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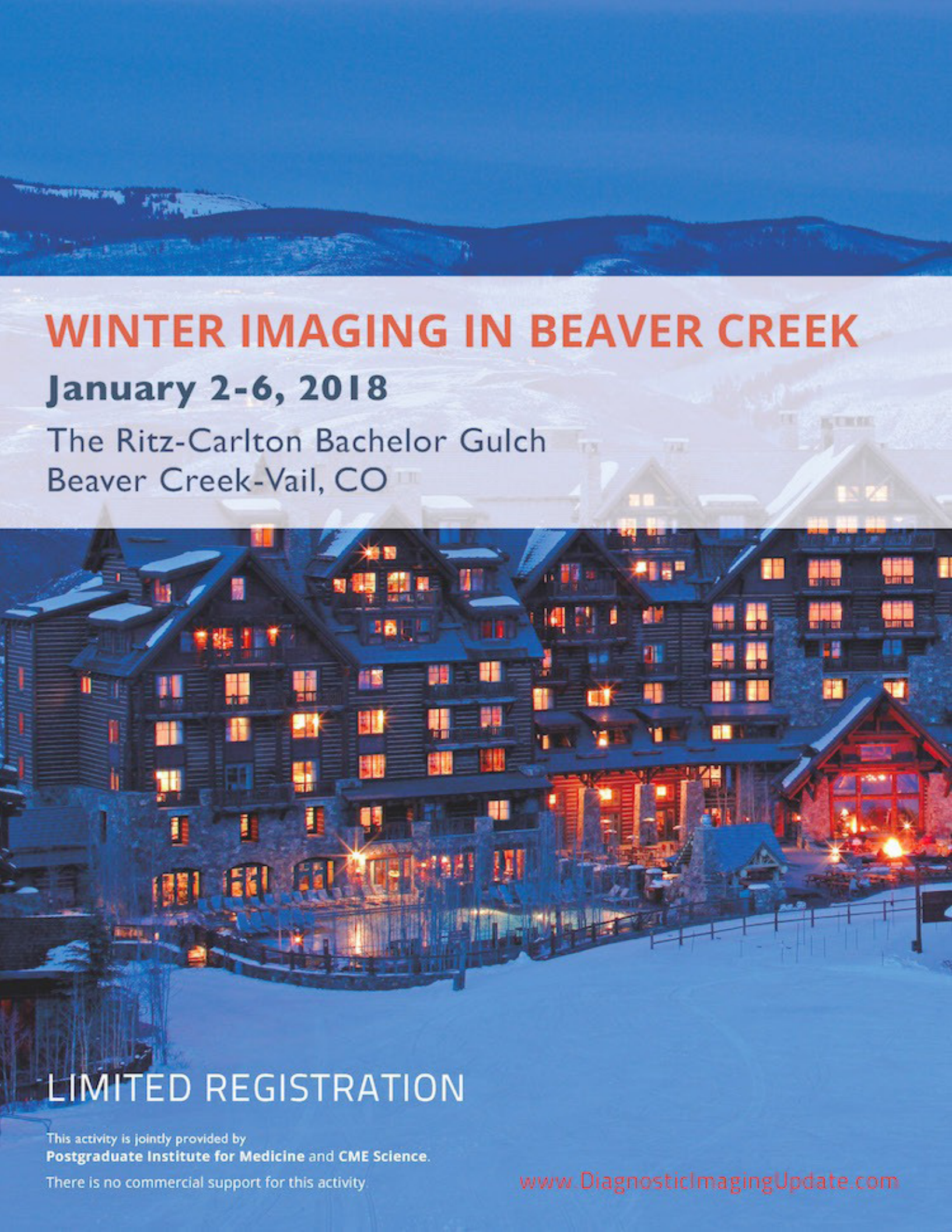

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REVIEW

Neurology

INTERNATIONAL JOURNAL OF BIOMEDICINE

Cognitive Disorders in Juvenile Myoclonic Epilepsy

Olga S. Shilkina*; Ivan P. Artyukhov, PhD, ScD; Polina Moskaleva; Irina Strotskaya; Natalia A. Shnayder, PhD, ScD

V.F. Voino-Yasenetsky Krasnoyarsk State Medical University
Krasnoyarsk, the Russian Federation

Abstract

Cognitive disorders are often associated with epilepsy and are a result of a combination of various factors. This review describes scientific advances in the field of cognitive disorders in patients with juvenile myoclonic epilepsy (JME), the most common form of idiopathic generalized epilepsy. Data in this review were collected through an extensive literature search of available full-text publications in PubMed and eLIBRARY.RU databases. We selected four theories of the origin of cognitive impairment in JME patients for discussion, based on the analysis of available studies. Our findings highlight the etiological heterogeneity of cognitive disorders in JME and the importance early screening for them. **(Int J Biomed. 2017; 7(1):9-14.)**

Key Words: juvenile myoclonic epilepsy • cognitive disorders • epileptiform discharges • antiepileptic drugs

Abbreviations

AEDs, antiepileptic drugs; **CBZ**, carbamazepine; **CDs**, cognitive disorders; **EDs**, epileptiform discharges; **EEG**, electroencephalography; **IQ**, intelligence quotient; **IGE**, idiopathic generalized epilepsy; **JME**, juvenile myoclonic epilepsy; **LTG**, lamotrigine; **LVT**, levetiracetam; **PHT**, phenytoin; **TPM**, topiramate; **VPA**, valproate.

Introduction

JME is the most important syndrome of IGE, which is accompanied by frequent myoclonic jerks, generalized tonic-clonic seizures and, less commonly, absences.[1] JME was first described as a syndrome by Janz and Christian in 1957.[2] The peak of disease onset is between ages 14 and 16, with a range of 8–26 years. Under the proposal for revised classification of epilepsies and epileptic syndromes, in 1989 the ILAE Commission on Classification and Terminology defined JME (impulsive petit mal) as follows:[3] "Impulsive petit mal appears around puberty and is characterized by seizures with bilateral, single or repetitive, arrhythmic, irregular myoclonic jerks, predominantly in the arms. Jerks may cause some patients to fall suddenly. No disturbance of consciousness is noticeable. Often, there are GTCS (generalised tonic-clonic seizures) and, less often infrequent absences. The seizures usually occur shortly after awakening and are often precipitated by sleep deprivation." A smaller proportion of patients report that seizures may be associated with "thoughts and concentration" (23%) or hand activities (20%).[4,5]

IGE is a socially significant disease with disadvantageous behavioral traits and poor social outcome. Social cognition is the ability to elaborate mental representations of social interactions, to use them correctly in social contexts; it includes the attribution of cognitive and affective mental states to self and others, and presumably relies on complex fronto-temporal interactions.[6]

Recent studies have demonstrated that individuals with JME who were followed over 25 years showed subtle anomalies in brain structure and cognition and poor long-term social outcomes when followed over 25 years, including depression, social isolation, and underemployment.[7,8] Cognitive impairments are frequent consequences of epilepsy.

***Corresponding author:** Olga Shilkina, MD; V.F. Voino-Yasenetsky Krasnoyarsk State Medical University, Krasnoyarsk. the Russian Federation. E-mail: olgabbn@mail.com*

Various factors can have a debilitating effect on cognitive function in epilepsy, including underlying structural lesions and disorders that cause epilepsy, seizure type and frequency, presence of interictal epileptic discharges, age at onset, duration of epilepsy, and AED treatment.[9] These impairments cause educational, vocational and management problems for patients. The systematic review of A. Loughman et al.[10] provides a quantitative synthesis of cognitive function outcomes in 26 peer-reviewed, case-controled studies published since 1989. Univariate random-effects meta-analyses were conducted on seven cognitive factor-domains and separately on executive function. Patients with IGE demonstrated significantly lower scores on tests across all cognitive factor-domains except visual-spatial abilities.

The results obtained in the study of J. O'Muircheartaigh et al.[1] provide convincing evidence for abnormalities in a specific thalamo-cortical circuit, with reduced structural and task-induced functional connectivity, which may underlie the functional abnormalities in JME. Neuropsychological studies revealed subtle cognitive deficits in JME patients, mainly implicating the frontal lobes.[11] Dysfunction of these areas results in deficits of concept formation, abstract reasoning, planning and self-regulation of behavior, and control of impulsivity and emotions. These functions have been summarized under the term "executive functions," which refers to processes of elaboration of cognitive and behavioral responses and strategies for the achievement of immediate or future goals.[12,13] However, a strong focus on hypothesis of selective executive deficits may have distracted attention from the pervasive nature of cognitive impairment in patients with IGE and, in particular, JME.[10] Authors highlight, "Since these impairments are likely to have significant educational, vocational and management impact for a proportion of patients, future studies of cognition in IGE and ME should ensure that cognitive abilities are sampled in a broadly representative fashion, so as not to underestimate the potential impact of these seizure syndromes."

Patients with epilepsy tend to have somewhat lower intellectual abilities than in the general population; however, verbal memory and language capacities are usually intact, whereas attention problems often associated with IGE do not depend on the intellectual level of the patients. Therefore, the natural reason for these complications and their connection with the epileptic process are the focus of study by many scientists. The goal of this review was to evaluate scientific literature about cognitive disorders (CDs) in JME and discuss the proposed theories of cognitive dysfunction with this disease.

Data in this review were collected through an extensive literature search of available full-text publications in PubMed and eLIBRARY.RU databases. We considered studies published from 2008 to 2014 and several earlier works (not earlier than 2000), referred by other authors. "Juvenile myoclonic epilepsy" and "cognitive disorders" were used as keywords.

Cognitive functions are defined as an intellectual process by which one becomes aware of, perceives and comprehends ideas. It involves all aspects of the ability to see and to hear, and the ability to think, perceive, reason, and remember.[14]

We considered four theories of CDs in JME:

1. Impact of EDs and disease-related characteristics
2. Adverse side effects of AEDs
3. Genetic predisposition theory
4. Cerebrocortical microdysgenesis

Impact of epileptiform discharges and disease-related characteristics

Human status epilepticus is consistently associated with cognitive problems. Seizures or abnormal EEGs are responsible for the cognitive deterioration. G. Holmes et al.[15] suggest that interictal spikes, particularly if frequent and widespread, can impair cognitive abilities, through interference with waking learning and memory, and memory consolidation during sleep. There are a number of studies using continuous EEG and video EEG monitoring, which indicated that patients without attacks have better results for some cognitive tasks compared to patients with epileptiform EEG activity. Some studies found greater cognitive problems in patients with generalized seizures than in patients with partial seizures;[16] others found it vice versa.[17] Complaints of memory difficulties are common among patients with temporal lobe epilepsy, where memory-related brain structures are directly involved in seizure activity.

In many patients, sensitive methods of observation, notably continuous psychological testing, show brief episodes of impaired cognitive function during EDs. C. Binnie[18] described the phenomenon of transitory cognitive impairment (TCI) in about 50% of patients who show subclinical EDs. These TCIs may be associated with behavioural disorders.

Lavandier et al.,[19] using continuous EEG and video monitoring, found that some frontal tasks were more impaired in patients with epileptiform EEG activity during rest than in those without discharges.

K. Carvalho et al.[20] studied CDs in 61 patients with specific endophenotypes of JME. Patients were divided into 4 groups: no reflex traits (Group 1), praxis induction (Group 2), eye-closure and/or photosensitivity (Group 3), and a combination of different reflex traits (Group 4). All patients underwent Neuropsychological and psychiatric assessment. the clinical variables, e.g. age at epilepsy onset, frequency of myoclonic seizures, total and sedative drug load were also controlled. Praxis induction was more common in groups with reflex traits (2, 3, and 4) presented higher rates of persistent myoclonia, polytherapy, clonazepam use (group 3), and more frequent psychiatric comorbidities. Group 4 patients performed worse in Trail Making Test B than the patients in Group 1. These findings were independent of clinical variables. The authors concluded that the combination of praxis induction and eye-closure/photosensitivity produces greater executive dysfunction, revealing an association between reflex ictogenic mechanisms and cognitive performance.

J.M. Lee et al.[12] showed that JME patients had memory and executive dysfunction and that these cognitive deficits were correlated with age at seizure onset and duration of epilepsy. These results may be consistent with the theory of disease-related characteristics. The authors also noted that

the impaired attention in patients was probably due to a high frequency of interictal EDs.

M. Motamedi et al.[13] investigated possible cognitive dysfunction in the patients with JME and its correlation to factors related to epilepsy and patients' demographic variables. The study showed significant differences between JME patients and healthy controls with respect to scores of mental control (*P=0.015*), forward digit span (*P=0.004*), total digit span (*P=0.008*) and IQ (*P=0.003*). In addition, age, education level, duration of epilepsy and medication showed an impact on several cognitive functions in the patients with JME. The authors concluded that JME is associated with impairment in specific cognitive domains and more specifically in the frontal, prefrontal and memory domains.[13] However, more investigations of JME patients should be performed to understand the associations between cognitive dysfunction and factors related to epilepsy

Adverse side effects of antiepileptic drugs

The deleterious effect of AEDs on cognition is well documented in epileptic patients and volunteer studies.[21,22] The major cognitive effects of AEDs are impaired attention, vigilance, and psychomotor speed, but secondary effects on other cognitive functions can be seen. Even in patients who do not report cognitive changes, neuropsychological tests have shown significant impairments.[21] Almost all AEDs have a negative impact on different aspects of cognition based on the type of therapy (mono or poly), doses, and drug generation.

Differential cognitive effects that are seen with various AEDs. CBZ,[23,24] PHT,[25,26] and VPA[27] can adversely affect cognition to a similar extent, which appears to be less than that of barbiturates and benzodiazepines.[28,29] The limited studies done to detect the effect of new AEDs on cognition revealed that new AEDs such as gabapentin (GBP),[30] lamotrigine (LTG),[31] zonisamide (ZNS),[32] and levetiracetam (LEV) [33] have fewer effects on cognition than do older drugs. Increased doses of AEDs, rapid initiation, and polytherapy entail an increased risk. In general, the cognitive effects of AEDs are less than the sum total of other factors and are usually reversible.[34]

R. Thomas et al.[35] examined 60 patients with drug-refractory JME and concluded that patients were profoundly impaired across the range of tests evaluating intellectual function, memory, language and naming, executive function, the impact of epilepsy, and AED side effects. Eighty-three percent of patients exhibited frank executive dysfunction, which was moderate to severe in 66%. A high prevalence of neurotoxicity symptoms such as fatigue and poorer functioning across intellectual and memory tests were also identified.

JME patients are usually treated with VPA or LTG, more rarely with TPM, and recently also with LVT. Few data exist as to possible cognitive side effects of LVT, but LVT is believed to be well tolerated, showing few cognitive side effects.[36]

In a study by R. Roebling,[37] 10 of 19 patients with JME took VPA either in monotherapy or in combination with another AED. The subgroup of patients treated with VPA scored significantly worse in the verbal memory test (*P=0.043*) and scored worse in most other tests but without reaching significance when compared to the patient subgroup taking either no medication or LTG in monotherapy. Withdrawal of VPA showed a trend toward an improvement of cognitive processing.[38]

It also must be noted that there are reports suggesting that patients with treatment-refractory seizures present a broader impairment related to cognitive deficits and impulsive traits than patients amenable to treatment.[39]

In general, the deleterious effect of AEDs on cognition could be attributed to: (1) Na+ channel blockade, (2) enhanced GABAergic activity, and (3) decrement in glutamate-mediated excitation.[40]

Simultaneously, there are also studies that describe a positive effect of AEDs on cognitive disorders. In general, the beneficial effects of AEDs on cognition could be due to: (1) reduction of seizure activity; (2) modulating effect on neurotransmitters, lowering excitotoxicity associated with a reduction in glutamate release from presynaptic terminals and preventing anoxic depolarization capacities; (3) inhibition of Ca2+-mediated cellular functions (protein phosphorylation and neurotransmitter release) and Ca2+-dependent depolarization; (4) scavenging of free radicals; and (5) their psychotrophic effect.[40,41]

It can be concluded that CDs in JME patients may at least partially be caused by side effects of medication. The basic mechanisms underlying AED-induced cognitive impairments require further investigation.

Genetic predisposition theory

One reason for a variation between results of different research groups may be the genetic and phenotypic heterogeneity of the disease. It is widely accepted that JME is a disease with a high genetic predisposition. JME has both Mendelian inheritance and complex genetic inheritance and accounts for 3% (population-based prevalence) to 12% (hospital/clinic-based prevalence) of all epilepsies.[42,43] Forty-nine percent of our JME families have clinical and EEG traits that are 'vertically' inherited over several generations, suggesting an autosomal dominantly inherited disease. In the other 51%, variants of JME genes, with small to modest effects, contribute to risk/susceptibility and to its complex genetics.[44] It therefore became necessary to carry out neuropsychological family studies to understand the role genes play in the development of cognitive impairment in children with JME.

M. Levav et al.[45] studied 65 families, in which one member had epilepsy. The disorders included childhood absence epilepsy, JME and temporal lobe epilepsy. JME relatives had lower scores than other relatives in tests of visual and auditory sustained attention and attentional flexibility, and showed greater variability in response time.

Iqbal et al.[46] examined expressive language, memory, and higher executive tasks in JME patients as compared with their siblings and a normal control group under video-EEG conditions. Eight sibling pairs, one in each pair with JME, were compared with 16 controls matched for age, sex, ethnicity, and educational level. The JME group differed significantly

from controls on measures of phonemic and semantic verbal fluency. In addition, they more frequently reported behavioral traits associated with executive dysfunction (i.e., impulsivity) on a behavior rating scale. Qualitative inspection of the data suggested a trend for JME patients and their siblings to perform worse than controls on some measures, notably those of expressive language and higher executive function, but on other measures the differences were not statistically significant.

B. Wandschneider[47] studied complex paradigms of the different phases of prospective memory (intention formation, intention retention, intention initiation, intention execution) in 19 JME patients, 21 siblings, and 21 healthy controls. Patients with JME and siblings showed specific deficits during intention formation and intention execution of prospective memory. Patients with JME were more impaired than both siblings and healthy controls.

Currently, five Mendelian JME genes are listed in OMIM or the "Online Mendelian Inheritance in Man" (http://omim.org and http://www.ncbi. nlm.nih.gov/omim/). These cause primarily channelopathies and are comprised as follows: CACNB4 (calciumchannel beta4 subunit),[48] CASR (calcium channel sensor receptor),[49] GABRa1 (GABA receptor alpha one subunit),[50] GABRD (GABA receptor delta subunit),[51] and Myoclonin1/EFHC1 (myoclonin1/ one EF-hand containing gene).[52]. Three SNP susceptibility alleles of putative JME genes in epistasis, namely, BRD2 (bromodomain-containing 2),[53] Cx-36 (connexin 36),[54] and ME2 (malic enzyme2),[55] have been reported to be major susceptibility alleles that contribute to the complex genetics of JME.[42,56] The effect of epistasis is due to influences of multiple genes. This complexity accounts for the obscured inheritance patterns, which must be present in JME.[58]

For example, T. Chachua et al.[59] found a highly significant dominance trait (aggression) in the Brd2+/- haploinsufficient mice compared with the wild type, more pronounced in females. Brd2+/- mice of either sex did not differ from wild-type mice in spatial learning and memory tests. Compared with wild-type littermates, it was found that there were decreased numbers of GABA neurons in the basolateral amygdala, which is consistent with the increase in aggressive behavior. Brd2+/- haploinsufficient mice showed no cognitive impairment but have behavioral traits similar to those found in patients with JME (recklessness, aggression).

It should be noted that Mendelian JME genes and non-Mendelian risk alleles have not been defined in over 90% of affected patients.[56]

Cerebrocortical microdysgenesis

The discovery of the interaction between the motor system and frontoparietal cognitive networks prompted scientists to conduct neuropathological studies of the brain in JME patients. Microdysgenetic lesions were found in the neocortex and subcortical white matter of the frontal lobes and the hippocampus, which suggested a disorder in neuron migration and cortical disorganization. Thus, HJ Mencke and Janz D,[60] in seven of the eight cases of primary generalized epilepsy, found marked microdysgenesis with varying regional distribution.

Woermann et al.,[61] using an interactive anatomical segmentation technique and volume-of-interest measurements of MRI, showed that 40% of JME patients had significant abnormalities of cerebral structure. The voxel-based statistical parametric mapping comparison between the group of JME patients and the control subjects showed an increase in cortical grey matter in the mesial frontal lobes of the patients. The authors concluded that obtained findings indicate a structural cerebral abnormality in JME, with involvement of mesiofrontal cortical structures.

On the other hand, Tae et al.[62] showed that the cortical thicknesses of superior/middle/medial frontal gyri, and superior/middle/inferior temporal gyri were decreased in JME patients. Moreover, cortical thicknesses of precentral gyrus and medial orbital gyrus of right hemispheres were negatively correlated with disease duration. These findings suggest that JME brains have cortical gray matter atrophy in the frontal and temporal lobes.

S. Kim et al.,[63] using the combined structural and diffusion tensor MRI analysis, found that 18 JME patients compared with 22 normal controls exhibited white matter alterations in the antero-superior corona radiata, both centro-parietal regions, and the left temporal lobe. JME patients also had reduced gray matter thickness (right paracentral lobule, precuneus, dorsolateral parietal and inferior temporal cortex; left dorsolateral frontal and anterior temporal areas). Furthermore, manual volumetry analyses revealed a significant volume reduction in the bilateral thalami and hippocampi. Thus, there is some evidence that microdysgenesis could be important in epileptogenesis, but the mechanisms involved remain unknown and difficult to investigate. A consensus on what histopathological criteria to use for the diagnosis of microdysgenesis is needed to explore this further.

In conclusion, the available evidence indicates a distinct cognitive impairment pattern in JME. However, many questions remain to be answered regarding the relationship between cognition and JME. In each case, it is difficult to determine whether cognitive and behavioral impairments in JME are caused by stable, disease-related factors or by the acute effects of paroxysmal epileptic activity, such as epileptiform EEG discharges. The harmful effects of an early onset of seizure and longer duration of disease on cognition have been well demonstrated. These results may be consistent with the theory of disease-related factors. The theory of the acute effects of paroxysmal activity on cognition has also an extensive evidence database. Neuropsychological studies of patients who are seizure free after receiving AEDs medication are needed to answer this question. Described microstructural abnormalities point to the supplementary motor area being a crucial hub in a thalamo-frontocortical network. The pathophysiologic concept of a genetically determined thalamo-frontocortical network dysfunction in JME is supported by family studies.

Thus, cognitive disorders in JME are etiologically heterogeneous.[64] However, all studies were performed in small cohorts of patients, in most cases not exceeding 30

patients. Therefore, this problem requires further and extensive investigation.

Competing interests

The authors declare that they have no competing interests.

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REVIEW

Critical Care Medicine

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Reduced Glutamatergic Neurotransmission as Possible Indicator of Unfavorable Prognosis

Elena V. Alekseeva, PhD

Central Clinical Hospital of the Presidential Administration of the Russian Federation
Moscow, the Russian Federation

Abstract

The paper summarizes the results of experimental and clinical studies showing a reduced function of the glutamatergic neurotransmitter system (GNS) in the development of critical states of the organism. Reduced function of GNS is considered as an unfavorable prognostic factor associated with key mechanisms of thanatogenesis. **(Int J Biomed. 2017;7(1):15-23.)**

Key Words: glutamate • glutamine • kynurenic acid • critically ill patients

Abbreviations

AAs, amino acid; **CNS**, central nervous system; **CVVH**, continuous veno-venous hemofiltration; **GNS**, glutamatergic neurotransmitter system; **GABA**, gamma-aminobutyric acid; **Glu,** glutamate; **Gln**, glutamine; **ICU**, intensive care unit; **IDO**, indoleamine 2,3- dioxygenase; **KP**, kynurenine pathway; **NMDAR**, N-methyl-D-aspartate receptor; **RGN**, reduced glutamatergic neurotransmission; **TDO**, tryptophan 2,3 dioxygenase.

Introduction

Modern methods of metabolomics research allow identifying a super-wide range of low molecular weight compounds. This presents a great potential for their use in critical care medicine for understanding the relationships between the factors of microorganisms and the course of the pathological process that is key to improving the disease outcomes.[1] Metabolomics promotes the development of "personalized" medicine based on the application of patient-specific profiles, incorporating genetic and genomic data as well as clinical and environmental factors, to assess individual risks and to improve diagnostics and the target treatment.[2,3]

GNS of humans and animals has a wide range of neurogenic and non-neurogenic functions, many of which are still little known. Through GNS components (Glu, Gln, its inactive metabolite and precursor, a wide range of Glu receptors, transporters, and enzymes), transmission of nerve impulses, glutamatergic neurotransmission, is carried out. Glu is an important signaling molecule and a major excitatory neurotransmitter in GNS; its receptors are widely prevalent in phylogenetically very distant species of living organisms.[4] Most, if not all, cells of the central nervous system (CNS) have at least one type of Glu receptor.[5] Under normal physiological conditions, Glu is released as a neurotransmitter into the synaptic cleft and initiates the propagation of action potentials. GNS components have been also identified outside CNS: in heart, liver, kidney, lung, thyroid gland, and skin, in the enteric nervous system, in the "gut-brain axis," in plasma and blood cells.[6-9] GNS is involved in the functioning of multiple organ systems: CNS, cardiovascular, respiratory, gastrointestinal, and immune systems, and the hypothalamic-pituitary-thyroid axis.[9-11] Glu-dependent activation of NMDARs in heart allows sufficient influx of calcium to increase myocardial contractility and systolic pressure.[12] Glu induces contraction of ductus arteriosus through GluR-mediated noradrenaline production. Supplementation of glutamate might help to prevent patent ductus arteriosus in extremely preterm infants.[13] Glu also modulates the motor function of the gastrointestinal tract.[14]

***Corresponding author:** Elena V. Alekseeva, PhD, anesthesiologist-resuscitator of the intensive care unit; the Central Clinical Hospital of the Presidential Administration of the Russian Federation. E-mail: aev_69@mail.ru*

Over the last 4 decades, a number of studies have shown that neurons release more than one neurotransmitter. It has been suggested that monoamine and cholinergic neurons use Glu as a co-transmitter. There is evidence of co-release of Glu and GABA, excitatory and inhibitory fast neurotransmitters, from a single axon terminal in neurons.[15-17] GNS is involved in synaptic and diffuse neurotransmission, the formation of neurons]18] and synaptic plasticity.[4] Glu and Gln are multifunctional AAs, which are involved in a large number of metabolic reactions aimed at the detoxification of ammonia, an increase in resistance to hypoxia, and the formation of the antioxidant glutathione, ATP, AAs, and other proteins.[19]

Evidence of Glu participation in the regulation of physiological and pathological processes in other organs and tissues (lung, kidney, liver, heart, gastrointestinal tract, and immune system) has been obtained only in recent years. Earlier studies on the GNS role in the development of patients' critical condition were (in the vast majority) experimental or postmortem studies and were focused on the action of Glu in CNS. According to E. Aleksandrova et al.,[21] the results of these studies are numerous debated concepts regarding the damaging effect of the increased or decreased extracellular Glu levels on the activity of brain neurons in experimental models of trauma, ischemia, and inflammation.

