing.

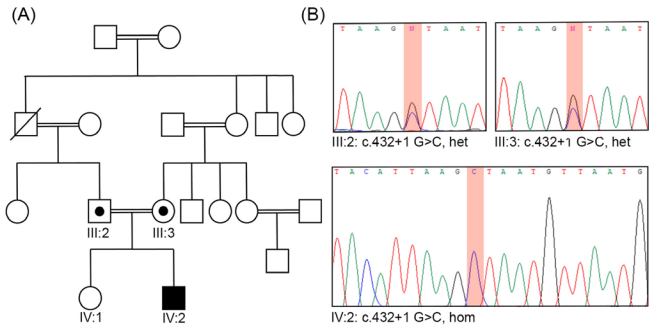

Fig 1. *(A) Family pedigree of the patient. The black square represents the patient. Parents have a family history of consanguinity. The unfilled square represents a male, and the circle represents a female. The diagonal line on the square represents a deceased male. The circle in the center of symbols represents heterozygosity. (B) Sequence analysis of the patient and parents revealed that in terms of inheritance, the patient's parents were heterozygous (III:2 and III:3) and the patient (IV:2) was homozygous. The chromatogram shows the splicing mutation, G to C change in the splice donor of intron 2 (c.432+1 G>C).*

Discussion

Known as Canavan disease, it is an autosomal recessive form of human spongiform leukodystrophy caused by an inborn error of the *ASPA* activity.[12] The substrate of aspartoacylase enzyme is NAA, which is exclusively synthesized in the brain. NAA is hydrolyzed by aspartoacylase to acetate which is necessary for myelin synthesis and aspartate.[13,14] Aspartoacylase deficiency leads to accumulation of NAA in the brain, causing pathological spongy degeneration of the white matter.[14,15] Clinically, two types of Canavan disease have been reported. The most common type of Canavan disease is the neonatal or infantile form that is more severe in clinical presentation in comparison with the juvenile type of the disease.[16,17] Clinical symptoms of neonatal or infantile Canavan disease commonly begin between the age of 2 to 6 months and appear with lack of neck holding in pull to sit maneuver, axial hypotonia, lethargy, spasticity as well as macrocephaly, poor feeding, developmental regression, and progressive hyperreflexia. Cortical blindness and optic atrophy are followed by seizures in later stages.[17] The clinical course of our patient was consistent with the infantile type of Canavan disease.

Extraction of human cDNA and the *ASPA* gene helps to explain the molecular basis of Canavan disease. *ASPA* is the only gene for Canavan disease that is located on chromosome 17p13.2.[16] The examination of mutations in patients with Canavan disease has revealed missense, nonsense, splice-Site, and frameshift mutations, deletions, or insertions.[18] Canavan disease is more prevalent in people of Ashkenazi Jewish than another ethnicity.[19]

Several mutations have been reported to have phenotypes associated with their genotypes, such as 698insC, X314W,

P181L, 244delAT, 923delT, C152W, V14G, D249V, and E214X with a severe phenotype. A stop codon or frameshift occurs in many of these mutations that is related to the onset during the first few months after birth (2 or 3 months of age). In Jewish populations, E285A and Y231X mutations are correlated with a severe phenotype as well.[20] The mutation that was found in our patient also cause a severe phenotype.

Deficiency of the *ASPA* activity caused by the nonsense tyr231ter, the missense ala305glu mutation, or the glu285ala mutation establishes that the three coding-sequence mutations are the cause of Canavan disease.[21] The 433-2 A to G transition in intron 2 (in the splice acceptor site) would result in skipping of exon 3. Additionally, skipping of 94-base exon 3, in the final transcript will change the reading frame. A frameshift accompanied by an exon-skipping would result in the aspartoacylase deficiency.[22]

In 2012, Durmaz et al. reported a novel heterozygous mutation Y88X (T to A nucleotide change at codon 88 in exon 2) within the aspartoacylase gene in a consanguineous family with an affected child diagnosed as Canavan disease. This mutation converts the codon for tyrosine (TAT) into a premature termination codon (TAA).[20] Also, in 2015, Ashrafi et al. indicated a novel homozygous missense mutation (c.202G>A) in the *ASPA* gene in exon 1 which was found in an Iranian patient.[23] In our case, we presented a novel homozygous pathogenic mutation in *ASPA* gene (c.432+1 G>C) related to Canavan disease. This mutation was at the 5' splice-site beginning intron 2, which can cause mis-splicing and alter the reading frame, and consequently, it will probably result in a serious alteration in ASPA protein conformation and leads to the Canavan phenotype. This type of mutation has not been reported in other populations.

Conclusion

In the present study, we report a 2-year-old Iranian boy with severe Canavan disease who harbors a novel pathogenic homozygous mutation (c.432+1 G>C) in the *ASPA* gene. Homozygous mutation as in the intron 2 of *ASPA* gene in the present case is a novel splice-site mutation that was not reported elsewhere. The mutation that leads to the Canavan disease has been defined in the family; it would make prenatal diagnosis possible and suggest parents with such disorder plan for the next pregnancy.

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Competing Interests

The authors declare that they have no competing interests.

Disclaimers

The views expressed in this article are the author's own and do not reflect the official position of the institutions.

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