Glu is not only the primary excitatory neurotransmitter in the adult brain, but also a critical transmitter for signaling neurons to degenerate following stroke. Excitotoxicity, the specific type of neurotoxicity mediated by Glu, is a primary contributor of ischemic neuronal death and other cellular components of the neurovascular unit. Cerebral ischemia leads to a massive release of Glu, which stimulates NMDARs and induces calcium influx through these ionotropic receptors; the calcium-dependent activation of death-signaling proteins that are immediately downstream of the receptors triggers a plethora of signaling cascades that work synergistically to induce neuronal death.[22,23] As scientists begin to understand the critical role of NMDARs and calcium input in excitotoxicity, several strategies have been developed against glutamate excitotoxicity; however, none of them have shown positive results in clinical practice so far, and all NMDAR antagonists failed in clinical trials.[24]

In the case of acute processes such as stroke or traumatic brain injuries, glutamate excitotoxicity is thought to cause harm within a narrow timeframe after which the neurotransmitter reassumes its normal function. Therefore, the use of agents acting on NMDAR may have not only missed the window for therapeutic efficacy but also led to undesired side effects from prolonged receptor blockades.[24] Nowadays, the concept of blood/brain glutamate grabbing or scavenging is well recognized as a novel and attractive protective strategy to reduce the excitotoxic effect of excess extracellular glutamate that accumulates in the brain following an ischemic stroke.[25]

It is important to note that Glu can produce both adverse (neurotoxic) and positive (anti-ischemic) effects in cerebral ischemia. A study performed by T. Gan'shina et al.[26] found an interaction between excitatory and inhibitory systems on the level of cerebral vessels.

Analysis of the results of a number of experimental and clinical studies allows us to consider reduced glutamatergic neurotransmission (RGN) as a possible indicator of unfavorable prognosis of pathological conditions, as well as a factor associated with the development of key mechanisms of thanatogenesis[27] and main pathological processes with self-dependent thanatological value: systemic hypoxia, sepsis, and acute kidney injury (Figure 1).

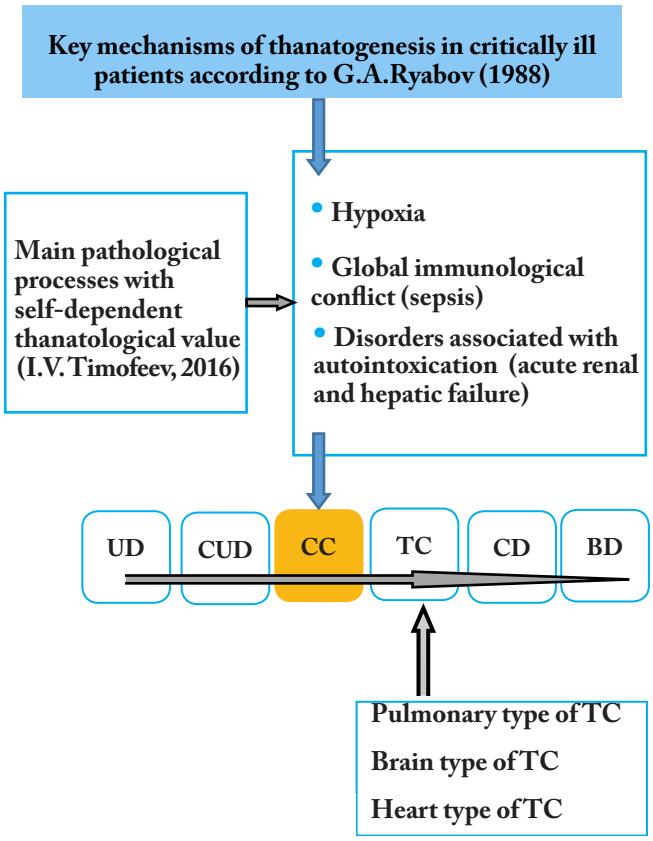

Fig. 1. *Key mechanisms of thanatogenesis in critically ill patients according to G.Ryabov (1988) and the schema fragment of pathogenesis and thanatogenesis by I.Timofeev (2016)*

UD - underlying disease; CUD- complication of underlying disease; CC- critical condition; TC - terminal condition; CD - clinical death; BD - biological death

The neurochemical criteria of GNS hypofunction

The main neurochemical criteria indicating hypofunction of GNS can be: 1) low level of Glu (or Glu+Gln) in the plasma and investigated tissues in comparison with reference values; 2) an increased level of kynurenic acid (KYNA), a neuroactive metabolite of tryptophan catabolism in enzymatic cascade, known as KP.

Currently, Glu concentrations in cerebrospinal fluid (CSF) and blood plasma are readily available criteria for indirect evaluation of Glu homeostasis in the body.[8] These available biofluids are interpreted as an average representation of the surrounding tissue.[29] The content of mediators and products of their inactivation in plasma and CSF depends on the circulation of neurotransmitters in the tissues and reflects the intensity of neurotransmitter processes.[30]

Maintaining a relatively constant concentration of extracellular AA pool is one of the functions of the interorgan AA exchange.[31] According to B.Bein and A. Ezhova,[32] the constant maintenance of a certain amount of free AAs in the blood is carried out due to transfer from the gastrointestinal tract and tissue during protein decay, as well as redistribution and consumption of AAs in organs and tissues. The exact mechanisms that lead to a change in the plasma Glu levels remain unknown, but in general, it is release and redistribution of Glu between organs or the activation of neutralizing natural mechanisms.[33] From CNS, neurotransmitters and their metabolic products are released in the blood and CSF.[29] The diffuse and synaptic types of neurotransmission have complex mechanisms of interaction. Researchers have described the movement of Glu from synoptic slit and its participation in the diffuse type of neurotransmission, as well as interactions of "diffuse" Glu with the synaptic (presynaptic and postsynaptic) receptors.[34] For regulation of extracellular concentrations of Glu, its transporters also have a significant effect. Glu transfer by transporters is carried out in both directions of the cell membrane, depending on the gradient of the ions Na+/K+ and amino acid itself and may change as a result of metabolic processes. Another major source of extracellular Glu is its release by neurons and glial cells through exocytosis. This process can be triggered by activation of glial glutamate receptors and represents Ca2+-dependent process.[34]

In should be noted that Glu level is closely associated with plasma concentration of KYNA, which is one of the end products of tryptophan formed in KP. KYNA in supraphysiological concentrations (micromolar levels) has an antagonistic effect on all three ionotropic Glu receptors and α7 nAChR.[35-37] An increase in KYNA concentration is accompanied by a decrease in Glu release, and a decrease in extracellular levels of dopamine[38] (Glu via ionotropic receptors indirectly stimulates the dopamine release).[39]

The modern methods of metabolomic research are characterized by high manufacturability analysis and, accordingly, the degree and accuracy of determination of low molecular weight substances.[30] Among the analytical methods used in metabolomics research, liquid chromatography/mass spectrometry (LC/MS) has been shown to be one of the best techniques in terms of selectivity, sensitivity, and reproducibility.[40,41] It provides the highest level of metabolite coverage, using a unified analytical technique. In LC-MS, limit of sensitivity is about 5nM to 10nM and the number of detectable characteristics - from 5000 to 20000.

In magnetic resonance spectroscopy (MRS), where the detection limit is usually 5μM to 10μM, a number of recognizable compounds are from 40 to 200, depending on the biological material to be analyzed.[42] Measuring the Glu concentration by MRS with the usual values of the magnetic field causes difficulty in distinguishing between Glu and Gln; for this reason, a composite index is often used.[43] Another aspect of modern metabolomic research is the application of multivariate statistical analysis [40] and the construction of predictive models.[30]

Thus, the levels of metabolites in the blood plasma reflect the systemic reactions of the body and are considered as a body's response to the disease.[44,45] As will be presented later, with hypofunction of GNS, likelihood of an unfavorable outcome of the pathological process in the following period of time increases, and the development of each thanatogenesis mechanism is associated with a reduced functioning of GNS.

RGN as a risk factor for an unfavorable course of the pathological processes in critically ill patients

According to M. Poeze et al.[46] and T. Hirose et al.[47], relatively lower values in plasma Glu concentrations may be an independent predictor of poor outcome in patients with sepsis. E.V. Alexandrova [21] found that the syndrome of RGN is prognostically less favorable than glutamatergic redundancy in patients with severe traumatic brain injury.

In our studies, the risk of an adverse outcome in the 28-day period in ICU was significantly higher (5 times) in critically ill patients (with different underlying pathology) with initially reduced plasma Glu levels than in patients with baseline Glu within reference levels. Prognostic significance of positive testing for reduced plasma Glu level in critically ill patients was 82%, and specificity - 84%.[48,49] The 28-day survival rates were less in critically ill patients with reduced plasma Glu levels compared to reference values (Gehan's Generalized Wilcoxon test, P=0.01544; Cox's F-test , P=0.00163; Cox-Mantel test , P=0.00243; Peto & Peto's and Prentice's generalized Wilcoxon, P=0.00738; the logrank test, P=0.00507). We found direct correlations between reduced plasma Glu levels and the Apache II Score and the SOFA scores for the cardiovascular, hepatic, coagulation, renal and neurological systems (Table 1); and we identified inverse correlations between the reduced plasma Glu levels and hemoglobin oxygen saturation in the superior vena cava (ScvO2).

Table 1.

Correlations between reduced plasma Glu levels and the Apache II Score and the SOFA scores

	Gamma correlations (P < 0.05)					
Reduced plasma Glu level	SOFA (total score) r = 0.564	SOFA scores				
		Cardiovascular system r = 0.614	CNS r = 0.542	Liver r = 0.569	Kidneys r = 0.583	Coagulation r = 0.362
	APACHE II score r = 0.400	Plasma procalcitonin level r = 0.368	Plasma lactate level r = 0.373	$S_{cv}O_2$ r = 0.621		

The impact of the reduced plasma Glu level on 28-day survival rate of critically ill patients was shown in regression models built using the software package Statistica 12 (adequate models: Cox's proportional hazards model, P=0.00619, exponential regression, P=0.00323; lognormal regression, P=0.01944; normal regression, P=0.00524) and Predictor Screening on basis of Statistica Data Miner Recipes (Chi-square=6.946742, P=0.0084.)[48; 49]

Currently, in contrast to previous studies, H. Buter et al.[50] have shown an association between plasma Gln levels and severity of clinical condition calculated on the APACHE-IV scale. According to Y. Lin et al.,[51] Gln reduces apoptosis of cardiomyocytes and increases their functional activity at low pH. Under experimental conditions, Gln increased the lifespan of rats after asystole.

The prognostic role of the high KYNA content in patients with unfavorable outcome has been confirmed in several clinical trials. In study by W. Dabrowski et al.,[52] the concentration of KYNA in the plasma of septic patients with good clinical outcomes decreased gradually over the course of CVVH. In contrast, the concentration of KYNA in the plasma of septic patients with poor clinical results did not decrease over the course of CVVH. In fact, an increase in KYNA concentration was observed. At the same time, the concentrations of CRP, PCT and lactate decreased during CVVH in this group of patients. In the study of G. Ristagno et al.,[53] which included 245 patients resuscitated in the first day after cardiac arrest, high KYNA levels were independently associated with ICU death and with 12-month death. L. Darligton et al.[54] showed that KYNA levels were significantly raised in patients with acute stroke who died within 21 days compared with those who survived; KYNA levels were significantly higher at all study time points (the first, second, third, fourth and seventh days after the stroke) in this group of patients.

RGN and hypoxia

In critically ill patients with signs of systemic hypoxia, reduced levels of plasma Glu concentrations were observed 5 times more often (P=0.019478, r=0.833) than in critically ill patients without systemic hypoxia. Direct correlation was found between reduced plasma Glu content and each criterion of systemic hypoxia: an increased level of lactate in the blood plasma (r=0.710) and reduced ScvO2 level (r=0.621). The relationship between the formation of systemic hypoxia and reduced Glu content in the blood plasma was shown in the logistic regression model built using Predictor Screening on basis of Data Miner Recipes Data Miner (Statistica 12).[55]

The above data of clinical trials are consistent with the results of experimental work. Changes in the metabolism of Glu and Gln in hypoxic conditions have been presented by several authors as a phenomenon of "metabolic reprogramming". The increased consumption of Glu and/or Gln in hypoxia is shown as a tissue adaptation to anaerobic conditions. Glu has been described as a more "preferred" substrate for fatty acid synthesis than Gln.[56-60] In a pilot study by H. Baran et al.,[61] an increase in KYNA level had a direct correlation with the severity of hypoxia. KYNA levels in tissues increased by 44% after 5min of asphyxia and 302% after 20min of asphyxia (the critical time limit of survival). According to G. Ceresoli-Borroni and R. Schwarcz,[63] up to 6 h, asphyxia caused 160-267% increases in KYNA levels in neonatal rats. Changes in the metabolism of Glu, Gln and KYNA in the body during hypoxia, which were identified in the experimental and clinical studies, are shown in Figure 2.

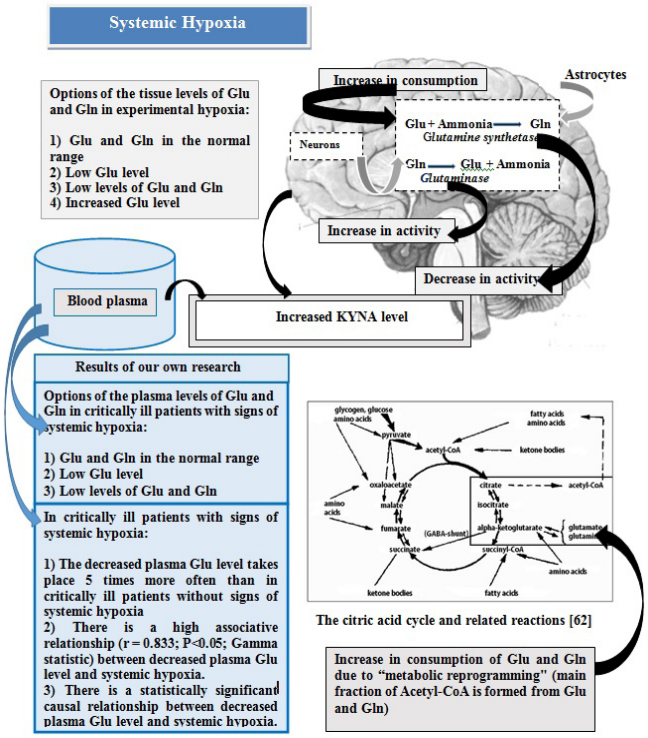

Fig. 2. *Changes in the metabolism of Glu and Gln in hypoxic conditions*

RGN and acute kidney injury

In a study conducted by H. Chua et al.,[64] critically ill patients with acute kidney injury (AKI) in more than 50% of cases had plasma Glu levels below the reference value. In our early study,[65] we found more frequent reduction in levels of Glu and Gln in the plasma of critically ill patients with AKI compared to critically ill patients without AKI. Frequency Glu reduction in plasma increased with the increasing severity of AKI: 50% for AKI-I and 73% for AKI-III (r=0.481; P=0.03). Relationships between the AKI development and reduced plasma Glu level, as well as with the degree of AKI severity, were shown in models of logistic regression and artificial neural networks. These data are consistent with the results of studies carried out on animals. In an experimental study performed by M. Duran et al.,[66] after the initiation of acute renal failure, which was modeled experimentally in two ways (ischemic and chemical forms), reduced Glu levels in the renal cortex and plasma were found in both cases. According to R. Goldstein et al.,[67] significantly decreased plasma Glu concentrations were found in cats with various stages of chronic renal failure. In experimental study of I. Montañés et al.,[68] the cortical concentrations of glutamine and glutamate in dogs were lower

in the recovery phase (48 hours) after acute renal ischemia than in control kidneys. Changes in the metabolism of Glu, Gln and KYNA in the body during AKI, which were identified in the experimental and clinical studies, are shown in Figure 3.

KYNA is a known uremic toxin.[69] Its content in patients with uremia exceeds the reference values by many times and correlates with the development of uremic symptoms. Toxic effects of the relevant concentrations of KYNA have been confirmed by experimental studies.[70] In the study of D. Pawlak et al.,[71] in spite of haemodialysis, plasma KYNA concentration was elevated in uremic patients in comparison with healthy volunteers. The high concentrations of KYNA positively correlated with degree of the renal insufficiency in rats with experimental chronic renal failure.[72]

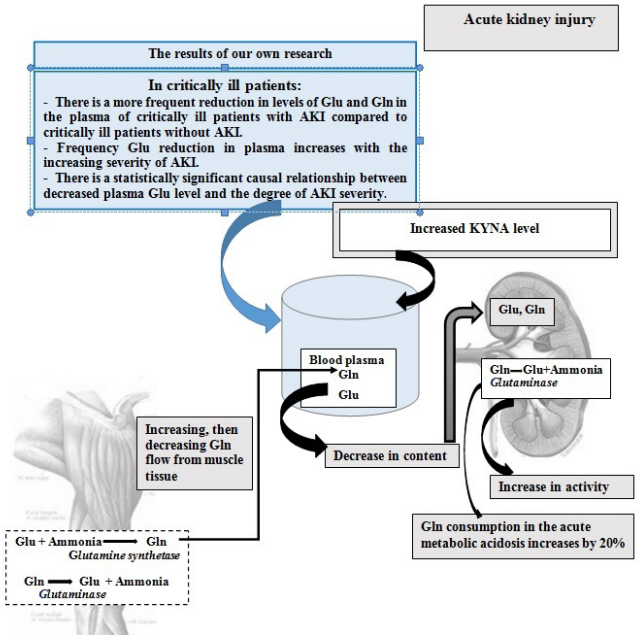

Fig. 3. *Changes in the metabolism of Glu, Gln and KYNA in AKI*

RGN and sepsis

Septic shock is a major cause of death in critically ill patients who are treated in ICUs. In patients with sepsis, according to R. Langley et al.,[73] the levels of Glu and Gln at enrollment to ICU and 24 hours later after treatment were significantly lower than in uninfected patients. Similar results were obtained in the study of W. Mickiewicz et al.,[74] thus, in the first 24 hours of admission to ICU, the plasma Glu concentration in patients with septic shock was lower than that of ICU patients with the systemic inflammatory response syndrome but not suspected of having an infection (P=0.00048)].

Our studies were carried out in 2013–2015 independently and simultaneously with foreign studies. In our studies and studies of foreign researchers, the "classical" diagnostic criteria for sepsis were applied (without revisions adopted in 2016). To enable comparison, all data are presented in the original version. According to our results,[75] the decreased plasma Glu level was observed 9.8 times more frequently in ICU patients with sepsis compared to ICU patients without criteria for sepsis (P=0.00028, ML chi-square test; r=0.890, Gamma statistic). With increasing severity of septic process (from severe sepsis to septic shock), frequency of the decreased plasma levels of Glu and Gln increased by 2 and 4 times, respectively (P=0.0208, ML chi-square test; r=0.625, r=0.730, Gamma statistic). The causal link between the reduced Glu levels and the development of sepsis, as well as its severity, in ICU patients was shown in statistical models.

Clinical findings are consistent with experimental results. According to C. Boutry et al.,[76] with experimental endotoxemia almost all circulating AAs, including Glu, decreased. A supplementation with 4% monosodium glutamate (MSG) or an isomolar amount of glutamine failed to restore Glu concentrations in plasma and muscle. A significant reduction in the concentration of extracellular Glu was determined during progressive inflammatory reaction induced by administration of LPS.[77] E.V. Sabadash and S.N. Skornyakova,[78] in a study on animals, showed a progressive decrease in the plasma Glu concentrations with an increase in the severity of infection.

H. Buter et al.[50] described a correlation between pre-operative plasma glutamine levels and the presence of a positive culture after cardiac surgery. In another study,[79] researchers also found that plasma Glu levels were determined by the severity of illness and the presence of an infection in ICU patients.

A considerable amount of evidence has accumulated as concerns interactions between KP and immune dysregulation in the development of septic shock. KYNA is one of the end products of tryptophan formed in KP. In the first step of this process, tryptophan is oxygenized by TDO or IDO into kynurenine, which is then transformed by kynurenine aminotransferases into KYNA.

Physiological concentration of the human plasma KYNA ranges between 25nM and 60nM.[80, 81] IDO occupies a key position connecting the immune system and KP.[82,83] As is well known, IDO is rate-limiting enzyme of tryptophan catabolism and plays a pivotal role in immune tolerance.

P.Tattevin et al.[84] showed that IDO activity gradually increased according to sepsis severity, and septic patients who died had higher IDO activity on admission than did survivors (P=0.013). IDO activity was markedly increased in patients with septic shock (0.235 [IQR, 0.152–0.481], +751%, P<0.001), in patients with severe sepsis (0.123 [IQR, 0.068–0.271], +344%, P<0.001), and in patients with sepsis (0.033 [IQR, 0.031–0.052], +20%, P=0.008), as compared with control participants (0.028 [IQR, 0.025–0.036]). In addition, IDO activity was correlated with SAPS II score and day LOD score. As was shown, increasing plasma KYNA concentration might predispose to sepsis and septic shock in patients after multi trauma and its level is related to severity of infection. [52, 85, 86]

Changes in the metabolism of Glu, Gln and KYNA in the body during sepsis, which were identified in the experimental and clinical studies, are shown in Figure 4.

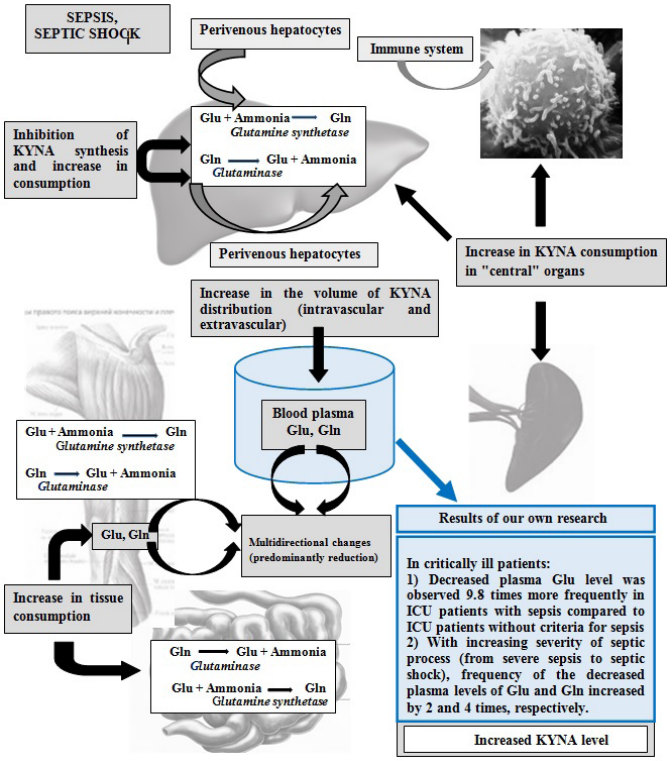

Fig. 4. *Changes in the metabolism of Glu, Gln and KYNA in the body during sepsis.*

Factors of the "generalized" hypoaminoacidemia and increased KYNA content in the development of a patient's critical condition

Factors of the "generalized" hypoaminoacidemia in the development of a patient's critical condition are the increased AA distribution due to vasodilation and increased permeability of the endothelium, an inhibition of the synthesis of a number of AAs in the liver and their increased consumption in central tissues (immune system, liver, spleen, wound). [46, 87]

Experimental data have shown that stress factors increased the KYNA formation and other metabolites of KP of tryptophan catabolism by activating the secretion of glucocorticoids and a significant increase in the activity of TDO[88] and IDO, which is regulated by cytokines.[89] The increased KYNA concentration after acute physiological stress was observed in clinical studies performed by E. Kotlinska-Hasiec et al.[90]

Conclusion

There are many factors that can influence the course of the pathological process in critically ill patients. One of them, apparently, is RGN. In this review article, we have attempted to summarize the currently available evidence on the connection between low Glu content and a high KYNA level with each of the key mechanisms of thanatogenesis and unfavorable outcome in critically ill patients. The mechanisms behind the association of low plasma Gln levels and low Glu levels with severity of illness or mortality in critical illness are not fully understood.[91,92] Whether plasma glutamine and glutamate levels can be used to identify critically ill patients with poor prognosis needs further study.

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REVIEW

Clinical Biofeedback

INTERNATIONAL JOURNAL OF BIOMEDICINE

Short-term HRV Biofeedback: Perspectives in Environmental Physiology and Medicine

Liliya V. Poskotinova, PhD, ScD*; Denis B. Demin, PhD, ScD; Elena V. Krivonogova, PhD

Federal Center for Integrated Arctic Research, the Russian Academy of Science Arkhangelsk, the Russian Federation

Abstract

This review demonstrates the effectiveness of short-term HRV biofeedback sessions in an evaluation of human adaptation to uncomfortable environments. A single HRV biofeedback session can be used as a test for the effectiveness of cortical-visceral connections in patients with cardiovascular disease. In addition, this method can be used as short-term assistance in adaptation to social stressful factors. **(Int J Biomed. 2017;7(1):24-27.)**

Key Words: heart rate variability • biofeedback • adaptation • HRV spectrum

Abbreviations

BP, blood pressure; **EEG,** electroencephalogram; **HR,** heart rate; **HRV,** heart rate variability; **HRVB,** HRV biofeedback.

The aim of this brief review was to determine the importance of short-term HRVB sessions in environmental physiology and medicine.

HRV analysis retains its relevance as a non-invasive and effective procedure to evaluate adaptation reserves of cardiac activity,[1] to predict biological age.[2] Currently, much attention is paid to relations between functions of the brain and autonomic nervous regulation of the heart rhythm.[3] Neuroimaging methods enabled identification of the brain areas where these relationships are the most important (the amygdala, ventromedial prefrontal cortex of the brain) HRV may be an indicator of how well "vertical integration" of cortico-visceral nervous connections are functioning, providing flexible control of behavior and function of internal organs.[4] During the emotional stress experienced by people with social phobias, a relationship was established between a decrease in the high-frequency component of the HRV spectrum and increased cerebral regional blood flow in certain Brodmann areas (in the right caudal nucleus, right anterior cingulate and bilateral medial prefrontal cortex as well as in the dorsolateral prefrontal cortex of the left brain hemisphere).[5] The neurogenic structures responsible for maintaining the high-frequency component of the HRV spectrum (vagal activity) were found in the same areas that are responsible for the different emotions (sadness, happiness, disgust) – the left insular part and caudal nucleus.[6] Arrhythmogenic changes in the bioelectric heart activity, which are considered as predictors of the sudden cardiac death, are correlated with changes in regional blood flow in the right brain.[7] In recent years, HRV analysis has been increasingly used not only for diagnosis, but also as an instrument for correction of an impaired relationship in the "brain-heart" system.

General biological laws of biofeedback have been identified on the basis of classical conditioning by Pavlov[8] and operant conditioning by Skinner.[9] Transmission paths for conditional visual signals are caused by training with modifications of discharges on the conditioned signal during HR control. These "variable" neurons receive information from the unconditioned signal and then modify their activity in accordance with the nature of the conditioned signal. These changes are found in the locus coeruleus of the brain.[10]

***Corresponding author:** Liliya V. Poskotinova, PhD, ScD. Department of Biorhythmology of Federal Center for Integrated Arctic Research, the RAS, Arkhangelsk, Russia. E-mail: liliya200572@mail.ru*

Registration of the activity of individual vagal preganglionic and postganglionic sympathetic cardiac neurons showed that heart motor neurons initially respond to conventional signals and determine the formation and magnitude of the response caused by the conditioned signal.[11] Thus, the conditioned reflex underlying HRVB has a specificity of formation at all levels of regulation: from the heart motor neurons to the cortical brain centers.

The method of HRVB is intended to increase the total HRV, vagal influences on HR, and the baroreflex activity.[12,13] It is shown that the application of HRVB helps to normalizing BP, increasing vagal reserve of autonomic regulation of cardiac activity.[13,14] Realization of the biofeedback effect is possible with the optimal functioning of the higher nervous activity mechanisms (motivation, memory, emotional and positive reinforcement) and cortical-visceral relations from the cardiovascular centers in the brain to the cardiac conduction system.

The total power (TP) of HRV spectrum as a controlled parameter is promising for use to enhance vagal influences on the heart rhythm. During short-term recording (5 minutes), this parameter is similar to standard deviation of RR interval in the physiological meaning and reflects the increase in vagal influences on the heart rhythm. A person manages the parameters of HR primarily through a voluntary change in the frequency and depth of breathing. Consequently, the successful increase in TP of HRV spectrum using biofeedback technology in persons with a normal cardiorespiratory interaction is possible during several sessions[15] or even within a single session. We have shown differences in the reactivity of brain structures in adolescents with sympathicotonia and normotonia after the first session of HRVB. Adolescents with sympathicotonia on the background of increased alpha EEG activity and decreased BP demonstrated the reduction in initially increased theta EEG activity, while the adolescents with normotonia had only increased alpha EEG activity. After 10 sessions of HRVB in adolescents with sympathicotonia, EEG indices and BP were comparable to those of adolescents with normotonia.[16] Krivonogova et al.[17] showed that people with high endurance, flexibility and tempo, and the moderate emotion demonstrated a persistent increase in TP after the first session and the next 10 sessions. Thus, these people have a high level of coordination of all sub-systems of the mental processes and body regulation. Individuals with the moderate endurance, plasticity, tempo and emotion demonstrated a significant increase in TP (in comparison with baseline values) after 4-5 sessions HRVB training. In individuals with low values for each of the mentioned properties, TP increased from session to session.[17] These data showed that since the first session of HRVB, the adjustment function of neural networks in humans will depend on a person's initial autonomic nervous tone and psychological status.

Kappes et al. [18] studied the possibilities of biofeedback technology for human adaptation to extreme climatic conditions. It was concluded that skillful training with bio-behavioral methods contributes to the survival and effectiveness of living in the Arctic. The cold pressor test is a demonstrative model that reflects the body's reactivity in conditions of hypothermia. Skills acquired via HRVB during immersion of hands in cold water help to reduce a pain in the hands' skin during local hypothermia.[19] A person's ability to raise his or her own HRV in a short time, and even during a single biofeedback session, can be seen as cognitive test of adaptation to uncomfortable environmental factors. Moreover, different versions of changes in the brain's bioelectrical activity reflect the various cognitive strategies during biofeedback training. In a study by Krivonogova et al.,[20] a single session of HRVB was carried out in healthy adolescents aged between 14 and 17 years in order to increase vagal effects on heart rhythm. Five different variants of EEG spectral power during the successful HRVB session were identified. These individual cognitive strategies during biofeedback sessions reflected the activation of the thalamic-cortical system or cortical-hippocampal system, as well as local cortical brain activity. It was shown that after a single session of HRVB in adolescents living in the polar areas (67°40'N), especially in patients with sympathicotonia, theta EEG activity decreased more intensively with a predominance of the dynamics in the right brain hemisphere compared to adolescents living in the subpolar region (64°30'N). Moreover, the changes of EEG activity continued after the biofeedback session in adolescents living in the polar areas.[21] After a single session of HRVB among adolescents living above the Polar Circle (67°40'N), a more prolonged increase in alpha EEG activity was identified on the background of a more pronounced decrease in theta EEG activity and decreased reaction of assimilation of photostimulation rhythm in comparison with adolescents living below the Polar Circle (64°30'N). Changes in EEG pattern are most characteristic for the right hemisphere with involvement of the frontal parts of the brain.[22] In adolescents, after a single session of HRVB in order to increase the TP, different versions of changes in the time of a long-latency auditory evoked potential (P300) were found. These data reflect the options of integrating neurons to optimize the balance of the sympathetic and vagotropic mechanisms. In the first variant, there is an optimization in the level of excitation and inhibition in neural networks, which is reflected in the decrease of P300 latency in the parietal, central, frontal and temporal brain regions. In the second variant, there is an increase in the internal differential inhibition to achieve a successful biofeedback control, which is reflected in the prolongation of the initially very short P300 latency.[23]

Demin et al.[24] studied the role of hormones of the pituitary-thyroid system in changing the brain bioelectric activity during a short HRVB session in adolescents aged from 15 to 17 years. In individuals with sympathicotonia, the changes of amplitude-frequency EEG characteristics were directly dependent on the serum thyrotropin levels during the HRVB session and after it.[24] It was shown that individuals with low serum serotonin levels after a single HRVB session had higher activation of brain structures than individuals with normal levels of serotonin.[25] Modern man must adapt to the uncomfortable factors of nature and social life. It was shown that the short-term HRVB session, increasing the total HRV, helps to reduce stress and anxiety.[26] Moreover, short-term HRVB training reduces anxiety more than walking.[27] There are a specific local changes in brain activity reflecting

an increase in internal attention combined with relaxation both during and after this procedure.[28] Thus, analysis of the literature showed the effectiveness of short-term HRVB training with regard to the optimization of autonomic tone and improvement of the functions of central nervous system. The main mechanisms for realization of HRVB include the resonance effect of certain HRV frequencies, activation of baroreflex and neuronal connections between the vagus nerve and the brain structures, especially, the frontal lobes.[12,13] It has been shown that a single HRVB session can significantly reduce subjective anxiety in musicians.[29] In addition, this method has certain advantages over passive relaxation.[30] Currently, more and more people are using computer games with biofeedback on various physiological parameters, including HR. Some authors have shown that most people are able to control their physiological functions after a single session of biofeedback. The game participants in pairs were more successful in biofeedback training than were single players.[31]

Researchers have found that a short-term HRVB session in adults with different BP levels has prognostic significance. In people with normal BP, the TP increased significantly, the stress indexes decreased, and oxygen saturation increased. In hypertensive individuals without drug treatment, a low success of HRVB on the background of high sympathetic reactivity was detected. In hypertensive people with drug correction, vagal reactivity was more pronounced than in those without drug correction, which was reflected in a significant increase in the TP compared to the baseline values. Oxygen saturation was not changed in comparison with the baseline values in those of two previous groups.[32-34] Thus, the ability of HRVB to increase the TP during the standard short-term recording (5 minutes) can be regarded as a test for the determination of the preserved vagal reserve in patients with high BP. However, the oxygen saturation in this case is a non-informative indicator of biofeedback effectiveness in people with hypertension. Previously, other authors on the example of skin temperature biofeedback training (single session) in persons with heart failure also showed minimal changes in oxygen saturation on the background of optimizing cardiac output and total vascular resistance.[35]

Despite the presence of a large database of evidence supporting the efficacy of HRVB training, there exist debatable questions of the sustainability of the biofeedback effect sustainability.[36] It has been shown that after a single 20-minute HRVB session, persons with psychotic disorders could increase the perceived control. However, there were no differences in HRV indices and symptoms of stress and paranoia in people who carried out the biofeedback session and in people from the control group did.[37] Other authors have observed that psychological stress, fear and anxiety significantly decreased in difficult-to-wean patients requiring prolonged mechanical ventilation after four HVRB sessions. However, HRV indices after this intervention did not differ significantly from baseline values.[38]

In conclusion**,** **t**his brief review demonstrates the effectiveness of short-term HRVB sessions in an evaluation of human adaptation to uncomfortable environments. A single HRVB session can be used as a test for the effectiveness of cortical-visceral connections in patients with cardiovascular disease. In addition, this method can be used as short-term assistance in adaptation to social stressful factors. However, there are certain limitations in the application of this method. First, low reactivity of some levels of vascular regulation and blood gas parameters during the first HRVB session was detected in people with impaired vascular tone. Secondly, the effects of HRVB training on HRV and clinical manifestations of disease did not always coincide in time. In the above cases, the use of long-term courses of HRVB will be more advisable.

Competing interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

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Evaluation of Placental Blood Flow in Patients with Placental Insufficiency

Julia E. Dobrokhotova, PhD, ScD[1]*; Sophia A. Zalesskaya[1]; Elena A. Zubareva, PhD, ScD[1]; Andrey R. Zubarev, PhD, ScD[1]; Tatiana A. Rumyantseva, PhD[1,2]

[1]N.I. Pirogov Russian National Research Medical University
[2]Central Research Institute of Epidemiology
Moscow, the Russian Federation

Abstract

Background: Placental insufficiency is a major problem of modern obstetrics due to its link to maternal and perinatal morbidity and mortality. Placental microcirculatory disorders play a decisive role in the pathogenesis of this condition. Thus, an evaluation of placental blood flow is of particular importance and crucial for appropriate diagnosis. ***The aim*** of this study was to evaluate placental blood flow in patients with placental insufficiency. SMI (superb microvascular imaging) was compared to color Doppler for that purpose.

Materials and Methods: Primigravida patients (n=91) at 15 to 16 weeks of gestation were enrolled. Inclusion criteria were spontaneous singleton pregnancy, age from 18 to 45 years. All participants were divided into 2 groups: Group 1 – control group (n=27) and Group 2 – threatened miscarriage group (n=64). Transvaginal ultrasound and color Doppler were performed to assess uteroplacental circulation. Placental blood flow was evaluated using a Toshiba Aplio™ 500 machine equipped with an SMI tool.

Results: Placental blood flow assessment in patients with normal pregnancy revealed homogenous placental tissue, normal distribution of vessels, and active blood flow; in patients with pregnancy complications, we found inhomogeneous placenta, decreased blood flow, sporadic vessels, and avascular areas. SMI demonstrated several benefits compared to color Doppler imaging. Color Doppler allows us to assess superficial vessels only, whereas SMI provides more comprehensive data on the overall vascularization of the placenta.

Conclusion: SMI by Aplio™ 500 (Toshiba) may be an effective tool in the assessment of placental blood flow and the diagnosis and prognosis of placental insufficiency. **(Int J Biomed. 2017; 7(1):28-31.)**

Key Words: superb microvascular imaging • placental circulation • threatened miscarriage • placental insufficiency

Introduction

Spontaneous abortion in early pregnancy remains an urgent problem in modern obstetrical care.[1] According to previous studies, spontaneous miscarriage occurs during the first trimester of pregnancy in 50% cases, during the second trimester of pregnancy - in 20% cases, and during the third trimester of pregnancy - up to 30% cases.[2] Uterus hyper tone is especially unfavorable in the first trimester of pregnancy when trophoblast invasion and placental formation are initiated. Deterioration of those processes may influence the further course of pregnancy. Spiral arteries are of major importance in the course of placental development. Spiral arteries are the terminal parts of uterine vessels, which penetrate the implantation and placentation area. Conversion of spiral arteries implies 5-10 fold dilation at the mouth of the vessels, which are temporarily blocked by extravillous trophoblast. By the 12th week of gestation, these vessels are unblocked, thus initiating maternal blood flow in the intervillous space. The absence of trophoblastic plugs along with deterioration of trophoblast invasion leads to pathological placentation.[3,4] Pathological placentation leads to the formation of preterm maternal blood flow and the increase in oxygen concentration in the intervillous space. Those problems in early pregnancy may affect chorionic villi, leading to retroplacental or subchorionic

***Corresponding author:** Professor Julia E. Dobrokhotova, PhD, ScD. Head of Department of Obstetrics and Gynecology, Medical Faculty, N.I. Pirogov Russian National Research Medical University, Moscow, the Russian Federation. E-mail: pr.dobrohotova@mail.ru*

hematomas and spontaneous abortion.[4,5] Placental insufficiency is a major problem of modern obstetrics due to its link to maternal and perinatal morbidity and mortality.[1,2] Placental insufficiency is classified according to the period of its onset (primary placental insufficiency, arising before 16 weeks of gestation, and secondary, arising after 16 weeks of gestation). Violations of the implantation process (defective trophoblast invasion and disruption of placental angiogenesis activity) cause pregnancy to take an abnormal course.[6,7] Microcirculatory disorders in the intervillous space play the major role in the pathogenesis of placental insufficiency, eventually leading to circulatory disorders in the mother-placenta-fetus system and to intrauterine fetal hypoxia and fetal retardation.[8] According to some studies, intraplacental circulatory disorders are accompanied by circulatory disorders in certain areas of the placenta.[9] Thus, an evaluation of placental blood flow is of particular importance and crucial for appropriate diagnosis. Ultrasound is one of the methods that offer a great perspective in the field. Color and pulsed Doppler as well as 3D reconstruction of vessels in the placenta are widely used in obstetrics and gynecology, while placental microcirculation evaluation is still under study.[10,11] The ultrasound SMI (superb microvascular imaging) technique has been recently introduced. SMI allows visualization of the smallest vessels characterized by low-velocity flows. Moreover, SMI implies high-definition techniques, which minimize the risk of registration of artifacts. Considering the benefits of the method, we tried to apply it to the visualization of utero-placental blood flow as a diagnostic tool for placental insufficiency.

The aim of this study was to evaluate placental blood flow in patients with placental insufficiency. SMI was compared to color Doppler for that purpose.

Materials and Methods

Primigravida patients (n=91) at 15 to 16 weeks of gestation were enrolled. Inclusion criteria were spontaneous singleton pregnancy, age from 18 to 45 years. Exclusion criteria were vaginal infections, congenital anomalies of the genitalia, ovarian and adrenal hyperandrogenism, thyroid gland diseases, other extragenital diseases, multiple gestation, and pregnancy as a result of assisted reproductive treatment. Last normal menstrual period was used to calculate the gestational age.

All participants were divided into 2 groups: Group 1 – control group (n=7) and Group 2 – threatened miscarriage group (n=64). Informed consent was signed by each participant. Threatened miscarriage was diagnosed by ultrasound. Pain and discomfort in the lower abdominal region were the leading complains (75%) in Group 2 upon admission. Vaginal bleeding was registered in 25% patients in Group 2. Myometrial hypertonus (78%), chorion abruption accompanied by retrochorial hematoma (12%) and cervical shortening (17%) were identified by ultrasound. Clinical data were collected and pelvic exam performed for all patients. The two groups did not vary significantly in terms of age, extragenital diseases, or sociodemographical characteristics.

Transvaginal ultrasound and color Doppler were performed to assess uteroplacental circulation. Placental blood flow was evaluated using a Toshiba Aplio™ 500 machine equipped with an SMI tool. Vascularization index (VI) was automatically calculated in all cases.

Examination and analysis included the following steps:

1. Visualization of placental vascularization by SMI.

Several parameters were evaluated:

a) uniformity of vascularization,

b) intensity of vascularization

c) avascular areas.

2. Visual assessment of intensity of vascularization in a certain area.

3. Evaluation of VI

4. Comparison of SMI and color Doppler.

The statistical analysis was performed using the statistical software «Statistica». (v6.0, StatSoft, USA). For data with normal distribution, inter-group comparisons were performed using Student's t-test. A probability value of $P<0.05$ was considered statistically significant.

Results

Mean age of participants was 23.9±3.5 years. Placental blood flow assessment in patients with normal pregnancy revealed homogenous placental tissue, normal distribution of vessels, and active blood flow (Fig.1); in patients with pregnancy complications, we found inhomogeneous placenta, decreased blood flow, sporadic vessels, and avascular areas (Fig.2).

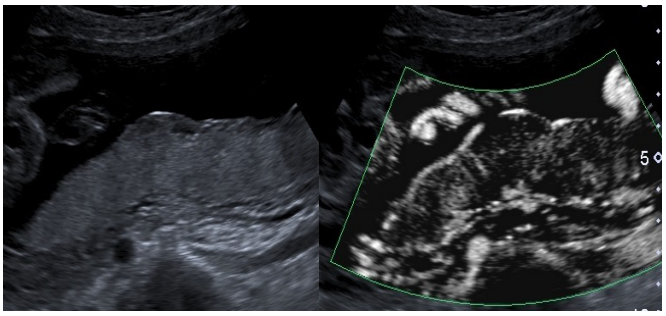

Fig. 1. *Normal placenta (left – B-mode, right – SMI).*

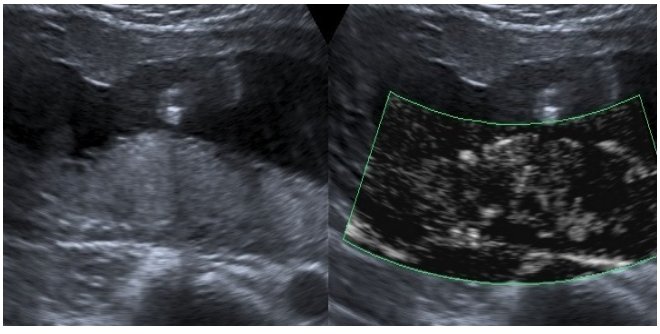

Fig 2. *Pathological placenta (left – B-mode, right – SMI).*

Evaluation of SMI results (Fig. 3-8) in a selected area included the pattern and distribution of vessels. The amount of spiral arteries in the area is a marker of normal/pathological

placentation. The vascularization index (VI) was calculated automatically. VI values were from 15.2% to 44.1% for all cases; mean values varied significantly in two groups (Table 1).

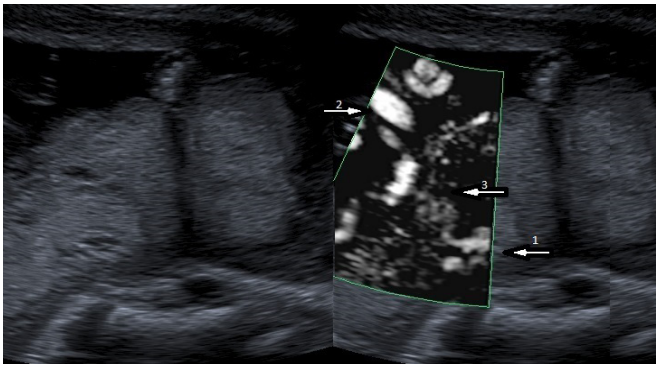

Fig 3. *An area of normal placenta and umbilical cord (left – B-mode, right – SMI. 1 – spiral arteries (SAs), 2 – umbilical cord, 3 – microvessels).*

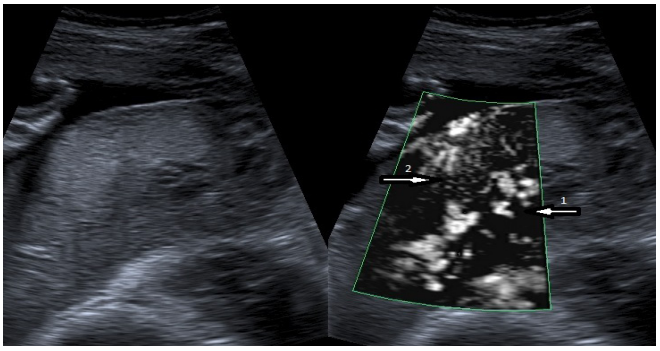

Fig 4. *An area of normal placenta (left – B-mode, right – SMI. 1 – SAs, 2 - microvessels).*

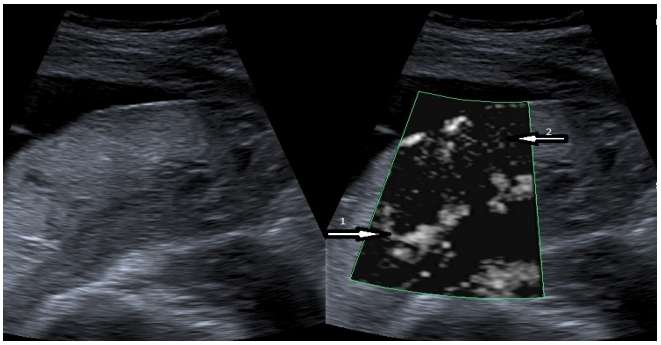

Fig 5. *An area of normal placenta (left – B-mode, right – SMI. 1 – SAs, 2 - microvessels).*

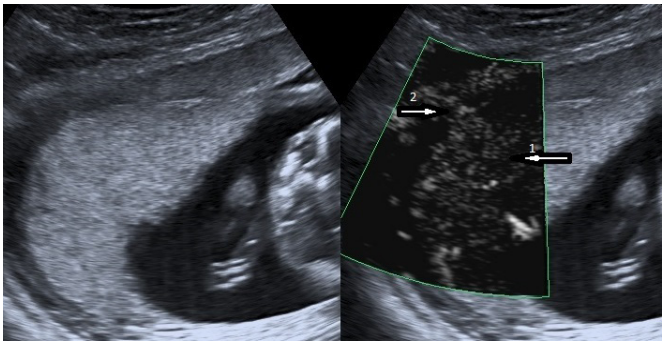

Fig. 6. *An area of placenta in patient with threatened miscarriage (left – B-mode, right – SMI. 1 – SAs, 2 - microvessels).*

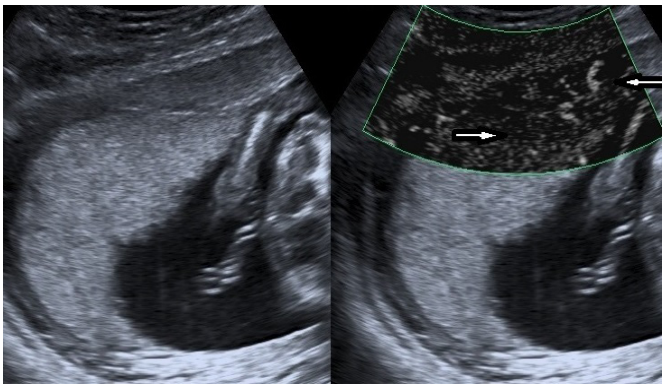

Fig 7. *An area of placenta in patient with threatened miscarriage (left – B-mode, right – SMI. 1 – SAs, 2 - microvessels).*

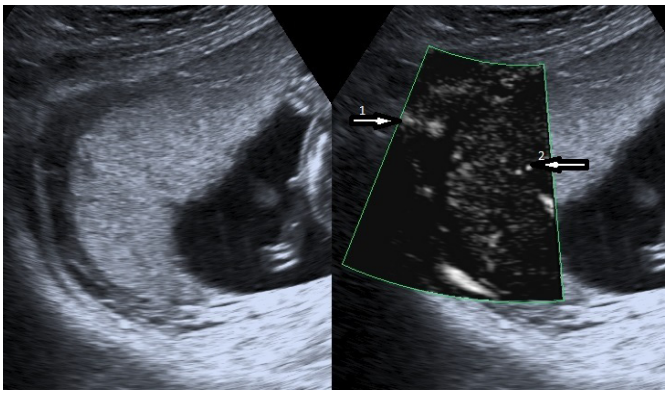

Fig 8. *An area of placenta in patient with threatened miscarriage (left – B-mode, right – SMI. 1 – SAs, 2 - microvessels).*

Table 1.

Vascularization index in control and threatened miscarriage groups

Parameter	Group 1	Group 2	P
VI, % Mean (range)	33.6 (16.1-44.1)	20.3 (15.2-24.2)	<0.05

Placental blood flow was also assessed by SMI as well as by color Doppler in the same areas of the placenta. SMI demonstrated several benefits compared to color Doppler imaging (Table 2).

Table 2.

Benefits of SMI compared to color Doppler imaging

Criteria	SMI	Color Doppler imaging
Maximum spatial resolution	+	-
Lower resistance vessels and microvessels visualization	+	-
High frame rate and scale	+	-
Artifacts	-	+

Color Doppler allows us to assess superficial vessels only, whereas SMI provides more comprehensive data on the overall vascularization of the placenta (Fig. 9-12).

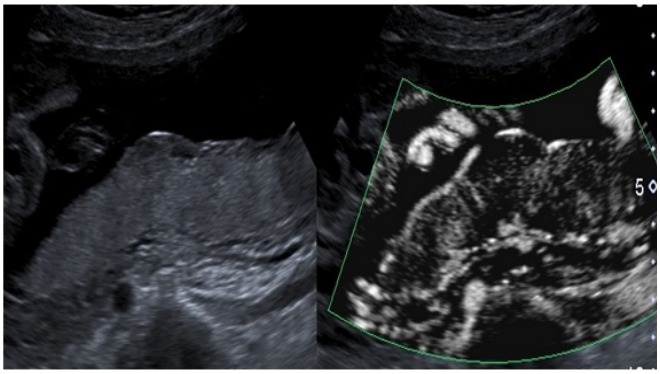

Fig. 9. *Normal placenta (left – B-mode, right – SMI).*

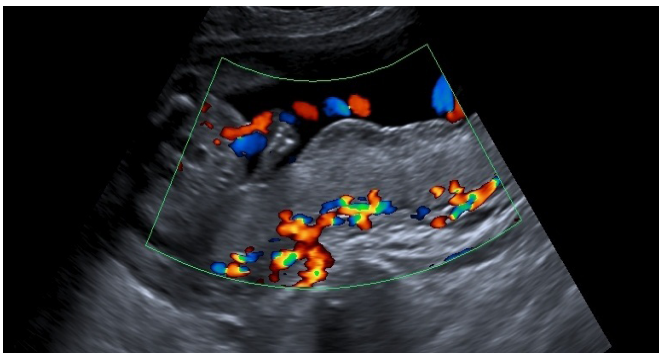

Fig. 10. *Normal placenta (color Doppler imaging)*

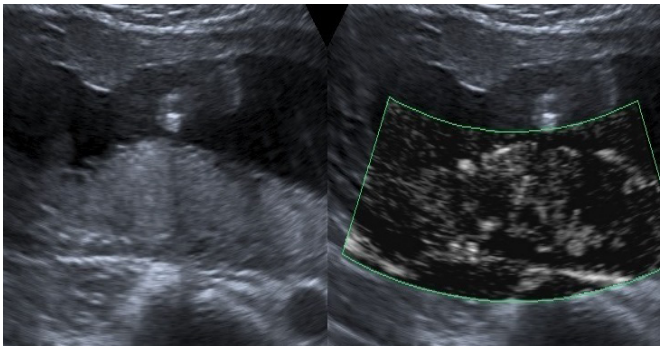

Fig. 11. *Placenta in patient with threatened miscarriage (left – B-mode, right – SMI).*

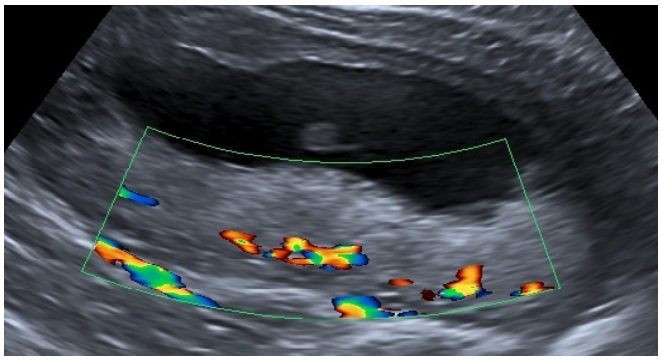

Fig. 12. *Placenta in patient with threatened miscarriage(color Doppler imaging).*

Conclusion

The SMI technique is beneficial in patients with complications during pregnancy due to the ability to detect microcirculatory abnormalities and vascular homomorphism, as well as abnormal transformation of spiral arteries. Those features are typical for placental insufficiency. Results of the current study demonstrate that SMI allows controlling the placental circulation. Assessment of the patterns of placental vessels may provide an opportunity for early diagnosis of blood flow alterations and early onset of treatment. Compared to color Doppler imaging SMI demonstrated several beneficial characteristics: color Doppler imaging is inappropriate for lower resistance vessels and for visualization of microvessels. This means that color Doppler does not provide enough data on placental blood flow, thus making early diagnosis of placental insufficiency impossible. SMI by Aplio™ 500 (Toshiba) may be an effective tool in the assessment of placental blood flow and the diagnosis and prognosis of placental insufficiency.

Competing interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

Pathophysiology

INTERNATIONAL JOURNAL OF BIOMEDICINE

Pentose Phosphate Pathway and Glutathione System of Red Blood Cells at the Exacerbation of Chronic Cytomegalovirus Infection during Pregnancy

Michael T. Lucenko, PhD, ScD*; Irina A. Andrievskaya, PhD, ScD

Far Eastern Scientific Center of Physiology and Pathology of Respiration,
Siberian Branch of Russian Academy of Sciences
Blagoveshchensk, the Russian Federation

Abstract

Objective: To determine the activity of the pentose phosphate pathway (PPP) and glutathione system (GS) in red blood cells (RBCs) of pregnant women at 28 to 30 weeks of gestation during the exacerbation of chronic CMV infection (CMVI).

Methods: The study included 50 pregnant women at 28 to 30 weeks of gestation: 25 CMV-seropositive pregnant women (the main group) with CMVI exacerbation and 25 CMV-seronegative pregnant women (the control group). We determined the activity of G6PD, GR, GP, and the amount of reduced glutathione and NADP; fatty acid peroxide levels in RBCs; the amount of degenerative forms of RBCs; RBC deformation ability; and indicators of the oxygenated form of hemoglobin and 2,3-DPG.

Results: In RBCs of pregnant women with CMVI exacerbation, cytophotometry analysis of blood smears showed a 2.16-times reduction in the intensity of histochemical reaction to G6P, a 3.3-times reduction in the intensity of histochemical reaction to reduced glutathione, and a 3.1-times reduction in the intensity of histochemical reaction to reduced NADP, compared with the control group. There were disorders in functioning and structural equivalence in both the disulfide reductase system and oxidative systems of PPP in RBCs, which was reflected in lower activity of GR and GP, 1.9 times and 2.5 times, respectively, in the main group. At the same time, the content of 2,3-DPG was 1.24 times more, whereas RBC deformability was 2.25 times less than in the control group.

Conclusion: CMVI exacerbation at 28 to 30 weeks of gestation causes the inhibition of the metabolic activity of the enzymes of PPP and GS, an initiation of membrane destruction oxidative processes that enhance the deformability of RBCs and decrease oxygen metabolism, which creates the risk of anemia and hemic hypoxia in pregnant women. **(Int J Biomed. 2017; 7(1):32-36.)**

Key Words: cytomegalovirus infection • pregnancy • red blood cells • pentose phosphate pathway • glutathione system

Abbreviations

CMV, cytomegalovirus; **2,3-DPG**, diphosphoglycerate; **G6P**, glucose-6-phosphate; **G6PD**, glucose-6-phosphate dehydrogenase; **GR**, glutathione reductase; **GP**, glutathione peroxidase; **GS**, glutathione system; **PPP**, pentose phosphate pathway; **RBCs**, red blood cells.

Introduction

CMV is an extremely common virus that can infect almost any one. During pregnancy, a direct effect of CMV on the structural and functional state of immune and hematopoietic cells is very pronounced.[1] RBCs are actively involved in the interactions of CMV through glycophorin receptors,[2,3] which lead to the initiation of the hydrolytic and proteolytic processes, altering the redox state and the energy balance of RBCs by inhibiting enzymatic reactions in the initial phase of PPP and GS. Disturbances in the erythrocyte metabolism lead to anemia, which is often diagnosed during pregnancy.[4] However, there are no data on the role of chronic CMVI in the formation of anemia in pregnant women during the third trimester of gestation, a period which is extremely important for fetal development.

***Corresponding author**: Prof. Michael T. Lucenko, Academician of the RAS, Far Eastern Scientific Center of Physiology and Pathology of Respiration of the RAS. Blagoveshchensk, Russia. E-mail: Lucenkomt@mail.ru*

The aim of this study was to determine the peculiarities of PPP function and GS activity in RBCs of pregnant women at 28 to 30 weeks of gestation during the exacerbation of chronic CMV infection (CMVI).

Study Design

We performed a prospective case-control study. The study was performed at a single institution for one year. The study included 50 pregnant women at 28 to 30 weeks of gestation: 25 CMV-seropositive pregnant women (the main group) with CMVI exacerbation and 25 CMV-seronegative pregnant women (the control group). The two groups were matched in age (24.8±0.3 years and 23.9±0.4 years). Written informed consent was obtained from all patients.

Inclusion criteria for the main group were a relapse of CMVI with clinical signs of acute respiratory viral infections and herpes virus infection (HHV-1,2) remission during the entire gestation period.

Exclusion criteria were primary CMVI, an aggravation of other inflammatory diseases of extragenital localization and sexually transmitted infections.

The clinical diagnosis of primary CMVI was determined by the presence in the peripheral blood of anti-CMV IgM antibody, low-avidity IgG (avidity index <65%), and the presence of CMV DNA in the samples of blood or urine; the relapse of CMVI was diagnosed by the presence of anti-CMV IgM, high-avidity IgG (avidity index > 65%), and the presence of CMV DNA in samples of buccal epithelium and cervical mucosa.

Description of intervention

We determined the activity of G6PD, GR, GP, and the amount of reduced glutathione and NADP; fatty acid peroxide levels in RBCs; the amount of degenerative forms of RBCs; RBC deformation ability; and indicators of the oxygenated form of hemoglobin and 2,3-DPG.

At 28 to 30 weeks of gestation, blood samples were collected from the ulnar vein to perform histochemical reactions, PCR, ELISA and spectrophotometric assays. Samples of urine, buccal epithelium, and cervical content were collected for PCR analysis. All samples of biological materials were examined simultaneously.

Primary endpoints: the activity of G6PD, GR, GP, and the amount of reduced glutathione and NADPH, fatty acid peroxide levels, the amount of degenerative forms of RBCs, and RBC deformation ability.

Secondary endpoints: the indicators of oxygenated form of hemoglobin and 2,3-DPG.

Methods of measuring outcomes

Blood samples (5 ml) were collected from the ulnar vein in standard vacuum tubes with EDTA to obtain the samples of mononuclear cells. For serological tests, we used blood that does not contain anticoagulants. Mononuclear cell isolation for PCR was carried out with Ficoll-Urografin (d-1.077g/ml) ("DNA-Technology", Russia). Serological studies were performed in paired serum samples at intervals of 10-14 days. The morning urine specimens for PCR analysis were collected in a sterile container (60 ml). Buccal epithelium and contents of the cervical canal were collected in standard sterile plastic tubes (0.5 ml) with a physiological solution.

G6PD activity and an amount of reduced NADP were determined by the cytochemical methods in native erythrocytes. The cytochemical assay is based on the reduction of water-soluble colorless tetranitro blue tetrazolium via the electron carrier 1-methoxyphenazine methosulfate, in its water-insoluble dark-colored formazan by NADPH. Dark-purple granules are present in erythrocytes that contain G6PD activity, whereas G6PD-deficient erythrocytes remain unstained. The same principle has been used for detection of glutathione according to the modified method of MT Lucenko and IA Andriyevskaya.[5]

The levels of the fatty acid peroxides were determined according to Winkler-Schulze. For control, RBCs were incubated in a medium containing adequate amounts of the substrate instead of a phosphate buffer. Reactions were performed according to the prescriptions listed in Table 1. The obtained smears were studied with the digital microscope MEJI (Japan) connected to a computer according to the Scion program (USA). The activity of reaction products was calculated automatically in a cytophotometric study and expressed in pixels/μm^2.

Table 1.

Composition and quantity of reagents used in histochemical reactions

Name of reaction	Composition of incubation solution	Amount
G6PD	0.1 M phosphate buffer, pH 7.5	1 ml
	Nitroblue tetrazolium (ICN Biomedicals, USA)	1 mg
	NADP (Appilichem, Germany)	2 mg
	1 M Na-G6P (ICN Biomedicals, США)	0.3 ml
	$MgSO_4$ (Reachim, Russia)	2 mg
NADP	0.1 M phosphate buffer, pH 7.4	1 ml
	NADP (Appilichem, Germany)	1 ml
	Nitroblue tetrazolium (ICN Biomedicals, США)	1 mg
Glutathione	10% aqueous solution of sodium nitroprusside (Biochemreactiv, Russia)	0.25 ml
	2% ammonia solution (Reahim, Russia)	0.25 ml
Fatty acid peroxides	576 mg of α-naphthol (ICN Biomedicals, USA) in 6 ml of 1N NaOH (Reachim, Russia) filled up to 50 ml with distilled water	0.25 ml
	691 mg of n-amino-N,N-dimethyl-phenylen-ediamine (Laverna, Russia) in 50 ml of distilled water	0.25 ml
	Lugol's Solution of Iodine 2%	0.5 ml
	0.005% solution of lithium carbonate (ICN Biomedicals, USA)	0.5 ml

RBC shape was determined by cytofluorimetric analysis using the MEKOS device (registration certificate of Ministry of Health of the Russian Federation 29/10010198/1282-01).

The erythrocyte membrane density was measured using a digital camera according to the BioVision Pixera program (USA) at a constant of 60 pixels, which corresponds to the morphofunctional state of degenerative forms of RBCs. RBC deformation properties were calculated using the cytophotometric device Mekos according to the method of MT Lucenko and IA Andriyevskaya.[6]

Oxyhemoglobin was determined spectrophotometrically by the method of Malloy and Evelyn.[7] 2,3-DPG level was determined by NS Luganov and MN Blinov.[8] The activity of GR and GP was determined spectro-photometrically using standard reagent kits (Sentinel Diagnostics, Italy).

Statistical analysis was performed using StatSoft Statistica v6.0. The mean (M) and standard error of the mean (SEM) were calculated. For data with normal distribution, inter-group comparisons were performed using Student's t-test. Two-tailed $P<0.05$ was considered statistically significant.

Results

Primary endpoints

In RBCs of pregnant women with CMVI exacerbation at 28 to 30 weeks of gestation, cytophotometry analysis of blood smears showed a 2.16-times reduction in the intensity of histochemical reaction to G6P (P<0.001) (Fig.1), a 3.3-times reduction in the intensity of histochemical reaction to reduced glutathione (P<0.001) (Fig.2), and a 3.1-times reduction in the intensity of histochemical reaction to reduced NADP (P<0.001) (Fig.3), compared with the control group, indicating suppressed PPP activity in RBCs caused by alteration in the acceptor properties of NADP, in the main coenzyme of dehydrogenases, and in thiol-disulfide reactions of the redox system defined by glutathione.

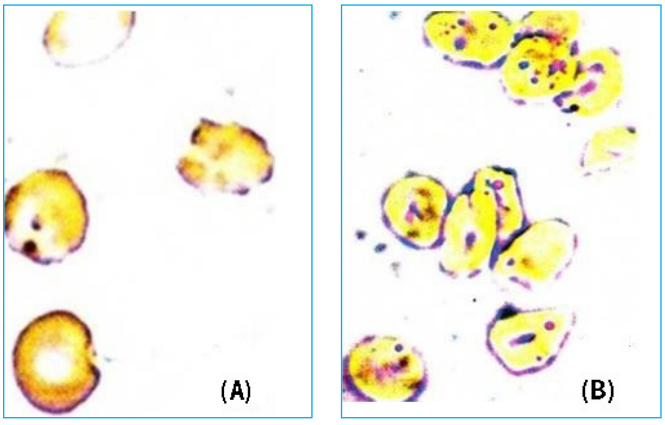

Fig. 1. *RBCs of a pregnant woman with CMVI exacerbation at 28 weeks of gestation. Histochemical reaction to G6PD according to R. Lilly. (A) – main group, (B) – control group. Magnification: 15x100.*

At the same time, there were disorders in functioning and structural equivalence in both the disulfide reductase system and oxidative systems of PPP in RBCs, which was reflected in lower activity of GR and GP, 1.9 times and 2.5 times, respectively, in the main group (Table 2). Moreover, changes in the oxidative status of RBCs of CMV-seropositive pregnant women were characterized by increased (4-fold) cytophotometric indicators of the histochemical reaction products to fatty acids peroxide (Fig.4, Table 3). Subsequent morphological analysis of blood smears revealed structural changes in RBCs, which manifested in increased specific density of the erythrocyte membranes determined at a resolution of 60 pixels (Fig.5). These changes were consistent with degenerative forms, the number of which increased by 3.3 times (Table 3). Automatic calculation of RBC deformability revealed its reduction by 2.25 times (Table. 3), which also indicated the alterations in the structural and functional properties, including those associated with PPP oxygen metabolism.

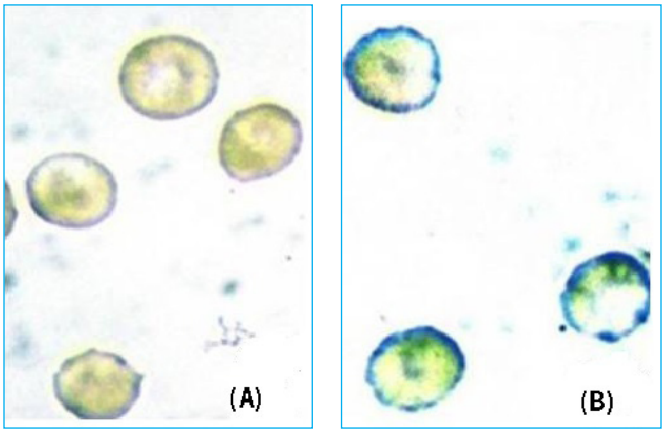

Fig. 2. *RBCs of a pregnant woman with CMVI exacerbation at 28 weeks of gestation. Histochemical reaction to reduced NADP according to Lloyd. (A) – main group, (B) – control group. Magnification: 15x100.*

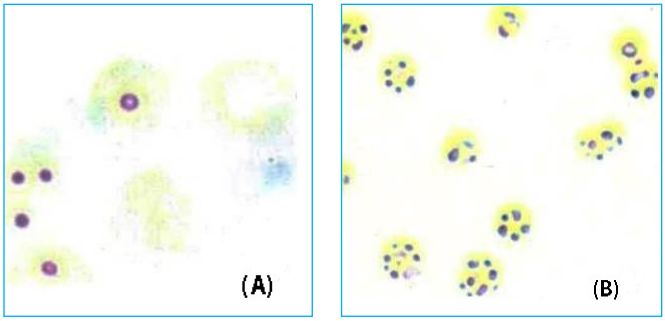

Fig. 3. *RBCs of a pregnant woman with CMVI exacerbation at 28 weeks of gestation. Histochemical reaction to reduced glutatione according to MT Lucenko and IA Andriyevskaya. (A) – main group, (B) – control group. Magnification: 10x90.*

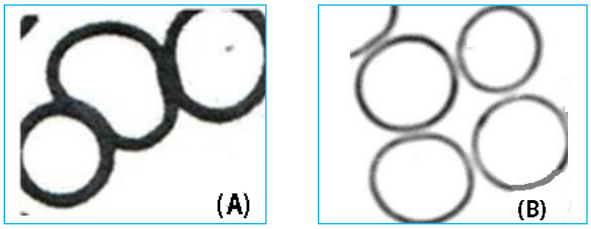

Fig. 4. *RBCs of a pregnant woman with CMVI exacerbation at 28 weeks of gestation. Histochemical reaction to* fatty acid peroxides *according to* Winkler-Schulze. *(A) – main group, (B) – control group. Magnification: 15x90.*

Table 2.

Parameters of the functional activity of PPP and GS in RBCs during CMVI exacerbation at 28 to 30 weeks of gestation

Parameters	Main group	Control group	P value
G6PD, pixel/μm^2	23.0±0.40	49.75±0.95	<0.0001
Reduced NADP, pixel/μm^2	14.5±0.25	45.5±0.8	<0.0001
Reduced glutatione, pixel/μm^2	17.6±1.2	58.6±0.8	<0.0001
GR, U/gHb	4.48±0.22	8.36±0.13	<0.0001
GP, U/gHb	5.81±0.13	14.66±0.36	<0.0001

Table 3.

Parameters of morphostructure and oxygen metabolism in RBCs during CMVI exacerbation at 28 to 30 weeks of gestation

Parameters	Main group	Control group	P value
Fatty acid peroxides, pixel/μm^2	95.0±1.9	23.5±0.85	<0.0001
Degenerative forms of RBCs,%	15.0±1.2	4.5±0.07	<0.0001
RBC deformability, CU	0.02±0.0012	0.045±0.0018	<0.0001
2,3-DPG, μmol/ml	7.05±0.05	5.7±0.09	<0.0001
Oxyhemoglobin, %	89±0.8	98±1.0	<0.0001

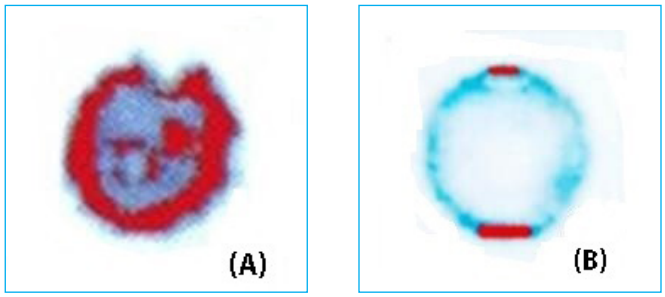

Fig. 5. *RBCs of a pregnant woman with CMVI exacerbation at 28 weeks of gestation. Increased specific density of the erythrocyte membranes determined at a resolution of 60 pixels to the BioVision Pixera program. (A) – main group, (B) – control group. Magnification: 10x90.*

Secondary endpoints

The amount of oxyhemoglobin in the main group was 1.1 times less (P<0.01), whereas the content of 2,3-DPG was 1.24 times more (P<0.001) than in the control group. Decreasing oxygen metabolism in RBCs during CMVI exacerbation at 28 to 30 weeks of gestation is a result of disturbances in enzyme activity of PPP and GS. In turn, the initiation of oxidation processes contributed to the appearance in the bloodstream of a large number of degenerative forms of RBCs with low capacity for oxygenation, creating the risk of developing hemic hypoxia.

Discussion

In summary, the present study analyzed the metabolic activity of the enzymes of PPP and GS, and the reduction state of NADP and glutathione in RBCs of women with CMVI exacerbation at 28 to 30 weeks of gestation. We identified the role of CMVI in the development of disorders in functioning and structural equivalence, in both the disulfide reductase system and the oxidative systems of PPP, which determine the processes of erythrocyte oxygenation and the development of hemic hypoxia during pregnancy.

The study of the functional activity of the initial stage of the PPP during CMVI exacerbation at 28 to 30 weeks of gestation was chosen because of known significant changes in the metabolism of RBCs, defined by destabilization and decline in the active state of G6P and reduced NADP that disrupt the thiol-disulfide activity of glutathione.[9-12]

The depletion of the pool of reduced glutathione in RBCs causes an energy crisis.[13] The result of this is enhanced proteolytic processes and the processes of lipid peroxidation, increased membrane microviscosity, and reduced deformation properties of membranes, all of which cause destruction of membranes and appearance in the bloodstream of a large number of degenerative forms with altered oxygen metabolism. It should also be noted that formed deficiency of G6PD and GR activity in RBCs may influence the enzymatic transfer and incorporation of iron into heme and thereby may disrupt hemoglobin-forming processes. On the other hand, the accumulation of reactive oxygen species in a form of hydroperoxides leads to an increased oxidation of the globin part that with high 2,3-DPG concentration disrupt oxygenation process and the formation of oxygenated form of hemoglobin, creating a risk of anemia and hemic hypoxia during CMVI exacerbation.

Conclusion

CMVI exacerbation at 28 to 30 weeks of gestation causes the inhibition of the metabolic activity of the enzymes of PPP and GS, an initiation of membrane destruction oxidative processes that enhance RBC deformability and decrease oxygen metabolism, all of which create the risk of anemia and hemic hypoxia in pregnant women.

Competing interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

Dermatology

INTERNATIONAL JOURNAL OF BIOMEDICINE

Role of Cytokines in the Pathogenesis of Acne

Olga M. Demina, PhD[1]*; Anatoly V. Kartelishev, PhD, ScD[2];
Elena I. Karpova, PhD, ScD[1]; Olga I. Danischuk[1]

[1]*N.I. Pirogov Russian National Research Medical University*
[2]*Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology and Immunology*
Moscow, the Russian Federation

Abstract

The aim of this study was to investigate the role of cytokines in light of recently discovered aspects of acne immunopathogenesis.

Materials and Methods: The study included 276 patients aged between 16 to 44 years with various forms of acne vulgaris. Severe manifestations of the disease were identified in 126/45.6% patients; disease lasted 1 to 5 years in 157/56.9% patients. The serum levels of cytokines were determined by ELISA using standard kits. The cells were characterized using flow cytometry.

Results: The obtained data on an excessive secretion of pro-inflammatory cytokines (IL-1α, IL-2) and VEGF on the background of decreasing the content of anti-inflammatory cytokines (IL-4, IL-10) indicate an insufficient activity of anti-inflammatory immune response. It was concluded that acne is a model of chronic immunodeficiency inflammatory dermatoses with the activation of innate immunity and the subsequent development of the adaptive T-cell immune response. **(Int J Biomed. 2017;7(1):37-40.)**

Key Words: acne vulgaris • cytokines • innate immunity • secondary immunodeficiency

Introduction

Acne vulgaris, also known as acne, is a chronic relapsing inflammatory disease of the pilosebaceous units (hair follicles and their accompanying sebaceous glands).[1-4] Acne has a multifactorial pathogenesis, of which the key factor is genetic predisposition. Acne develops as a result of an interplay of the following factors: follicular epidermal hyperproliferation with hyperkeratosis, excess sebum production, the presence and activity of the commensal bacteria *Propionibacterium acnes (P.acnes*), androgen excess states, and inflammation.[1,5,6] Previously, it was reported that the release of the cytokine IL-1α by keratinocytes of the sebaceous duct was pivotal in the life cycle of the comedone, mediating both its development and its spontaneous resolution.

There is clear evidence that *P.acnes* is responsible for the local inflammatory response of acne. After sebum production, *P.acnes* colonizes sebaceous follicles and releases lipase and proinflammatory mediators. *P.acnes* may trigger an innate immune reaction via the activation of TLR2. Toll-like receptors (TLRs) are a component of the innate immune system involved in host defense against invading micro-organisms [7-9] and their activation ultimately triggers the expression of immune response genes, including those coding for various cytokines and chemokines that stimulate recruitment of host immune cells.[10] TLRs can activate innate immune responses through keratinocytes, neutrophils, monocytes/macrophages, natural killer cells, and dendritic cells. There are nearly a dozen different TLRs, but TLR2 and TLR4 appear to be specific for acne pathogenesis.[11]

Stimulation of TLR2 by *P.acnes* increases concentrations of IL-8 and IL-12.[4,12,13] *P.acnes* can activate several pathways that ultimately converge to activate nuclear factor (NF)-κB transcription factor. Downstream release of inflammatory cytokines (such as IL-1, IL-6, IL-8, IL-10, IL-12, and TNF-α) mediate pathogen destruction via effector cells.[9,10]

Macrophages surrounding the pilosebaceous unit with TLR2 receptors were histologically described in biopsy material of patients with acne.[14]

In addition to innate immunity, also adaptive immunity, and especially the Th17 pathway, may contribute significantly to the inflammatory response in acne.[15,16]

***Corresponding author:** Olga M. Demina, PhD. N.I. Pirogov Russian National Research Medical University. Moscow, Russia. E-mail: demina.75@mail.ru*

Several converging lines of evidence indicate that inflammation may be present throughout the development of acne lesions, both during the latter stages where inflammatory papules, pustules, and nodules are present, and also during the early stages of lesion development, in microcomedones and comedones.[7, 17]

It has been found that the delayed resolution of inflammatory acne lesions takes place due to opportunities of *P.acnes* to stimulate the production of antibodies and histamine vasoactive peptides, as well as their resistance to the neutrophilic and monocytic phagocytosis.[18,19] It has been shown that the inflammatory process in patients with acne is supported by polynuclear neutrophils, which produce a large number of free radicals, by prostaglandins, leukotrienes B4 and complement.[1,20]

It has also been revealed that *P.acnes* antigens activate the complement system and provide the migration of free radicals, neutrophils, and macrophages in PSUs with the following production of proteolytic enzymes, IL-1α, IL-1β, IL-8, and TNF-γ, which causes inflammation and a complex cascade of pathogenetic mechanisms of disease.[5,20-22] However, the role of cellular and humoral immunity and cytokine activity in acne is still a subject of studies.

The aim of this study was to investigate the role of cytokines in light of recently discovered aspects of acne immunopathogenesis.

Materials and Methods

A study performed between 2010 and 2014 included 276 patients (86/31.1% men and 190/68.9% women) aged between 16 to 44 years with various forms of acne. Severe manifestations of the disease were identified in 126/45.6% patients; disease lasted 1 to 5 years in 157/56.9% patients. Written informed consent was obtained from all patients. An investigation of the dynamics of clinical and paraclinical parameters was carried out under the scheme developed by the individual protocol of clinical and laboratory examination (standard and special parameters), taking into account gender, age, onset and duration of the disease and the nature of its course. The acne severity was identified according to FDA guidance (2005). The serum levels of cytokines (IL-1α, IL-2, IL4, IL-6, IL- 8, IL-10, TNF-α, INF-γ and VEGF) were determined by ELISA using standard kits (BioSource International, Inc. hIL-1-10 kit, Inc.INF-γ kit, Inc.TNF-α kit, Inc. hVEGF kit) in the range of detectable concentrations (of 1 to 13 pg/ml) and test-system Bio-Plex Pro™ Human Cytokine 8-plex Assay (BenderMedSystems, Austria). The cells were characterized using flow cytometry. The statistical analysis was performed using the statistical software «Statistica». (*v6.0*, *StatSoft*, *USA*).The mean (M) and standard error of the mean (SEM) were calculated. 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 chi-square. Pearson's Correlation Coefficient (r) was used to determine the strength of the relationship between the two continuous variables. A probability value of $P<0.05$ was considered statistically significant.

Results and Discussion

Parameters of cellular immunity before treatment in patients with various forms of acne severity are shown in Fig. 1.

Analysis of parameters of cellular immunity showed that in all degrees of acne severity, there was a tendency to increase the content of neutrophils, lymphocytes; in severe forms of the disease, we found a tendency to increase in the content of white blood cells and reduction in lymphocyte level due to the chronic abscessed course of the disease. In patients with mild to moderate severity of acne, CD3+CD4+-T-cells were within normal range (42.9±3.9%), but their content significantly decreased in the severe form of acne (35.9±4.8%; $P<0.05$). We found a significant increase in the content of CD95+ lymphocytes in patients with mild to moderate degrees of the disease and a significant decrease in the content of CD95+ lymphocytes with severe degrees. The immunoregulatory index (CD4/CD8) decreased significantly in patients with a severe form of acne (1.38±0.3; $P<0.05$).

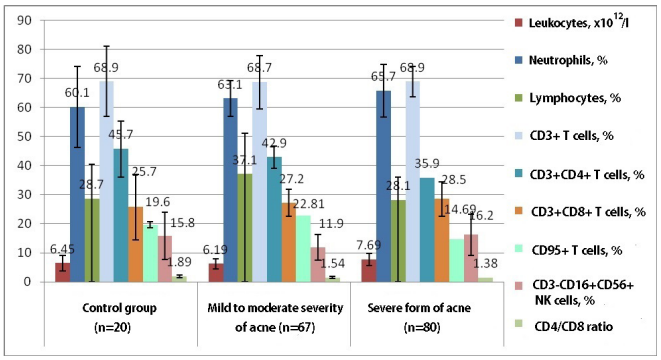

Fig. 1. *Parameters of cellular immunity in patients with various forms of acne severity*

The parameters of humoral immunity before treatment are presented in Figure 2. Thus, in various forms of acne severity, the initial levels of B-lymphocytes (CD19+) and immunoglobulin levels (IgA, IgM IgG) were in the normal range. At the same time, we found a significant decrease in the content of large circulating immune complexes (CIC) for all degrees of acne severity; middle CIC were in the normal range, while the content of small CIC was significantly increased in all degrees of acne severity, ensuring the maintenance of the aseptic inflammation.

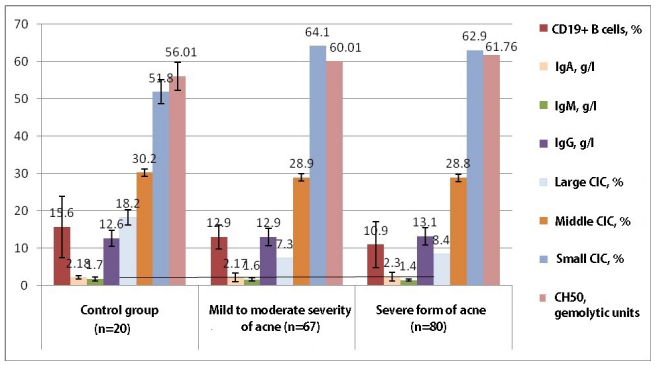

Fig. 2. *The parameters of humoral immunity in patients with various forms of acne severity.*

The serum levels of cytokines in patients with various forms of acne severity are shown in Figures 3 and 4. The serum level of IL-1α tended to increase with mild to moderate severity, while the serum level of IL-1α significantly increased in the severe form of acne. It should be noted that serum IL-1α level exceeded the reference values more than 3 times. These changes directly correlated with the acne severity and clinical symptoms of severe skin inflammation (r=+0.88). Serum IL-2 level in patients was also significantly elevated in all degrees of acne severity, but more significant in mild to moderate severity. This fact is due to the maximum functional activity of IL-2 in the early stages of the inflammatory process and less pronounced increase during chronic inflammation. Serum VEGF level was also significantly elevated in all monitored patients regardless of the severity compared to control values.

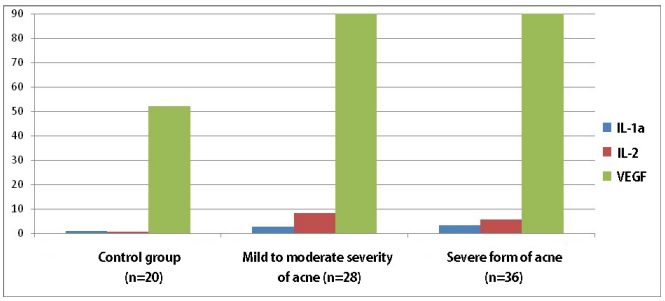

Fig. 3. *Serum levels of IL-1α, IL-2 and VEGF (pg/ml) in patients with various forms of acne severity*

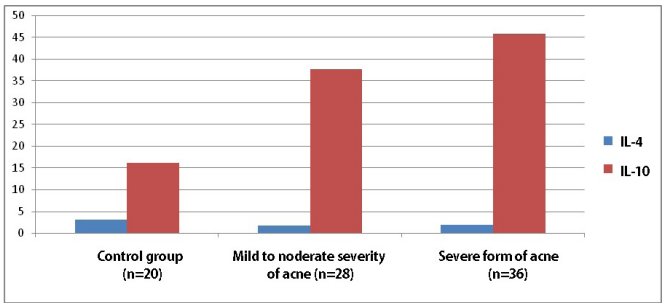

Fig. 4. *Serum levels of IL-4 and IL-10 (pg/ml) in patients with various forms of acne severity*

Serum IL-4 level was significantly reduced in all degrees of severity, indicating the suppression of anti-inflammatory cytokine secretion. Serum IL-10 level was increased in all degrees of severity, indicating the prolonged activation of inflammatory systems but was insufficient to provide the regression of clinical symptoms of the disease. Thus, the obtained data on an excessive secretion of pro-inflammatory cytokines (IL-1α, IL-2) and VEGF on the background of decreasing the content of anti-inflammatory cytokines (IL-4, IL-10) indicate an insufficient activity of anti-inflammatory immune response. Clinically, these changes correlated (r=+0.89) with the persistent torpid course of acne.

Findings:

• In all patients with acne, regardless of the disease severity, there is a pronounced secondary immunodeficiency with the predominant deficiency in T-cell immunity that is manifested on the background of significant changes in the level of large and small CIC, which indicates an imbalance in humoral immunity, underlying the recurrent protracted nature of acne.

• An excessive secretion of pro-inflammatory cytokines (IL-1α, IL-2) and VEGF on the background of decreasing the content of anti-inflammatory cytokines (IL-4, IL-10) indicates an insufficient activity of anti-inflammatory immune response.

• It can be assumed that acne is a model of chronic immunodeficiency inflammatory dermatoses with the activation of innate immunity and the subsequent development of the adaptive T-cell immune response.

• The revealed immune-related markers of acne pathogenesis are a scientific basis for the development and introduction of innovative technologies with the universal corrective mechanism of action, including photodynamic therapy in combination with low-level laser therapy.

Competing interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

Nephrology

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Experimental Testing of the New Biomedical Substance of Pig Kidneys for the Treatment of Nephrolithiasis

Aleksandr Yu. Zharikov, PhD, ScD*; Igor P. Saldan, PhD, ScD; Valery I. Kiselev, PhD, ScD; Ganna V. Pavlyashik

Altai State Medical University, Barnaul, the Russian Federation

Abstract

The aim of our study was to test anti-lithogenic activity of the new biomedical substance from pig kidneys (PK-BMS) on the experimentally induced nephrolithiasis.

Materials and Methods: The experiments were performed with 30 male Wistar outbred rats weighting 220-280g. The experimental animals were divided into two groups. In the disease-control group (DCG), the animals received a 1% solution of EG for 6 weeks. The rats in therapy group (TG), beginning from the fourth week, in addition to the daily EG intake, received PK-BMS in the amount of 1.0 per rat. The production of this substance was carried out at Altaivitaminy ZAO (Biysk, Altai Krai, Russia) by the method of cool dehumidification. In daily urine, we determined the concentration of oxalate, phosphate and calcium ions, creatinine excretion every 3 or 4 days in both groups; the activity of LDH, GGT, and NAG was measured every 7 days in the injured kidney epithelium. For the study of free-radical oxidation activity and for morphological examination of animal kidneys, after 6 weeks of the experiment, 5 rats from each group were euthanized. The occurrence of calcium compounds was determined by von Kossa's staining method.

Results: After a 6-week EG intake, we found the typical biochemical and morphological symptoms of experimental nephrolithiasis in rats of DCG: urine supersaturation with oxalate ions, significant increase in the activity of marker enzymes, activation of free-radical oxidation in the kidneys, and formation of calcium deposits in the kidneys. In TG, the new PK-BMS intake resulted in a significant alleviation of the experimental nephrolithiasi: significant decrease in the level of oxalate ions and activity of marker enzymes, reduction of free-radical oxidation in the kidneys, decrease in the number and size of calcium deposits in the area of renal papillae.

Conclusion: It was established that during the experimental nephrolithiasis, a three-week intake of the new PK-BMS is accompanied by a significant anti-lithogenic effect. **(Int J Biomed. 2017; 7(1):41-45.)**

Key Words: experimental nephrolithiasis • anti-lithogenic activity • biomedical substance • pig kidneys

Abbreviations

CAT, catalase; **EG**, ethylene glycol; **GGT**, gamma glutamyl transpeptidase; **GPO**, glutathione peroxidase; **LDH**, lactate dehydrogenase; **MDA**, malondialdehyde; **NAG**, N-acetyl-beta-D-glucosaminidase; **SOD**, superoxide dismutase; **TAA**, total antioxidant activity; **TPO**, total prooxidant activity; **TBRPs**, thiobarbiturate-reactive products.

Introduction

Previous research has shown that the long-term use of the raw material from pig kidneys (PK-RM) results in significant alleviation of experimentally induced nephrolithiasis in rats.[1] The typical diagnostic indicators were alleviation of the urine supersaturation with oxalate ions, a significant reduction in the activity of marker enzymes of the injured kidney epithelium, suppression of free-radical oxidation, and significant reduction of the number and size of calcium deposits.[1] These results allowed us to assume that PK-RM can be a very promising source for the development of a new effective anti-lithogenic remedy.

***Corresponding author:** Prof. Alexander Yu. Zharikov, PhD, ScD. Altai State Medical University, Barnaul, the Russian Federation. E-mail: zharikov_a_y@mail.ru*

The aim of our study was to test anti-lithogenic activity of the new biomedical substance from pig kidneys (PK-BMS) on the experimentally induced nephrolithiasis.

Materials and Methods

The experiments were performed with 30 male Wistar outbred rats weighting 220-280g. The animals were kept in separate cages designed for urine accumulation in the conditions of standard laboratory nutrition. Modeling of nephrolithiasis was made according to the generally accepted ethylene-glycol model: lithogenesis was provoked by the continuous intake of a 1% solution of EG.[2]

The experimental animals were divided into two groups. In the disease-control group (DCG), the animals received a 1% solution of EG for 6 weeks. The rats in therapy group (TG), beginning from the fourth week, in addition to the daily EG intake, received PK-BMS in the amount of 1.0 per rat. The production of this substance was carried out at Altaivitaminy ZAO (Biysk, Altai Krai, Russia) by the method of cool dehumidification that included the following stages:

1. The preparation of biomaterials: defrosting the raw pig kidneys at room temperature; removing surface fat; homogenizing by the laboratory homogenizer at 10,000 rpm for 5 minutes; weighing and distributing the homogentisate on the calibrated trays as a smooth layer not more than 0.5cm thick.

2. Freezing (vitrification) of the product at a temperature of -45°C for 4 hours.

3. Primary drying: drying of the product in the vacuum chamber at a temperature of -40°C and under a pressure of 10Pa for 48 hours.

4. Secondary drying: drying of the product at a temperature of 20°C for 6 hours.

As a result of the process described, 80% of the moisture was removed from the biological material while preserving its structural and functional integrity.

In daily urine, we determined the concentration of oxalate, phosphate and calcium ions, creatinine excretion every 3 or 4 days in both groups; the activity of LDH (indicates the level of cell cytolysis), GGT (indicates the level of cellular membrane damage), and NAG (indicates the functional disorders in nephrocytes) was measured every 7 days in the injured kidney epithelium.

The oxalate-ions in the urine were determined by high-performance liquid chromatography in accordance with the methods we developed earlier.[3] The 80%-acetonitrile aqueous solution at gradient from 0 to 100% and 0.1% sulphuric acid solution were used as eluents. The eluent feed rate was 100 µl/min, the volume of elution – 1000 µl, and the temperature of the chromatographic column – 35 C°. The detection was carried out at λ=210 nm. A calibration curve was built using the standard oxalate ion solution with a concentration of 1mg/ml (Fluka Chemicals Ltd). The calculation of phosphate ions was carried out by the method of photo-electric colorimetry at λ=440 nm. The method is based on the reaction of formation of the phosphoric-molybdenic-vanadic complex having a typical yellow color. The calcium ions in the urine were also calculated by photoelectric colorimetry in a reaction with o-cresol-phtalein complexon at λ=590 nm.

The activity of LDH was determined by spectrophotometry at λ=340 nm. This method is based on the pyruvate reduction reaction to lactic acid. This reaction is catalyzed by LDH and its rate is proportional to the enzyme activity. The catalytic activity of GGT, for the calculation of which we used the method of photoelectric colorimetry, was calculated proportionally to the amount of n-nitroaniline, which is formed in the reaction between L-γ-glutamyl-3-carboxy-4-nitroaniline and glycylglycine. The detection of n-nitroaniline was carried out using the photoelectric colorimeter at λ=400 nm. The calculation of NAG was carried out according to the modified Maruch method.[4] According to this method, NAG activity is proportional to the amount of n-nitrophenol, which is formed as a result of the hydrolytic reaction of n-nitro-N-acetyl-β-glucosamine catalyzed by this enzyme. The measurement of the n-nytrophenol amount was carried out by spectrophotometry at λ=400 nm. The activity of all enzymes was calculated in relation to the urine creatinine concentrations (mg/l) and was presented in standard units (U/mg of creatinine).

For the study of free-radical oxidation activity, which is a significant indicator of lithogenesis,[5,6] and for morphological examination of animal kidneys, after 6 weeks of the experiment, 5 rats from each group were euthanized taking into account the requirements of the World Medical Association Declaration of Helsinki (2000). Free-radical oxidation activity was determined based on the indicators of oxidant/antioxidant status. The indicators of oxidant status were determined in the homogenate of the kidney cortex. The total index of the concentration of all pro-oxidants and free-radical methabolites (TPO) was estimated by the color intensity of the florescent complex, which is formed by the interaction between Tween-80 peroxidation products and thiobarbituric acid. In addition, we estimated the concentration of MDA and other TBRPs.

The activity of the antioxidant system was studied in the homogenate of kidney cortex. To estimate the antioxidant status in cells, we determined TAA and activity of CAT, SOD and GPO. TAA was estimated by the degree of inhibition of Fe2+/ascorbate-induced oxidation of Tween-80 by the tissue homogenate (erythrocyte hemolysate). CAT activity was estimated by the reduction of the sodium molybdate oxydation by hydrogen peroxide. SOD activity was estimated by the level of nitroformazan, a colored product of nitrotetrazolium reduction by superoxide radicals. The marker of GPO activity was the estimation of unoxidized glutathione in the color reaction with Ellman's reagent.

The morphological study of the rat kidneys was conducted using light-optical microscopy. A 10% formalin solution was used as a preservative. For the estimation of disorders in the kidney cortex and medulla, the 4-6-micron-thick tissue sections were colored by H&E. On the 10-15-micron-thick sections, the occurrence of calcium compounds was determined by von Kossa's staining method. They were identified as calcium deposits of black color. In this histochemical reaction, the nuclei turn red and the other tissue

structures turn pink (magnifications are x100 and x400).

The obtained results were statistically processed.[7] The mean (M) and standard error of the mean (SEM) were deduced. The Wilcoxon criterion was used to compare the differences between the paired samples. A probability value of *P<0.05* was considered statistically significant.

Results and Discussion

After a 6-week EG intake, we found the typical biochemical and morphological symptoms of experimental nephrolithiasis in rats of DCG: urine supersaturation with oxalate ions, significant increase in the activity of marker enzymes, activation of free-radical oxidation in the kidneys, and formation of calcium deposits in the kidneys. Thus, on the third day of the experiment, the animal urine had already shown a consistently high level of the oxalate-ion concentration, which had been preserved during the whole experiment (Table 1).

Table 1.

Parameters of excretory kidney function in rats of DCG

Days	Oxalate (mg/ml)	Phosphate (mg/ml)	Calcium (μmol/ml)	Diuresis (ml/day)	Creatinine (mmol/day)
Initial level					
	-	9.1±0.38 (n=15)	1.8±0.10 (n=15)	5.3±0.36 (n=15)	7.1±0.38 (n=15)
Experimental nephrolithiasis					
3	1.3±0.29* (n=12)	9.0±0.47 (n=12)	1.9±0.19 (n=8)	5.9±0.65 (n=12)	9.3±0.66* (n=8)
7	1.2±0.14* (n=15)	8.0±0.44 (n=15)	1.3±0.09* (n=15)	5.4±0.48 (n=15)	8.5±0.61 (n=15)
10	1.3±0.10* (n=9)	8.5±0.40 (n=9)	Not determined	6.5±0.64 (n=9)	Not determined
14	1.4±0.13* (n=15)	8.2±0.33 (n=15)	1.6±0.22 (n=15)	5.9±0.43 (n=15)	6.3±0.44 (n=15)
17	1.1±0.10* (n=15)	8.1±0.21 (n=15)	1.9±0.13 (n=15)	7.7±0.96* (n=15)	10.9±0.63* (n=15)
21	1.3±0.13* (n=15)	7.4±0.46* (n=15)	1.9±0.23 (n=15)	6.7±0.55* (n=15)	7.2±0.49 (n=15)
24	1.6±0.17* (n=15)	5.9±0.43* (n=15)	1.4±0.14 (n=15)	7.6±1.41 (n=15)	9.2±0.84 (n=15)
28	1.6±0.16* (n=15)	6.2±0.40* (n=15)	1.5±0.10 (n=15)	7.7±0.78* (n=15)	8.6±1.00 (n=15)
31	1.3±0.12* (n=8)	7.9±0.63 (n=8)	1.5±0.28 (n=8)	9.4±1.73* (n=8)	Not determined
35	1.3±0.12* (n=15)	6.1±0.50* (n=15)	1.6±0.06 (n=15)	8.2±1.01* (n=15)	8.4±0.74 (n=15)
38	1.7±0.21* (n=9)	8.2±0.17 (n=8)	Not determined	5.6±0.64 (n=9)	9.8±0.68* (n=9)
42	1.3±0.14* (n=15)	6.2±0.45* (n=15)	1.6±0.17 (n=15)	9.0±1.19* (n=15)	8.3±1.03 (n=14)

** - P<0.05 in relation to the initial level*

Moreover, DCG rats showed a continuous growth of activity of the marker urine enzymes. By the end of the sixth week of the pathology modeling, LDH activity increased by 2.9 times, GGT – by 1.6 times, and NAG – by 3.8 times (*P<0.001* vs. the initial levels in all cases) (Table 2).

Table 2.

Enzyme activity in the urine of DCG rats

Days	LDH	GGT	NAG ($\times 10^{-3}$)
	U/mg creatinine per day		
Initial level			
	0.18±0.015	0.26±0.015	8.4±0.32
Experimental nephrolithiasis			
7	0.32±0.024	0.30±0.015	14.4±2.48 (P<0.01)
14	0.50±0.033 (*P<0.001*)	0.32±0.011	19.4±1.40 (*P<0.001*)
21	0.52±0.032 (*P<0.001*)	0.37±0.015	20.1±2.11 (*P<0.001*)
28	0.44±0.018 (*P<0.001*)	0.30±0.010	17.2±0.90 (*P<0.001*)
35	0.45±0.025 (*P<0.001*)	0.42±0.049 (P<0.05)	15.4±1.26 (*P<0.01*)
42	0.53±0.018 (*P<0.001*)	Not determined	31.9±2.86 (*P<0.001*)

P - in relation to the initial level; n=15 in all cases.

Simultaneously, we observed a distinct development of oxidative stress in the kidneys of DCG rats (Table 3).Thus, TBRP concentration increased 8.3 times in relation to this index in healthy rats (P<0.001). At the same time, the activity of GPO, the main antioxidant enzyme, decreased by 7.5%. The most significant evidence of the occurrence of lithogenic processes in the kidneys of DCG rats was the results of morphometry, according to which the number of calcium deposits in the area of renal papillae was 27.4±3.22 per field of view and their average size was 12.0±0.62 micron.

In TG, we also found the typical biochemical symptoms of nephrolithiasis during the first 3 weeks of the disease modeling (Table 4).

Table 3.

Activity of free-radical oxidation in the kidneys of DCG and TG rats

TBRP (μmol)	TAS (%)	CAT (%)	SOD (%)	GPO (%)
Healthy rats				
2.9±0.18	75.5±2.71	11.9±0.79	14.9±1.61	37.4±1.88
DCG				
24.1±0.62* (*P<0.001*)	78.2±2.71	13.6±1.50	11.6±1.26	29.9±2.45* (*P<0.05*)
TG				
6.5±0.64** (*P<0.001*)	73.0±2.24	28.5±2.66** (*P<0.001*)	23.4±1.03** (*P<0.01*)	39.7±1.80

**- in relation to the healthy rats; **- in relation to DCG*

Table 4.

Parameters of excretory kidney function in rats of TG

Days	Oxalate (mg/ml)	Phosphate (mg/ml)	Calcium (μmol/ml)	Diuresis (ml/day)	Creatinine (mmol/day)
Initial level					
	-	9.0±0.20 (n=15)	2.2±0.10 (n=15)	4.0±0.49 (n=15)	5.8±0.52 (n=15)
Experimental nephrolithiasis					
3	1.5±0.13 (n=15)	9.4±0.91 (n=13)	2.1±0.18 (n=15)	3.5±0.56 (n=15)	5.9±0.54 (n=15)
7	1.8±0.19 (n=11)	8.6±0.20 (n=12)	1.4±0.13* (n=15)	4.1±0.16 (n=15)	6.5±0.69 (n=14)
10	1.6±0.11 (n=15)	7.6±0.15 (n=15)	1.8±0.08* (n=15)	4.3±0.72 (n=15)	4.9±0.52 (n=14)
14	1.6±0.12 (n=13)	7.7±0.31 (n=10)	1.8±0.17* (n=12)	4.2±0.73 (n=14)	6.6±0.63 (n=14)
17	1.4±0.09 (n=15)	8.3±0.36 (n=15)	1.5±0.06* (n=15)	5.3±0.84 (n=15)	7.2±0.77 (n=15)
21	1.5±0.11 (n=12)	11.3±0.28* (n=14)	2.0±0.21 (n=13)	4.1±0.67 (n=15)	7.1±0.52 (n=15)
Treatment with the new PK-BMS					
24	1.2±0.10 (n=14)	11.5±0.46* (n=14)	1.1±0.05* (n=14)	4.5±0.64 (n=14)	6.4±0.78 (n=14)
28	1.0±0.07 (n=14)	10.8±0.40* (n=14)	2.4±0.16 (n=14)	5.9±0.93 (n=14)	8.3±0.95* (n=14)
31	1.1±0.09 (n=15)	11.2±0.38* (n=15)	1.6±0.06* (n=15)	4.7±0.69 (n=15)	6.4±0.92 (n=15)
35	1.1±0.05 (n=14)	11.4±0.32* (n=15)	1.5±0.09* (n=15)	4.8±0.39 (n=15)	7.1±0.63 (n=15)
38	0.9±0.07 (n=12)	11.5±0.34* (n=15)	1.2±0.06* (n=15)	5.4±0.73 (n=15)	7.8±0.90 (n=15)
42	1.0±0.08 (n=15)	11.2±0.41* (n=15)	1.3±0.06* (n=15)	5.2±0.83 (n=15)	7.3±0.84 (n=15)

**- P<0.05 in relation to the initial level*

These symptoms included urine supersaturation with oxalate ions and an increase of fermenturia that led to increased levels of LDH- and NAG activity in the urine by 2.1 and 1.6 times, respectively (*P<0.001* in both cases). Nevertheless, the new PK-BMS intake resulted in a significant alleviation of the experimental nephrolithiasis (Table 4). Thus, the level of oxalate ions in the urine decreased by 1.3-1.5 times in relation to the 21st-day level. In addition, the urine enzyme activity significantly decreased, and by the end of the experiment, LDH activity was 2.1 lower than on the initial level. The same dynamics were observed in relation to NAG activity (Table 5). Furthermore, we discovered significant changes in the development of oxidative stress in the kidney tissue. By the end of the course of intake of the new biomedical substance from pig kidneys, the concentration of TBRP decreased compared to DCG by 3.7 times (*P<0.001*), while the activity of all antioxidant enzymes increased significantly compared to their levels in DCG and healthy animals (Table 5). Finally, according to the results of morphometry, the number of calcium deposits forming in the area of renal papillae of the treated rats decreased by 12.5 times (from 27.4±3.22 to 2.2±2.00 per field of view) in comparison with DCG, and their average size decreased by 3.2 times (from 12.0±0.62 μm to 3.7±3.55 μm; *P<0.001*).

Table 5.

Enzyme activity in the urine of TG rats

Days	LDH	GGT	NAG ($\times 10^{-3}$)
	U/mg creatinine per day		
Initial level			
	0.21±0.023 (n=11)	0.33±0.015 (n=10)	9.8±0.46 (n=15)
Experimental nephrolithiasis			
7	0.35±0.025 (n=14)	0.36±0.015 (n=14)	10.5±0.48 (n=14)
14	0.44±0.034 (n=14)	0.42±0.014 (n=14)	15.3±0.97 (n=14)
21	0.45±0.013 (*P<0.001*; n=13)	0.36±0.011 (n=8)	Not determined
Treatment with the new PK-BMS			
28	0.36±0.020 (n=14)	0.38±0.016 (n=14)	7.3±0.33 (n=14)
35	0.09±0.014 (*P<0.001*; n=15)	0.36±0.018 (n=13)	6.5±0.52 (n=15)
42	0.10±0.008 (*P<0.001*; n=15)	0.37±0.013 (n=14)	8.5±0.37 (n=15)

P - in relation to the initial level

Thus, the obtained results during the conducted experiments demonstrated that the new PK-BMS had a high anti-lithogenic activity. Also, it should be noted that there is a multipurpose nature of the therapeutic effect of this substance at experimental nephrolithiasis. Such components of oxalate nephrolithiasis pathogenesis as oxidative stress in kidney tissue, damage of urothelium, urine supersaturation with lithogenic ions and crystallization of renal concrements can also be the targets for correction. All of these resulted in a significant decrease of the number and sizes of calcium deposits in the kidneys of experimental rats.

It is entirely possible that the multipurpose effect of the new PK-BMS is determined by the complex of its biomedical properties depending on the organic structure of pig kidneys. The research of these properties and the search for the active ingredient of this substance will be the object of our further investigations. Currently, with some certainty it is possible to suppose that the technological processing of pig kidney tissues using the gentle methods does not decrease their anti-lithogenic activity, which creates favorable conditions for the development of the optimized technological production scheme for the new anti-lithogenic drugs.

In conclusion, it was established that during the experimental nephrolithiasis, a three-week intake of the new biomedical substance from pig kidneys is accompanied by a significant anti-lithogenic effect equal to the raw material from pig kidneys.

Competing interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

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Effects of Heart Rate on the Pump Function and Electrophysiological Characteristics of the Heart in the Frog *Rana temporaria*

Natalya A. Kibler, PhD[1*]; Vladimir P. Nuzhny, PhD, ScD[1]; Svetlana V. Achmetzhynova[2]; Dmitry N. Shmakov[1]

[1]*Institute of Physiology, Komi Science Center, Urals Branch of the RAS*
[2]*Syktyvkar State University named after Pitirim Sorokin*
Syktyvkar, Komi Republic, the Russian Federation

Abstract

The aim of the study was to investigate the electrical activity and contractility of the heart ventricle in frogs *Rana temporaria* (n=14) under different heart rates. The activation time (AT, as dV/dt_{min} during QRS complex), the repolarization time (RT, as dV/dt_{max} during ST-T wave), and the activation-recovery intervals (ARIs, as difference between RT and AT) were measured. The hemodynamic variables were determined with the Prucka MacLab 2000 system. Heart rate (HR) was changed by the use of right atrium pacing from 0.6 Hz to 1.1 Hz with step 0.1 Hz. The increasing HR from 0.6 Hz to 1.1 Hz led to the increased duration of ARIs on the ventral and dorsal fragments of ventricular epicardium as compared with initial sinus rhythm. During the high HR, more prolonged ARIs were observed on the ventral side of the epicardium than on the dorsal surface (exclusion is supraventricular rhythm with rate of 1.1 Hz). The repolarization dispersion of epicardium on the whole, as well as repolarization of both epicardial sides separately, decreased under the higher rate. Repolarization sequence depended on the activation sequence and the distribution of local repolarization durations only at supraventricular rhythm with a frequency of 1.1 Hz. The indexes of pump function decreased under high HR. Thus, the increased HR resulted in a decrease in the dispersion of repolarization and ARIs; the repolarization duration of ventricular epicardium at supraventricular rhythms was shortened as compared with sinus rhythm. During an increase in HR, repolarization sequence is formed in association with the level of ARI dispersion and changes of the repolarization duration. **(Int J Biomed. 2017; 7(1):46-50.)**

Key Words: ventricular pump function • heart rate • repolarization • activation-recovery intervals • frog *Rana temporaria.*

Abbreviations

CO, cardiac output; **EDP**, end-diastolic pressure; **HR**, heart rate; **LV**, left ventricle; **LA**, left atrium; **RA**, right atrium; **SV**, stroke volume; **VE**, ventricular epicardium; **VEDV**, ventricular end-diastolic volume.

Introduction

The structural and hemodynamic changes during the evolution of the vertebrate heart are not reflected in obvious changes in the overall electrical patterning of the heart.[1] The total duration of the electrical events of a given heart beat is shorter in endothermic birds and mammals with a high HR [1,2] than in ectothermic vertebrates.[3,4] This difference in HR is connected with a low degree evolutionary development of cardiac conduction system (CCS) among groups of poikilotherm vertebrates;[5,1] as a result, ectothermic animals are unable to develop a high HR in nature.[1]

High HR leads to the mechanical changes of the indexes of cardiac pump function in endothermic animals. Early investigations showed that rising HR resulted in the elevation of LV preload and pressure in LA and a decrease in systolic LV pressure and isovolumic indexes (dP/dt_{max}, dP/dt_{min}), LVEDV and CO in dogs.[6,7] However, an increased HR resulted in increasing the isovolumic indexes [8] and decreasing SV,

***Corresponding author:** Natalya A. Kibler., PhD; Laboratory of Cardiac Physiology, Institute of Physiology, Komi Science Center, Ural Branch of the RAS, Syktyvkar, Komi Republic, Russia. E-mail: natanadya@mail.ru*

LVEDV, and LV preload in pigs.[9] In the lowest vertebrates, a functional organization of the ventricular myocardium under electrical stimulation at different frequencies has not been sufficiently studied. It should be noted that early data about pump function and electrical properties were based on specific anatomo-physiological cardiac characteristics in amphibians (presence of the single ventricle, poor development of CCS, sequential type of the activation of ventricular myocardium, low HR and others) in conditions of natural habitat.

The dependence of heart contractility on HR[10] is a very important cardiac inotropic mechanism of the majority of species of animals; therefore, amphibians are a convenient model for research of the features of changes in the electrical properties and cardiac contractile function. The study of the electrophysiological and contractile properties of the ventricle in ectothermic vertebrates **(**amphibians) with specific structural cardiac organization is necessary for understanding the patterns of cardiac evolution and adaptation of the heart to different habitats of these animals. The analysis of the electrophysiological and hemodynamic cardiac properties under different HRs will promote the further clarification of the adaptation mechanisms of the lowest ectothermic vertebrates to changing environmental conditions.

The aim of the study was to investigate the electrical activity and contractility of the heart ventricle in frogs *Rana temporaria* under different HRs.

Material and methods

2.1. Animals and surgical procedure

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Experiments were performed on 14 adult (2-3 years old) frogs (*Rana temporaria*) of both sexes weighing from 34g to 47g. All animals demonstrated normal sinus rhythm on an ECG. The frogs were anaesthetized by placing them for 3 minutes in a jar containing 40% ethanol. After that the ventral thoracic wall was removed and the pericardium was cut open.

During the experiment, the body temperature of each animal was in the range of 18 to 20 ºC and the heart was flushed with warm Ringer's solution (18-20ºC). In the laboratory, their body temperature equilibrated rapidly with the ambient temperature that corresponds to the data of other researchers. At the end of the experiment, the animals were euthanized by the intravenous injection of an overdose of alcoholic solution.

2.2. Hemodynamic recording

The hemodynamic variables were determined with the Prucka MacLab 2000 system (GE Medical System, GmbH). The pressure in the ventricle was measured with a catheter (internal diameter, 1 mm) filled with the heparinized 0.9% saline inserted via the free wall into the ventricle cavity. Invasive monitoring of the pressure was carried out using transducers, transforming blood pressure inside of the vessels as the transducer registered mechanical changes.

Heart performance was evaluated as follows: inotropism was evaluated in terms of maximal systolic ventricular pressure (MSVP in mmHg, index of contractile activity) and maximal value of the MSVP derivative [$+(dP/dt)_{max}$, mmHg/s; index of maximal ventricle contraction rate]]. Lusitropism was assessed on the basis of the maximal rate of MSVP decline [$-(dP/dt)_{min}$, mmHg/s]] and EDP (mmHg).

2.3. Electrocardiographic recording and analysis

Standard bipolar limb lead electrocardiograms were recorded with an application of subcutaneous steel needle electrodes. Registration of an epicardial electrogram was performed by using a matrix (5mm x 5mm) containing 64 electrodes at the sinoatrial and supraventricular rhythms. The matrix was alternately superimposed on the central portion of the ventral and dorsal sides of the epicardium, in such a way that the cephalic border of the matrix grasped the basal part and the inferior border – part of the ventricular apex.

In every epicardial lead, the activation time (AT), the repolarization time (RT), and the activation-recovery intervals (ARIs) were obtained. The latter was used to assess local repolarization durations. AT, RT and ARIs were determined as dV/dt_{min} during QRS complex, dV/dt_{max} during ST-T wave, and the difference between RT and AT, respectively. [15] The values were determined automatically, inspected by the observer and corrected manually if necessary. In each set of simultaneously recorded electrograms, the beginning of the QRS complex in the II limb lead was chosen as a reference time point with respect to which ATs and RTs were measured in a given set of electrograms.

The dispersion of ATs, ARIs, and RTs of the ventricle were taken as the difference between the maximal and minimal AT, RT and ARI values in a set of recorded electrograms, respectively.

In order to construct isochronal activation maps, the zero point was assigned to the timing of the epicardial activation breakthrough. Similarly, the zero points in the repolarization maps identify for the earliest repolarization on the epicardium. At the end of the experiment, the heart was removed for weight measurements.

2.4. Stimulation

HR was changed by the use of right atrium pacing from 0.6 to 1.1 Hz with step 0.1 Hz. RA was stimulated (supraventricular rhythm) with cathodic impulses (duration of 1 ms; twice diastolic threshold) at a cycle length 1200 ms. Amplitude of impulses was 4-8 V. The duration of the pacing period was 1 min.

2.5. Statistical examination was done using statistical package Primer of Biostatics 4.03 and SPSS 11.5 using Wilcoxon test for paired comparisons and Fridman test followed by the Wilcoxon test with Bonferroni correction for multiple comparisons. Values are given as M±SD. A probability value of $P<0.05$ was considered statistically significant.

Results

I. Ventricular epicardial activation, repolarization and ARIs under the spontaneous sinus rhythm

At the sinus rhythm, HR was 38±7 bpm and duration of QRS complex - 78.2±7.4 ms. The depolarization wave of VE on the ventral and dorsal parts spread from the base (20.4±3.7

ms and 27.4±2.1 ms, respectively) to apex (36.6±6.0 ms and 42.2±7.3 ms; *P<0.05*), and from the right (24.5±4.2 ms) to the left (38.6±5.3 ms; *P<0.05*) on the ventral side.

Repolarization sequence on the ventricular epicardial surface of both sides was similar to the activation sequence; however, the apicobasal gradient was not statistically significant. Under sinus rhythm, dispersions of activation and repolarization increased in the dorsal-to-ventral direction (*P<0.05)* (Table 1). With that, ARIs were increased on the ventral side of VE as compared with dorsal side *(P<0.05)*. On the ventral side, ARIs were significantly longer on the left part of VE than on its right part (894.8±204.2 ms vs. 813.2±193.0 ms; *P<0.05*). Dispersion of ARIs was increased in the dorsal-to-ventral direction.

II. Hemodynamics, ventricular epicardial activation, repolarization and ARIs under the electrical stimulation of RA

Duration of the QRS complex did not significantly change under the increase in HR from 0.6 Hz to 1.1 Hz, but the duration of the QT interval was decreased as compared with initial sinus rhythm. The indexes of the pump function (SPV, dP/dt_{max}, and dP/dt_{min}) are presented in Table 2. During RA pacing (1.1 Hz), the activation dispersion (duration of activation) was increased *(P<0.05*) on the dorsal side of VE (Table 1). Under the increase in HR from 0.6 Hz to 0.9 Hz, the difference in time depolarization was insignificant between the ventral and dorsal sides of VE. However, during the next increase in HR up to 1.1 Hz, the ventral side was depolarizated earlier as compared with dorsal side *(P<0.05*)

Table 1.
Effect of HR on the dispersion of AT, ARI, and RT (n=14)

Parameters		Sinus rhythm	HR (Hz) / Duration of cardiac cycle (ms)					
			0.6 / 1570	0.7 / 1360	0.8 / 1250	0.9 / 1140	1.0 / 1000	1.1 /937
HR, bpm		38±7	38	44	48	55	60	64
Dispersion of ATs	GVD	85.2±20.0	74.5±14.6	86.4±25.7	77.8±17.4	80.2±25.6	63.4±14.1	88.3±23.3
	Ventral side	97.4±18.7^	80.6±23.6	105.4±32.2	63.4±21.4	94.9±25.3	74.2±11.0	70.7±20.3^
	Dorsal side	73.3±25.3	70.3±20.3	87.1±16.9	95.6±22.4	67.3±18.4	55.4±11.3	106.8±29.2*
Dispersion of RTs	GVD	584.5±181.3	485.0±174.6	422.2±142.3	273.4±105.6*	289.7±85.8*	204.4±76.2*	241.3±108.4*
	Ventral side	600.2±113.7^	627.7±153.6	501.1±107.4	215.2±53.4*	293.5±72.2*	194.7±85.8*	204.6±103.2*
	Dorsal side	569.6±94.4	576.4±105.9	429.5±102. 6	215.6±89.0*	285.7±96.8*	211.0±81.7*	277.6±88.3*
Dispersion of ARIs	GVD	599.2±133.7	451.6±106.4	452.4±153.6	326.2±111.7	295.2±82.0*	217.3±73.4*	259.4±109.6*
	Ventral side	611.1±109.6^	408.3±102.2	322.1±92.0	355.4±104.5	293.5±84.9*	184.7±44.9*	203.0±51.1*
	Dorsal side	585.0±106.7	484.3±117.1	354.0±107.2	322.1±110.9	299.4±74.5*	242.6±73.7*	315.7±72.9*
Duration ARIs	GDV ARI	800.4±33.9	617.2±27.0*	528.7±40.5*	386.3±81.0*	290.2±73.9*	274.3±12.8*	273.7±19.6*
	Ventral side	828.6±96.2^	640.0±98.0^*	565.2±85.1^*	308.3±96.4^*	352.5±91.9^*	279.7±100.0^*	259.4±90.2 ^*
	Dorsal side	772.2±101.1	593.4±107.1*	492.1±94.0*	463.0±112.4*	291.4±100.9*	269.3±104.0*	288.7±95.0*

GVD - General ventricular dispersion**;** GDV ARI **-** General duration ventricular ARI; ^ *P<0.05* values vs dorsal side; **P<0.05* values vs sinus rhythm.

Table 2.
Haemodynamic indexes, electric parameters and cardiac mass under the i*ncrease in HR*

Parameters	Sinus rhythm	HR (Hz) / Duration of cardiac cycle (ms)					
		0.6 / 1570	0.7 / 1360	0.8 / 1250	0.9 / 1140	1.0 / 1000	1.1 /937
HR, bpm	38±7	38	44	48	55	60	64
MSVP, mmHg	24.3±3.4	18.5±3.4*	16.2±5.0*	15.4±5.3*	14.6±4.8*	14.7±5.9*	14.4±5.3*
EDP, mmHg	1.7±0.5	1.0±0.2	1.1±0.5	1.1±0.6	1.3±0.4	1.4±0.2	1.4±0.3
$+dP/dt_{max}$, mmHg/s	126.5±36.7	83.2±16.7*	83.4±20.3*	83.2±21.9*	82.2±19.8*	81.1±16.6*	73.7±17.9*
$-dP/dt_{min}$, mmHg/s	110.2±38.7	86.2±21.7*	83.4±18.5*	68.2±13.5*	67.2±15.4*	61.2±17.6*	61.7±17.8*
QRS, ms	78.2±7.4	80.2±9.9	80.0±8.2	82.3±9.4	82.4±11.1	82.3±10.7	80.9±15.3
QT, ms	959.4±62.2	924.6±100.7	736.6±113.3*	624.2±89.8*	555.4±86.9*	488.7±33.2*	428.1±59.4*
Mv, mg	93.5±17.2						
Cv, mg	121.0±18.6						

**P<0.05 – values vs sinus rhythm; Mv - ventricular mass; Cv - cardiac mass.*

Table 3.

Correlation (r) between the processes of depolarization and repolarization of VE in frogs

Parameters	Sinus rhythm	HR (Hz) / Duration of cardiac cycle (ms)					
		0.6 / 1570	0.7 / 1360	0.8 / 1250	0.9 / 1140	1.0 / 1000	1.1 /937
HR, bpm	38±7	38	44	48	55	60	64
AT - RT	r=0.072	r=0.043	r=0.021	r=-0.076	r=0.034	r=-0.033	r=0.581 *P<0.05*
AT - ARI	r=0.074	r=-578 *P<0.05*	r=-0.031	r=-0.567 P<0.05	r=-0.561 *P<0.05*	r=-0.551 *P<0.05*	r=-0.012
RT - ARI	r=0.9996 *P<0.001*	r=0.984 *P<0.001*	r=0.998 *P<0.001*	r=0.944 *P<0.001*	r=0.970 *P<0.001*	r=0.964 *P<0.001*	r=0.941 *P<0.001*

The repolarization dispersion of epicardium on the whole, as well as repolarization of both epicardial sides separately, decreased under the higher rate **(***P<0.05***)**. The increasing HR from 0.6 Hz to 1.1 Hz led to the increased duration of ARIs on the ventral and dorsal fragments of VE as compared with initial sinus rhythm (Table 1). Additionally, the higher rate resulted in a different dorso-ventral distribution of the repolarization durations. We observed the shortest durations (*P<0.05*) of ARIs on the dorsal fragment of VE compared to ventral side under the higher rate (1.1 Hz); repolarization duration decreased on VE of the frogs (Table 1).

Thus, the increased HR resulted in a decrease in the dispersion of repolarization and ARIs; the repolarization duration of VE at supraventricular rhythms was shortened as compared with sinus rhythm. During the high HR, more prolonged ARIs were observed on the ventral side of the epicardium than on the dorsal surface (exclusion is supraventricular rhythm with rate of 1.1 Hz). The indexes of pump function decreased under high HR.

Under the sinus rhythm, we found a high positive correlation between the end of repolarization timing and the ARI duration, and the negative dependence of these parameters on AT (Table 3). Under HRs from 0.6 Hz to 1.1 Hz, we found a positive correlation between the end of repolarization timing and durations of ARIs. Also, we observed a relationship between the time of depolarization and the end of repolarization timing at an HR of 1.1 Hz. At the same time, we observed an inverse relationship between the AT and duration of repolarization at HRs of 0.6 and 0.8-1 Hz.

Thus, under high HR, the repolarization sequence in VE of frogs does not repeat the activation sequence, especially, during sinus rhythm, and depends on the ARI distribution. An HR of 1.1 Hz is exclusion, where the repolarization sequence depends on the depolarization sequence and repolarization duration. During an increase in HR, the repolarization sequence is formed in association with the level of ARI dispersion and changes of the repolarization duration. The indexes of pump function decrease under high HR.

Discussion

HR in amphibians is directly related to the ambient temperature, and provides a fair estimate for the influence of ambient temperature on metabolism,[13,16] as compared with endothermic vertebrates, when a high HR is a pivotal requirement for the high cardiac output needed to sustain the high metabolism associated with endothermy.[1,2] In our research, the experiments were performed at 18-20 ºC. It was shown, that all cardiomyocytes have already been activated at 18-20°C, which corresponds to the preferred activity temperature of this species ectothermic vertebrates.[17]

In our study in frogs with increasing HR, we identified a slight increase in VEDP, and a significant decrease in systolic blood pressure and isovolumic indexes. According to R.Berger, the rapid atrial pacing of the heart led to potentially important mechanical alterations in canine heart.[6] For example, as heart rate increases, atrial contraction occurs earlier in diastole and can become synchronous with the onset of LV filling. This could augment initial diastolic filling pressures and thereby influence pressures throughout a rate-limited diastolic period. Thus, the increased diastolic blood pressure in mammals as the HR increased led to the deterioration of coronary reserve and high energy phosphate metabolism, and, consequently, dysfunction of Ca2+ homeostasis in cardiomyocytes.[6]

It is known that regulation of the functional activity of the myocardium, rhythm and strength of heart contractions in frogs and mammals, is carried out with the participation of Ca2+, which generates a signal that triggers excitation and contraction of the heart.

Intracellular Ca2+ concentration is subjected to significant fluctuations, which has a modulating effect on the strength of heart contractions and HR.[18] This effect is realized through a change in the action potential (AP) aimed at unloading cardiomyocytes at the excess load. It is possible that high HR in our study blocked Ca2+ - canals in cardiomyocytes during diastole, which has been accompanied by changes in the structural and functional organization of myocardium, directed at the support of vital activity in more extreme environmental conditions. As a result, the AP amplitude drops and myocardial activity decreases during systole, the value of which, in lower vertebrates, is determined by Ca2+ predominantly.[18] Therefore, we can assume that an increase in HR in frogs at poor development of Ca2+ channels of T-type and sarcoplasmic reticulum may be an indirect cause of reduction in cardiac isovolumic indexes and duration and dispersion of ARIs of VE, that we observed in our study. The Na+/Ca2+-mechanism in frog cardiomyocytes operates to remove Ca2+ from cardiomyocytes.[18] Under increased HR beyond the

physiological norm for frogs, this mechanism seems to stop working, resulting in the phenomenon of dedifferentiation of cardiomyocytes and switches their activities to the fetal phenotype. The above-mentioned situation was observed in our study: the repolarization sequence depended on the activation sequence and the distribution of local repolarization durations only at supraventricular rhythm with a frequency of 1.1 Hz.

The HR variability should be considered as a factor that provides the heart function in extreme conditions beyond the usual habitat of the organism. At the same time, we have shown that an increase in heart rate in amphibians is not accompanied by an increase in myocardial contractility. The natural variability of HR as a factor in increasing the contractile function is realized through an increase in the heterogeneity of the myocardium, whereas atrial artificial stimulation reduces the electrical heterogeneity of the myocardium. Most likely, the electric homogeneity of the myocardium is the reason for the change of contractile properties of the heart.

Thus, the increase in HR in amphibians is limited by a physiologically reasonable limit, specific to a particular type of animal, beyond which, the heart activity becomes ineffective and is accompanied by structural and functional remodeling. Also, initially, the structure and heterogeneity of the myocardium determine the maximal and functional properties of the heart in each cycle and the level of security of maximum HR.

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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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Biosafety Assessment of Microbial Strains Used in Biotechnology According to Their Taxonomy

Natalia I. Sheina, PhD, ScD[1]*; Elena V. Budanova, PhD[2]; Yuri P. Pivovarov, PhD, ScD[1]; Lyubov I. Mjalina, PhD[1]; Lyubov P. Sazonova, PhD[1]

[1]*N.I. Pirogov Russian National Research Medical University, Moscow, Russia*
[2]*I.M. Sechenov First Moscow State Medical University, Moscow, Russia*

Abstract

A great variety of biotechnological products are now widely used in different ways in agriculture, medicine, food manufacturing and other areas of our life. Industrialized societies now more than ever depend on the use of genetically engineered products, with many of them synthesized using recombinant strains of microorganisms. There is an opinion that microbial strains used in biotechnology are potentially harmful for human health and the environment. Similar to many other countries, we have enacted environmental legislation in an effort to balance the risks and benefits of using biotechnological strains. Although environmental monitoring rules focus mainly on safety assessments of chemicals, the biosafety assessment of microbial strains used in biotechnology is a very important issue as well.

This article summarizes 15 years of research on the biotechnological strains of microbes widely used as producers of various biological substances for industrial purposes, and their environmental and biotechnological applications. In our survey, we tried to evaluate possible adverse effects (general toxicity and damage to the immune system, potential sensitizing effects, and damage to normal microbiota) caused by these microbes. It was shown that microscopical fungi of genera *Aspergillus*, *Penicillium* and *Candida*, and some gram-negative bacteria can affect the immune system and disrupt the normal balance of microbial flora of the intestinal tract in rats. The actinomycetes are less dangerous in that they cause fewer side effects. The investigation data obtained can be used to develop safety and hygienic standards for industrial microbes that will help decrease or minimize the occupational risk of infection or damage to the immune system when working with biotechnological strains of microbes. **(Int J Biomed. 2017; 7(1):51-56.)**

Key Words: biotechnology • microbial strains • biosafety standards • hygiene regulations

Introduction

Without any doubt, biotechnology and numerous biotechnological products are valuable for medicine, veterinary science, agriculture, the food industry and other spheres of our life. Industrialized societies now more than ever depend on the use of genetically engineered products. Achievements of modern genetic engineering and molecular biology promote expansion of a wide range of the microbial strains used in the industry.

However, there is a concern that microbial strains used in biotechnology are potentially hazardous for human health and can increase the risk of biological environmental pollution.[1-3] Numerous investigations in Russia testify to the extensive influence of biotechnological strains on human health represented by allergic diseases of the respiratory organs, skin, and other immune disorders; and disruption of the normal flora balance of the body common to people working in the microbiological industry and living in residential areas, and even to those people who simply use some products of biotechnology.

Determining the safety of biotechnological strains of microorganisms and assessing the potential risk of the emission of 'strain producers' and their effect on the population can help prevent the technogenic pollution of residential areas, and it is evidently an extremely important issue. Many countries have enacted environmental legislation in an effort to balance the risks and benefits of using chemicals and biotechnological strains. Many surveys in Russia have investigated potential adverse effects of industrial strains of microbes.[4-6] These

***Corresponding author:** Prof. Natalia I. Sheina, Department of Hygiene, N.I. Pirogov Russian National Research Medical University, Moscow, Russia. E-mail: ni_sheina@mail.ru*

studies help develop safety and hygienic standards.

The results of research into the nature of harmful effects of industrial microorganisms helped outline the main stages and the scheme for researching hygienic rationing of microorganisms. However, these studies were developed without considering biological features of different taxonomic groups of microorganisms. The principles of hygienic standardization require a comprehensive approach and improvement of methodology. Our 15-year research was aimed at studying possible adverse effects of different taxonomic groups of biotechnological strains of microbes experimentally.

Material and methods

We have tested 32 strains of microorganisms applied in biotechnology as producers of a variety of biological substances. They included members of different taxonomic groups: gram-positive and gram-negative bacteria, actinomycetes, molds, and yeasts (Table 1).

The experiments were carried out on conventional male and female white mice (20-25 g, body weight) and Wistar rats (290-320 g, body weight). Each test and control group included 8 animals. The Institutional Ethical Committee of Animal Care and Use of Pirogov Russian National Research Medical University approved all procedures involving animals. After finishing each experiment, the animals were euthanized to prevent unnecessary suffering according to recommendations of the Ethical Committee of Animal Care and Use of Pirogov Russian National Research Medical University using an overdose of sodium pentothal.

To determine the virulence of biotechnological strains, we injected each species (mouse/rat) intraperitoneally with a dose of microbes equal to DV_{50} and examined the ability of the microbes to reach the bloodstream within 30 min after inoculation. The possible risk of microbes causing a generalized effect was evaluated by inoculation of culture media with 'fingerprints' of kidney, liver and spleen of animals on the 3rd, 8th, 15th and 22nd day after injection of the strain tested. [6,7]

Table 1.

Taxonomy of biotechnological strains of microorganisms used in Russia for industrial purposes and their characteristics

Strain	Activity/application
1. **Gram-positive bacteria:** *Bacillus subtilis* 65, *B.subtilis* 72, *B.subtilis* 103, *B. subtilis subsp. subtilis* KO-1 BKM B-2716 D, *B.licheniformis* 60, *B.licheniformis* 103, *B.licheniformis* B-9608, *B.licheniformis* KO-2 BKM B-2717D, *B.amiloliquefaciens* B-1029	Produce full-range complex of heat-stable amylolytic and proteolytic enzymes
Bacillus licheniformis 1001	Bacitracin-producing bacterium
Bacillus thuringiensis	Component of insecticide
Lactobacillus casei 5-1/8, *L. plantarum* 435, *Micrococcus varians* 80	Bacteria are used as 'fermenting agents' in meat product manufacturing
2. **Gram-negative bacteria:** *Alcaligenes denitrificans* C-32	Produces nitrilase
Pseudomonas caryophylii KM 92-102/1	Stirol destructor
3. **Actinomycetes** *Rhodococcus corallinus*	Purification of tobacco wastes
Rhodococcus erythropolis КД	Purification of environment from oil pollution
Streptomyces aureofaciens 777	Produces Biovit and Chlortetracycline for veterinary use
Streptomyces avermitilis 3NN	Produces Avermectin, the broad-spectrum antiparasitic agent
Streptomyces fradiae БС-1	Produces tylosin used in veterinary medicine
4. **Micromycetes and Yeasts** *Candida tropicalis* Y-456	Xylitol producer
Yarrowia lipolytica Y3323	Produces lipase
Tolypocladium cylindrosporum	Component of insecticide
Aspergillus terreus 44-62	Produces lovastatin
A.awamori 120/177, *A.awamori* Nakazawa ВУД Т-2 1000-У	Produce glucoamylase
P. funiculosum 18.2, *P.funiculosum* BKM F-3668D, *P.verruculosum* PV2007 BKM F-3972D	Produce a group of carbohydrases
Penicillium funiculosum F-149	Produces dextranase
Penicillium canescens PhPl33 BKM F3867	Produces pectin lyase (pectolyase) used in food industries and phytase used as an animal feed supplement
Trichoderma viride 44-11-62/3, *T. longibrachiatum* TW-1, *T. longibrachiatum* TW-420 BKM F-3880D	Producers of a range of cellulolytic enzymes, β-glucanase and xylanase
Trichoderma reesei 18.2K	Produces Celloviridin G20X used as an animal feed supplement

The general toxicity of each microbial strain in intranasal inhalation of animals within one month (with concentration of microbial suspension from 10^3 to 10^8 CFU/m^3) was determined by monitoring the body weight gained or lost, behavioral reactions of the animals, and total weight of internal organs upon termination of the experiment. To determine the dose of inoculation we used the following formula [8]:

$$C = \frac{D}{m \times t \times v \times 10^6}$$

C – the number of microbial cells per 1 m^3 of ambient air measured in CFU/m^3;

D - the number of microbial cells inoculated to animal measured in CFU per animal;

m – weight of the animal, g;

t – time of exposure, in minutes (it is equal to 240 and 120 min per day for rats and mice, respectively);

v – the pulmonary ventilation rate expressed as cm^3/(g x min) (it is taken as 0.65 and 1.24 for rats and mice, respectively);

10^6 – cm^3 to m^3 conversion coefficient.

The experiments concerning possible adverse effects to the immune system included measuring the total weight of immunocompetent organs, calculating leukocytes in Giemsa-stained blood smears, and also detecting and counting the main populations of T- and B-lymphocytes.[6-8]

The sensitizing effect on the development of delayed Type IV hypersensitivity reaction was demonstrated by means of inoculation of 10ml of sonicated microbes in a concentration of 10^4 CFU/ml in Freund's adjuvant (1:1). The inoculation was given under the aponeurosis of the animals' back feet with subsequent measurement of thickness of both back feet of each animal to detect possible edema due to allergic reaction. The immediate type of hypersensitivity reaction was shown by the effect of degranulation of mast cells taken from peritoneal exudates of rats. [7,9]

We conducted a bacteriological examination of feces to demonstrate possible adverse effects to gut microflora by inoculating 10-fold dilutions of animal feces in sterile saline onto a set of general-purpose and selective culture media for Enterobacteriaceae members, staphylococci, enterococci, clostridia, bifidobacteria, lactobacilli, and fungi, with subsequent identification of the genus of the microbes. [10,11] The specimens were taken immediately after the animals received microbial strain inoculation and 2 weeks thereafter.

The results of experiments were analyzed with simple t-test using Statistica (v.6.0, Stat Soft, USA) and Microsoft Office Excel 2007. Results were considered statistically significant when $P<0.05$. [12]

Results and Discussion

Our experiments have shown that the biotechnological strains we tested were not virulent, toxigenic or dangerous for warm-blooded animals. The virulence of microbes examined is also very low and LD_{50} is higher than 10^{12} CFU/animal (Table 2).

Table 2.

Virulence of biotechnological strains of microorganisms in intraperitoneal injections to rats

Microorganisms	LD50, lg CFU/ml	Threshold dose (limit dose), lg CFU/ml	Isolation from internal organs	
			Dose, lg CFU/ml	TAI, days
Gram-positive bacteria *(Bacillus spp.), Actinomycetes (Rhodococcus spp., Streptomyces spp.)*	>12.0	10.0 - 11.0	9.0 - 12.0	2
Gram-negative bacteria *(Alcaligenes sp., Pseudomonas sp., E. coli)*	>12.0	8.0 - 9.0	8.0 - 12.0	5
Micromycetes and Yeasts *(Aspergillus spp. Penicillium spp., Tolypocladium sp., Candida sp.)*	>12.0	9.0 - 10.0	9.0 - 12.0	8

TAI- time after inoculation

The invasiveness of gram-positive bacteria, actinomycetes, and spores of microscopic fungi is low; they do not multiply in internal organs of rodents and cannot persist in the host organism. The limit dose of these taxonomic groups of microbes that induced their translocation into blood at intraperitoneal injection was also very high and amounted to 10^9 CFU of fungal spores per animal, 10^{10} CFU/animal for actinomycetes, and 10^{11} CFU/animal for gram-positive bacteria.

As for gram-negative bacteria, their invasiveness seems to be relatively higher and the dose of microbes that caused bacteriaemia was about 10^8-10^9 CFU/animal (Table 2). We isolated colonies of gram-positive bacteria and actinomycetes from internal organs (kidneys, liver and spleen) within 2 days after inoculation, unlike gram-negative bacteria and fungi that continue to persist for up to 8 days. This fact testifies to the low invasive properties of these microbes.

In 'subchronic experiments' there was no visible toxic effect if the concentrations of microbes were inoculated for one month. Long-term inhalation of microorganisms tested may result in a mild immonotropic effect or light changes in the composition and concentration of normal intestinal flora habitants. Such effect was caused by the taxonomic group of microbes.[13-39]

In the case of a hygienic regulation of industrial microorganisms, it is required to define a possible sensitizing effect as the limiting criterion of harmful action. Our experiments have shown that 75% of examined bacteria, 67% of actinomycetes, and 80% of fungi (micromycetes) possess a sensitizing effect. Molds and yeasts, for example, demonstrated an immediate type of hypersensitivity that was dose-dependent. *Penicillium funiculosum* and *Aspergillus awamori* in concentrations of $2x10^4$ and $2x10^5$ CFU/m^3, respectively, caused intense degranulation of mast cells (Figure 1). *Bacillus licheniformis* caused a similar effect only if they reached the concentration of $5x10^6$ CFU/m^3 (Table 3).

The delayed Type IV hypersensitivity was typical for *Bacillus licheniformis*, *Pseudomonas caryophylii, Alcaligenes*

denitrificans and micromycetes in concentrations similar to immediate reactions. Actinomycetes did not show any sensitizing effect.

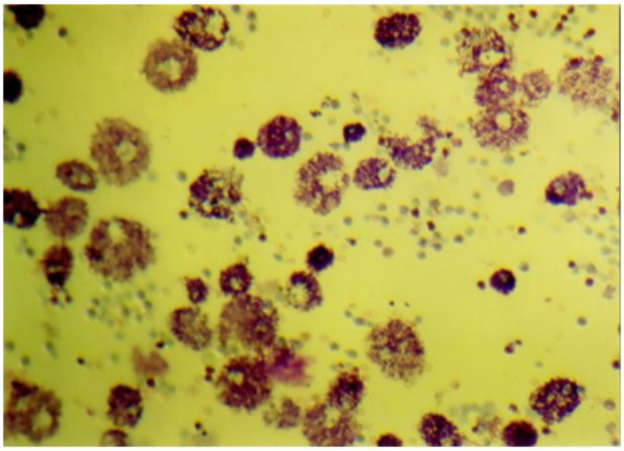

Fig. 1. *Degranulation of peritoneal mast cells in the case of A.awamori inoculation (2x10⁴ cells/m³), Giemsa Stain, x 400*

Furthermore, fungi and gram-negative bacteria affected the immune system, which resulted in decreasing the total amount of T-lymphocytes in peripheral blood and simultaneously increasing the number of B-cells (Table 3).

The microecological changes in the fecal microflora also varied greatly from strain to strain tested and was related to the taxonomic group of microorganisms (Table 4).

Table 4.

Characteristics of possible microecological disorders of large intestine caused by microbial strains used in biotechnology (ways of inoculation: oral and/or inhaling)

Microorganisms	Route of inoculation and dose of inoculation, lg CFU/m³	Microecological Changes
Gram-positive bacteria	5.0 – 7.0	Mild
Gram-negative bacteria	4.0	Manifest, severe
Actinomycetes	4.0 – 7.0	None
Candida sp.	3.0 – 4.0	Manifest, severe
Yarrowia sp.	3.0	None
Micromycetes (Aspergillus sp., Penicillium sp.)	4.0	Manifest, severe or intermediate
Micromycetes (Trichoderma sp.)	5.0	None

The most prominent visible effect was caused by *Candida sp.* and gram-negative bacteria in moderate inoculated concentrations – 10^3-10^4 CFU/m³.

Table 3.

Immunotropic activity of microbial strains used in biotechnology

Concentration of microorganisms, CFU/m³	T-lymphocytes, %	B-lymphocytes, %	Sensitizing effect		Eosinophils of Peripheral Blood, %
			Immediate type, %	Delayed type, mm	
R.erythropolis КД					
Control	44.2±1.5	17.8±1.5	4.7±1.2	0.142±0.007	2.5±0.6
Test 5x10⁷	39.6±2.3	20.2±1.4	5.7±1.0	0.151±0.010	2.6±0.4
B.subtilis 103					
Control	46.5±0.9	16.8±1.0	3.6±0.6	0.13±0.013	1.7±0.2
Test 1 - 6x10⁴	44.2±1.4	17.2±1.2	4.6±1.7	0.20±0.007	2.0±0.4
Test 2 - 6x10⁵	42.8±1.7	17.6±1.5	9.1±2.8	0.29±0.022	2.3±0.5
B.licheniformis 60					
Control	44.0±1.1	21.8±0.9	4.6±0.4	0.12±0.03	2.6±0.5
Test 1 - 5x10⁴	45.5±1.5	20.4±1.0	4.4±0.5	0.22±0.03	4.6±0.7
Test 2 - 5x10⁵	39.1±1.0*	25.4±1.7	9.0±0.9**	0.38±0.02**	5.5±1.1*
P. caryophylii KM 92-102/1					
Control	43.9±2.0	18.4±2.3	4.0±0.9	0.139±0.021	3.4±0.8
Test 1 - 7x10³	40.5±2.1	18.9±2.1	6.1±1.6	0.233±0.064	4.8±1.5
Test 2 - 7x10⁴	33.2±1.9**	22.5±1.7*	12.6±2.4**	0.403±0.034*	6.4±1.0*
P. canescens PhPl33 BKM F3867					
Control	41.6±0.8	21.1±0.8	3.5±0.6	0.19±0.03	3.4±0.3
Test 1 - 2x10³	39.8±0.4	22.2±1.8	4.2±0.5	0.24±0.04	4.2±0.8
Test 2 - 2x10⁴	36.3±1.2*	27.0±1.4*	12.5±2.7**	0.36±0.04*	7.2±1.2**
A. awamori 120/177					
Control	43.5±2.3	21.0±1.1	4.4±0.4	0.188±0.018	2.6±0.4
Test 1 - 1.2x10⁴	32.2±1.7**	24.6±0.9	11.0±1.1**	0.138±0.028	4.9±0.8*
Test 2 - 1.2x10⁵	29.6±3.5**	30.4±1.4**	24.4±2.7**	0.306±0.044*	4.4±0.5*
C.tropicalis Y-456					
Control	42.2±2.6	19.5±1.6	4.7±0.4	0.201±0.045	3.8±0.8
Test 1 - 3x10³	38.7±1.7	25.5±1.7*	11.2±1.8**	0.436±0.061*	8.3±1.1**
Test 2 - 3x10⁴	35.5±1.7	31.3±2.1**	20.0±3.3**	0.490±0.051*	10.0±1.5**

** P<0.05, ** P<0.01*

These changes observed in gut microbiota concerned the decreased level of normal lactose-positive *E.coli* and lactobacilli and simultaneously the increased concentration of opportunistic bacteria – staphylococci, enterococci, and gram-negative enterobacteria.

The mold strains of *A.awamori* and *P.funiculosum* at the level of $2x10^4$ CFU/m^3 also induced a decreased amount of normal fecal flora members and an elevated concentration of opportunistic enterobacteria, including *Proteus sp.* At the same time, the micromycetes of genus *Trichoderma* in higher inoculating concentrations did not demonstrate any visible side effects to gut microflora.

The gram-positive bacteria of genera *Lactobacillus, Micrococcus* and *Rhodococcus*, like *Bacillus sp,* and *Streptomyces sp.*, did not affect the immune system and gut microbiocenosis in very high concentrations (10^5 CFU/m^3) in inhaled air – 10^6, 10^7 and 10^8 CFU/m^3. The data obtained were used to improve the scheme of testing industrial strains and developing safety and hygiene standards for industrial use that will help decrease or minimize the occupational risk of infection and damage to the immune system when working with biotechnological strains of microbes. The strain producers of *Pseudomonas sp., Alcaligenes sp.,* fungi of genera *Aspergillus*, *Penicillium* and *Candida* make the list of priority industrial microorganisms for potential harm due to their sensitizing effect. We suggest testing any new biotechnological strain to determine its potential harmful effect according to standards developed. It is necessary to assess any possible adverse effects of newly prepared industrial strains of microorganisms.

The program of experiments can be shortened if performed for gram-positive candidates for industrial strains – including *Bacillus sp., Streptomyces sp.,* and *Trichoderma sp*. – that possess poorly expressed sensitizing properties. For the members of genera *Lactobacillus, Micrococcus, Rhodococcus,* etc., not showing harmful effects in the studied concentrations, we recommend performing group testing (for genus characteristics) on the basis of a study of their potential virulence.

Thus, our 15-year investigation allowed characterizing the degree of safety of microorganisms and creating a databank according to their toxicity and potential danger. The experimental justification for a maximum concentration limit of microorganism producers in the air of a working zone and atmospheric air of the occupied places was fixed in the State hygienic standards approved by the Federal Service for Supervision over Protection of Consumer Rights and Human Well-Being. [11, 32, 35-38]

Competing interests

The authors declare that they have no competing interests.

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SHORT COMMUNICATION

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Efficacy and Safety of Rebamipide in Prevention of NSAID-Gastropathy

Serhiy Tkach, PhD, ScD[1]; Liudmila Onischuk[2]*; Anna Balabantseva[3]

[1]*Ukrainian Scientifically Practical Center of Endocrine Surgery, Kyiv, Ukraine*
[2]*Bogomolets National Medical University, Kyiv, Ukraine*
[3]*Medical Academy named after S.I. Georgievsky, Simferopol, Republic of Crimea*

Abstract

Background: Nonsteroidal anti-inflammatory drugs (NSAID) are one of the most widely used drugs in medical practice. However, all medical benefits of NSAID are paid for by increased risk of developing numerous side effects. One of the most clinically significant side effects is a NSAID-induced gastrointestinal lesion that develops in, on average, 30% of NSAID users, even with the absence of ulceration; NSAID-induced ulcers and bleeding cause 61% of deaths related to side effects of these medicines. The main aim of this study was to compare the incidence of erosive and ulcerative lesions of the gastroduodenal zone as a result of patients receiving diclofenac on the background of concomitant prophylactic use of proton pump inhibitor (PPI) omeprazole or rebamipide.

Materials and Methods: To achieve this goal we have conducted a randomized comparative study, which included 118 patients aged from 25 to 65 years (mean age, 45±18 years) with osteoarthritis (94 patients) and rheumatoid arthritis (24 patients), who had taken a once-daily dose of 100 mg diclofenac (Dikloberl) over 1 month. Depending on the treatment, all patients were randomized into 3 groups by using the computer method of random numbers. Within 1 month, patients of Group 1 (n=42) received additionally a once-daily dose of 20 mg omeprazole (Omez), and patients of Group 2 (n=46) - rebamipide at a dose of 100 mg three times a day. Patients of Group 3 (n=30) received only diclofenac. The primary endpoint was the cumulative incidence of development of erosions and ulcers in the gastroduodenal zone, which was determined after the treatment according to data from the endoscopy. The secondary endpoint was the incidence of development of dyspeptic symptoms and side effects.

Results: During 1 month of continuous reception of diclofenac, peptic ulcers of stomach and duodenum were found in 2/4.8% and 2/4.8% patients of Group 1 and in 3/6.5% and 2/4.3% patients of Group 2, respectively. In Group 3, peptic ulcers of stomach and duodenum were found in 5/16.6% and 3/10% patients, respectively, and in 2 cases, these ulcers (1 gastric ulcer and 1 duodenal ulcer) have manifested into gastrointestinal bleeding. Thus, all peptic ulcers of the gastroduodenal area were detected in 4/9.5% patients of Group 1, 5/10.9% patients of Group 2, and 8/26.6% patients of Group 3. **(Int J Biomed. 2017; 7(1):57-59.)**

Key Words: NSAID • side effects • gastropathy • rebamipide

Introduction

Nonsteroidal anti-inflammatory drugs (NSAID) are one of the most widely used drugs in medical practice that tens millions of patients all over the world use every day. Their popularity and widespread use are due to their significant anti-inflammatory effect that relieves pain in various states, including arthritis, and other musculoskeletal pathology. However, all medical benefits of NSAID are paid for by increased risk of developing numerous side effects, including some that are life-threatening. For example, a prospective analysis has shown that in the United Kingdom, NSAID were the main class of drugs that cause side effects (on average in 30% over 18000 hospitalized patients); in the USA, side effects of NSAID were the 15th leading cause of death.[1] One of the most clinically significant side effects is a NSAID-induced gastrointestinal lesion that develops in, on average, 30% of NSAID users, even with the absence of ulceration; NSAID-induced ulcers and bleeding cause 61% of deaths related to side effects of these medicines.

***Corresponding author:** Lyudmila Onyshchuk, MD. Bogomolets National Medical University, Kyiv, Ukraine. Email: lyudmila.onishhuk@mail.ru*

Rebamipide (Otsuka Pharmaceutical Co., Japan) is a cytoprotective anti-ulcer drug that increases the protective mechanisms of gastric mucosa by increasing the production of gastric mucus and stimulation of endogenous production of prostaglandins. The efficiency of rebamipide in the prevention of NSAID-induced gastric damage was confirmed in healthy volunteers during their treatment with indomethacin.[2-4] Rebamipide was also found to be efficacious in reducing gastric injury in healthy subjects taking aspirin 1500 mg once a day.[5] However, there are not enough data concerning the efficiency of rebamipide in the prevention of NSAID-induced gastrointestinal lesion.

The main aim of this study was to compare the incidence of erosive and ulcerative lesions of the gastroduodenal zone as a result of patients receiving diclofenac on the background of concomitant prophylactic use of proton pump inhibitor (PPI) omeprazole or rebamipide.

Materials and Methods

To achieve this goal we have conducted a randomized comparative study, which included 118 patients aged from 25 to 65 years (mean age, 45±18 years) with osteoarthritis (94 patients) and rheumatoid arthritis (24 patients), who had taken a once-daily dose of 100 mg diclofenac (Dikloberl) over 1 month. The study excluded patients with concomitant severe liver or kidney disease, those with clinically significant upper gastrointestinal pathology confirmed during endoscopy (GERD, esophageal varices, peptic ulcers or tumors), patients after operations on the stomach, and patients who have taken over the last 4-weeks antisecretory drugs, cytoprotectors, prokinetics, NSAID, corticosteroids and anticoagulants.

Depending on the treatment, all patients were randomized into 3 groups by using the computer method of random numbers. Within 1 month, patients of Group 1 (n=42) received additionally a once-daily dose of 20 mg omeprazole (Omez), and patients of Group 2 (n=46) - rebamipide at a dose of 100 mg three times a day. Patients of Group 3 (n=30) received only diclofenac. Informed consent was signed by each participant.

All patients before the study underwent esophagogastroduodenoscopy, which was repeated at the end of the treatment after 4 weeks, or in the case of severe pain syndrome and/or dyspeptic symptoms that additional antacids did not stop, signs of overt or latent gastric bleeding, anemia. Ulcers are defined as breaks in the mucosal surface 3 mm or more in diameter measured by using biopsy forceps during endoscopy. Erosions were defined as superficial mucosal defects of less than 3 mm in diameter, and intramucosal hemorrhage – hemorrhagic lesions without superficial mucosal defects. Endoscopic mucosal damage was assessed using the modified Lanza scale (MLS) ranging from 0 to 5, during the screening and at the end of the study. In all cases during screening endoscopy, the rapid urease test was performed to find the presence of Helicobacter pylori infection, and photo and video documentation of this procedure were made.

The primary endpoint was the cumulative incidence of development of erosions and ulcers in the gastroduodenal zone, which was determined after the treatment according to data from the endoscopy. The secondary endpoint was the incidence of development of dyspeptic symptoms and side effects.

The obtained results were statistically processed. Group comparisons with respect to categorical variables are performed using chi-square tests with Yates correction or, alternatively, Fisher's exact test. A probability value of $P<0.05$ was considered statistically significant.

Results and Discussion

During 1 month of continuous reception of diclofenac, peptic ulcers of stomach and duodenum were found in 2/4.8% and 2/4.8% patients of Group 1 and in 3/6.5% and 2/4.3% patients of Group 2, respectively. In Group 3, peptic ulcers of stomach and duodenum were found in 5/16.6% and 3/10% patients, respectively, and in 2 cases, these ulcers (1 gastric ulcer and 1 duodenal ulcer) have manifested into gastrointestinal bleeding. Thus, all peptic ulcers of the gastroduodenal area were detected in 4/9.5% patients of Group 1, 5/10.9% patients of Group 2, and 8/26.6% patients of Group 3. Thus, the cumulative incidence of peptic ulcers in Groups 1 and 2 was not significantly different among themselves ($P=1.000$), but was significantly lower than in Group 3 ($P=0.037$). It should be noted that all patients with peptic ulcers had concomitant gastroduodenal erosive changes (from 1 to 5 according to the Lanza scale). The cumulative frequency of occurrence of gastroduodenal erosions, evaluated according to the Lanza scale, in Groups 1, 2 and 3 was 19%, 23.9% and 46.6%, respectively ($P=0.027$). Thus, the frequency of endoscopic visible damage of gastroduodenal area in groups with rebamipide and omeprazole (19% and 23.9%, respectively) was not significantly different among themselves ($P=0.614$), but was significantly lower than in the control group ($P=0.017$).

The vast majority of patients with NSAID-induced gastroduodenopathy in Groups 1, 2, and 3 (87.5%, 90.9% and 85.7%, respectively) were infected with *Helicobacter pylori* (*H. pylori*). These data support the preconceived notion that *H. pylori* infection and NSAID have a synergistic damaging effect on gastric mucosa, and the fact that eradication of *H. pylori* can act as one of the most effective strategies for the prevention of NSAID-gastropathy.

As can be seen, the frequency of development of NSAID-induced dyspeptic symptoms and complications in Groups 1 and 2, except diarrhea syndrome, did not differ significantly, but was significantly lower than in Group 3 patients receiving no omeprazole or rebamipide. In addition, 2 patients in Group 3 had gastrointestinal bleeding stopped by conservative treatment, which was not observed in Groups 1 and 2. Thus, the use of rebamipide in patients who require long-term use of NSAID should be considered as a safe method of preventing NSAID-gastropathy and its complications, and their effectiveness is not inferior to the preventive effect of PPI.

Our findings are consistent with 4 earlier foreign randomized placebo-controlled trials. S. Ono et al.[6] also showed that rebamipide significantly prevented low-dose aspirin-induced erythema in the antrum compared with

placebo (*P=0.0393*). In the study by Kawai et al.,[7] the number of gastric lesions were counted to evaluate low-dose ASA-induced gastrointestinal injuries compared among the placebo, omeprazole, and rebamipide group. The number of erythema, erosions, petechia were evaluated as changing their numbers compared with before starting study and after (after had three points, 24 h, 3 days, and 7 days). Thus, the number of erythema was increased in the placebo group at 3 days compared with the omeprazole and the rebamipide groups, 9.6±10.5 vs 1.4±6.8 (*P=0.0611*) vs 0.3±4.2 (*P=0.0327*). The number of petechiae was increased in the placebo group at 7 days compared with the omeprazole and the rebamipide groups: 8.3±8.8 vs 5.6±23.6 (*P=0.0213*) vs 1.6±5.2 (*P=0.0335*), respectively.

The efficacy of rebamipide in reducing NSAID-induced gastric injury has been reported in healthy volunteers on indomethacin treatment.[8] In the study by Kim et al.,[9] twenty healthy volunteers were randomized two groups. The placebo group took ibuprofen, 600 mg t.i.d., and placebo for 7 days. The rebamipide group took ibuprofen, 600 mg t.i.d., and rebamipide, 100 mg t.i.d., for 7 days. After 7 days of the administration of ibuprofen and either placebo or rebamipide, severe gastric mucosal lesions, MLS ≥3, were found in six (60%) of the placebo group and none (0%) of the rebamipide group (*P=0.011*).

In 2008, Naito et al.[10] investigated the efficacy of rebamipide and famotidine in *H.pylori*-negative healthy volunteers taking NSAID. This study was a randomized, two way crossover study comparing the preventive effect rebamipide 100 mg, t.i.d. and famotidine 10 mg, b.i.d against indomethacin (25 mg, t.i.d.)-induced gastric mucosal injury in *H. pylori*-negative healthy volunteers. The incidence of gastric lesions (MLS ≥2) was 17% (2/12) in the rebamipide group and 25% (3/12) in the famotidine group. Peptic ulcers did not occur in both groups.

Our study, which confirmed the efficacy and safety of rebamipide in the prevention of NSAID-gastropathy, has some drawbacks and limitations. They are a relatively small sample size, lack of comparisons with the placebo, short-term use of NSAID and rebamipide (1 month). Nevertheless, these results allow us to recommend using rebamipide more widely as an effective and safe method of prevention of NSAID gastropathy. To clarify the protective effects of rebamipide on the lower gastrointestinal tract, further research is needed.

Competing interests

The authors declare that they have no competing interests.

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SHORT COMMUNICATION

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Complex Dietary Supplements from Raw Plants Provide Nutrition for Athletes

Dmitrii M. Uvarov*; Albina V. Stepanova; Petr P. Vasilyev; Ksenia N. Naumova; Aigerim Sh. Smagulova; Vera V. Anshakova, PhD, ScD

M. K. Ammosov North-Eastern Federal University, Yakutsk, the Russian Federation

Abstract

The aim of this study was to investigate the effectiveness of mechanically activated complexes from plant substances to enhance athletes' adaptability to intense physical activity.

Methods: The object of the study was the dietary supplement Kladorod, which is based on the reindeer lichen *Cladonia rangiferina* and *Rhodiola rosea* in weight ratio of 10:1. To test the dietary supplement, 10 elite athletes (boxers and mixfighters) were divided into 2 groups. Athletes of the experimental group were given the dietary supplement Kladorod (capsule of 0.4 g by mouth between meals 4 times a day for 28 days). The control group was given placebo (Ringer-Locke powder) capsules in the same terms in a similar way. During the experiment, the athletes were medically examined 3 times: at the beginning, in the middle, and after the course of intervention. We measured muscle performance, fat mass, muscle mass, and serum concentrations of cortisol and total testosterone.

Results: Administration of Kladorod for 28 days stabilized the absolute and relative muscle mass, preventing its reduction, in comparison with the placebo group. At the same time, indicators of fat mass decreased significantly in the experimental group; we did not observe a significant decrease in testosterone/cortisol ratio, compared to the control group. Thus, the use of biologically active supplements based on lichen raw materials and complexes of lichen raw materials with different plant substances enables the body to increase its adaptive potential and physical capacity. **(Int J Biomed. 2017; 7(1):60-62.)**

Key Words: Cladonia rangiferina • Rhodiola rosea • dietary supplement • physical capacity

Introduction

In athletes, chronic overloading and psychoemotional stress lead to a decrease in adaptive capacities, a breach of immunological resistance, a reduction of general and special athletic performance, and also significantly reduce the effectiveness of the training process and sport performance.[1] In recent years, in order to recover and maintain the required physical condition of athletes, products that originate in plants have attracted an increasing interest due to their good tolerability and mild corrective effect on the body. In this regard, for specialized nutrition of athletes, we propose a plant complex from renewable raw materials with increased adaptogenic effect. This unique complex was developed in the laboratory "Mechanochemical biotechnologies" of NEFU.

***Corresponding author:** Dmitrii M. Uvarov, M. K. Ammosov North-Eastern Federal University, Yakutsk, Russia. E-mail: uvadmi91@inbox.ru*

The aim of this study was to investigate the effectiveness of mechanically activated complexes from plant substances to enhance athletes' adaptability to intense physical activity.

Methods

The object of the study was the dietary supplement Kladorod, which is based on the reindeer lichen *Cladonia rangiferina* and *Rhodiola rosea* in weight ratio of 10:1.

The make-up of the multicomponent dietary supplement Kladorod is based on the intermolecular complexes of lichen β-polysaccharides and bioactive substances (BAS) from roots and rhizomes of Rhodiola rosea: salidroside and flavonoid aromatic acids formed during mechanochemical activation of the above-mentioned mixture.

In the composition of reindeer lichen, we identified reindeer lichen, lichen acids (perlatolic, fumarprototcetraric, usnic), free sugars, amino acids, and oligo- and polysaccharides. In addition, the lichen genus Cladonia can serve as a source of

chemical elements, especially Ca, Mg, P, K, Na and microdoses of Li, I, and Se.[2] It is known that lichens and Rhodiola rosea stimulate the immune system and protect it by restoring the body's metabolic balance.[3,4] The collection of raw materials was carried out in an ecologically clean area.

Quantitative assessment of the flavonoid content was performed in a known manner by UV spectrophotometry in terms of quercetin.[5] UV spectra were recorded on the Libra S12 device (quartz cuvettes with 1 cm absorbing layer thickness). Lichen flavonoid content in the raw material in term to quercetin was 1.58±0.38%.

To test the dietary supplement, we developed a special scheme for the experiment and selected 10 elite athletes (boxers and mixfighters) aged from 22 to 34 years with sport experience from 5 to 16 years. The experiment was conducted in the condition of the planned educational and training process of preparations for rating fights. Athletes were divided into 2 groups and were under the same conditions (nutrition, medical monitoring, living conditions and training process). Written informed consent was obtained from all participants.

Athletes of the experimental group were given the dietary supplement Kladorod (capsule of 0.4 g by mouth between meals 4 times a day for 28 days). The control group was given placebo (Ringer-Locke powder) capsules in the same terms in a similar way. During the experiment, the athletes were medically examined 3 times: at the beginning, in the middle, and after the course of intervention. We measured muscle performance, fat mass, and muscle mass. Serum concentrations of cortisol (C) and total testosterone (T) were estimated by the method of solid-phase, competitive chemiluminescent enzyme immunoassay.

Statistical analysis was performed using the statistical software «Statistica». (v6.0, StatSoft, USA). The mean (M) and standard error of the mean (SEM) were calculated. The Wilcoxon criterion was used to compare the differences between the paired samples. A probability value of $P<0.05$ was considered statistically significant.

Results

The results obtained during experimental testing on athletes served as the basis for conclusions about the efficacy of this dietary supplement. It is known that at the stage of specialized training under conditions of high-intensity training (submaximal and maximal power) in athletes specializing in martial arts, their body composition undergoes regular changes in labile components.[6] At this stage of training (up to the beginning of the competition), lean body mass decreases significantly, while fat mass increase. This effect of intensive loads reflects a decrease in adaptation of the body to stress and negatively affects the efficiency of the whole cycle of preparing for a competition.

Based on these data, it is assumed that indicators of labile components of body composition are sufficiently reliable criteria (albeit indirect) of combat athletes' adaptation to training loads. It was established that during the intensive training of boxers and mixfighters for rating fights, administration of the dietary supplement Kladorod for 28 days stabilized the absolute and relative muscle mass, preventing its reduction, in comparison with the placebo group. At the same time, indicators of fat mass decreased significantly in the experimental group (Table 1).

Table 1.

Morphological body composition parameters during training process in experimental and control groups

Parameter	Experimental group		Control group	
	Day 1	Day 28	Day 1	Day 28
Body weight, kg	75.8±4.4	75.0±6.8	76.2±2.25	75.8±7.0
Muscle mass, kg	39.15±4.16	39.4±4.10	40.53±2.88	39.48±5.2*
%	53.7±0.1	54.0±0.3	53.2±0.1	52.3±0.4*
Fat mass, kg	9.15±0.12	9.09±0.24*	9.39±0.25	9.87±0.4*
%	12.9±0.1	12.7±0.2*	12.1±0.1	12.9±0.16*

**- P<0.05 vs. initial data in each group*

To monitor the athlete's response to exercise intensity, we investigated the T/C ratio. It is known that well-conditioned athletes have more controlled cortisol secretion during exercise; however, when an athlete is overtrained, the cortisol rises more and testosterone decreases, creating a low T/C ratio. The control and experimental groups did not differ in initial values of the T/C ratio. After administering the course of Kladorod, we did not observe a significant decrease in T/C ratio, compared to the control group (Table 2).

Table 2.

Serum levels of testosterone and cortisol during training process in experimental and control groups

Parameter	Experimental group		Control group	
	Day 1	Day 28	Day 1	Day 28
Testosterone, nM/l	28.9±3.7	29.9±4.0	25.2±5.2	16.3±2.1*
Cortisol, nM/l	141±16.2	179±11.0	137±17.4	281 ±10.8*
T/C ratio	0.21±0.04	0.18±0.06	0.18±0.04	0.11±0.01*

**- P<0.05 vs. initial data in each group*

Mechanochemical processing of the plant raw material destroys the cell walls, where the bulk of BAS is contained, and contributes to formation of ultrafine particles in the solid phase, thereby contributing to effective BAS release from the cells.[4]

We can conclude that during the period of training with high-intensity loads of submaximal power on the background of a course of the dietary supplement Kladorod in athletes there is stabilization of muscle mass with a parallel decrease in fat mass. This type of dynamics of morphological parameters of the body composition corresponds to a stable level of the adaptation of the organism. Thus, the use of biologically active supplements based on lichen raw materials and complexes of lichen raw materials with different plant substances enables the body to increase its adaptive potential and physical capacity. BAS contained in the plant lichen raw materials demonstrate a significant membrane-stabilizing effect and a positive effect

on the course of metabolic processes, and they stimulate the enzyme cascades of different biochemical reactions, which is important for enhancing the totality of an athlete's physical adaptations during high-intensity training.

Competing interests

The authors declare that they have no competing interests.

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CASE REPORT

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Second Fatal Case of Infective Endocarditis caused by *Gemella bergeriae*

Aijan Ukudeeva, MD[1]*; Hal K. Hawkins, MD, PhD[1,2]; Masood Ahmad, MD[2]; Peerawut Deeprasertkul, MD[2]; Liliana Rincon, MD[2]; Brandon K. Kimbrel, BS[2]; David H. Walker, MD[2]

[1] *Shriners Burns Hospital for Children, Galveston, TX, USA*
[2]*University of Texas Medical Branch, Galveston, TX, USA*

Abstract

Our case illustrates a fatal course of infection with *Gemella bergeriae* endocarditis that was complicated by cardiogenic shock due to perforation of the mitral valve with severe mitral regurgitation, extension of infection into the myocardium adjacent to the mitral valve, and coronary sinus thrombosis. **(Int J Biomed. 2017;7(1):63-66.)**

Key Words: Gemella bergeriae • infective endocarditis • mitral valve • cardiogenic shock

Abbreviations

AO, aorta; **AML**, anterior mitral leaflet; **IE**, infective endocarditis; **LA**, left atrium; **LV**, left ventricle; **MV**, mitral valve; **MR**, mitral regurgitation; **PML**, posterior mitral leaflet; **PCR**, polymerase chain reaction; **RV**, right ventricle

Introduction

The incidence of infective endocarditis (IE) in developed countries has increased dramatically during the last few decades.[1-4] Between 2000 and 2011, the incidence of IE in the United States increased from 11 per 100,000 people to 15 per 100,000 people.[1] *Gemella bergeriae* (or *bergeri*), one of the six species belonging to the genus *Gemella*, was isolated for the first time by Collins et al.[5] in 1998 from the blood cultures of six febrile patients, three of whom had endocarditis. Since then, only seven cases of *G.bergeriae* endocarditis have been reported.[5-8] This report describes a fatal case of IE caused by *G.bergeriae*, which is part of the normal flora of the human oral cavity, slow growing fastidious bacteria, which can insidiously lead to a fulminant fatal outcome.[5-7,9-14] We present the second fatal case of IE caused by *G.bergeriae*.

***Corresponding author**: Aijan Ukudeeva, MD, MS. Shriners Burns Hospital for Children, Galveston, TX, USA. E-mail: draijan@yahoo.com*

Case Presentation

A 63-year-old man with a medical history of hypertension and poliomyelitis was admitted to the emergency department with acute bilateral lower extremity weakness and pain, low back pain, and a 2-week history of influenza-like symptoms. On examination, his temperature was 96.8°F, blood pressure was 98/44 mmHg, heart rate was 112 beats/min, and respiratory rate - 26/min. Cardiac examination revealed holosystolic murmur at the apex with radiation to the axilla. A physical exam revealed the decreased movement and sensation in both lower extremities; they were cool to the touch and mottled; a Doppler posterior tibial pulse and a Doppler dorsalis pedis pulse were not present. Laboratory investigations revealed a white blood cell count of 31.4 x $10^3/\mu L$, lactic acid of 6.3 mmol/L, B-type natriuretic peptide of >3000 pg/mL, and troponin I of 1.710 ng/mL. Computed tomography of the abdomen and pelvis showed almost the complete occlusion of the abdominal aorta distal to the inferior mesenteric artery and the right common iliac artery. On the same day, the patient underwent aortoiliac thrombectomy through bilateral femoral

incisions. Transthoracic echocardiogram showed bileaflet prolapse with leaflet thickening and large mobile vegetation (1.1x1.4cm) on the posterior mitral leaflet (Figures 1 and 2). Blood cultures were collected before starting antimicrobial therapy. Starting therapy included once-daily doses of vancomycin 1500 mg, piperacillin-tazobactam 4500 mg, and levofloxacin 750 mg. On the next day, blood cultures yielded gram-positive cocci in clusters, and the patient was treated with cefazolin 1000 mg q8h. On the third day of his hospital stay, piperacillin/tazobactam 3375 mg q6h and vancomycin 1000 mg q12h were started, and cefazolin was discontinued. His condition declined, and he developed oliguria. On the fourth day, the patient underwent intra-aortic balloon pump placement for afterload reduction. Coronary angiography showed nonocclusive coronary artery disease. On the fifth day, he developed respiratory distress and lactic acidosis of 9.3 mmol/L and was intubated. A few hours after intubation, he developed hypotension and bradycardia; cardiopulmonary resuscitation was initiated but was not successful.

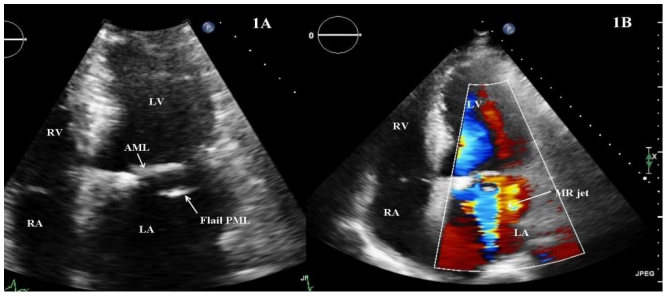

Fig. 1A. *Apical four-chamber view showing a large vegetation and a portion of flail posterior leaflet (arrow).*

Fig. 1B. *Apical four-chamber view showing mitral regurgitation jet (arrow).*

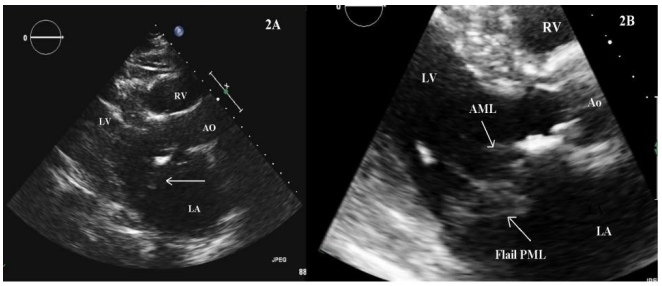

Fig. 2A. *Parasternal long axis view, arrow pointing to mitral valve leaflets prior to endocarditis (arrow).*

Fig. 2B. *Parasternal long axis view, arrow pointing to a large vegetation, and flail portion of PML.*

Two days post-mortem, the final results of the first blood culture obtained in the emergency department became available, and the Gram-positive cocci were identified as *Gemella bergeriae* by MALDI-TOF mass spectrometry (bioMerieux, Durham, NC). However, the FDA has not approved this mass spectrometry for *G.bergeriae* investigation. Therefore, additional identification was performed by using the VITEK®2 (bioMerieux, Durham, NC), which also identified the organism as *G.bergeriae*. It was later determined that the patient had had an extensive dental procedure three weeks prior to his clinical presentation.

Post-mortem examination revealed massive cardiomegaly (1100g), bilateral pulmonary congestion, and passive congestion of the liver. Both leaflets of the mitral valve were thickened. The posterior leaflet was perforated and had a 3x2x1cm vegetation attached to the superior surface (Figures 3A and 3B). The posterior-inferior wall of the left atrium and the posterior-superior wall of the left ventricle were inflamed. Inflammation involved the adjacent myocardium and pericardium. Adjacent to the inflamed myocardium, the coronary sinus contained a thrombus measuring 2x1x0.5cm. The upper lobe of the left lung had a small thromboembolus. Based on the autopsy findings and the clinical history, the cause of death was reported as cardiogenic shock due to perforation of the mitral valve with severe mitral regurgitation, extension of infection into the myocardium adjacent to the mitral valve, and coronary sinus thrombosis.

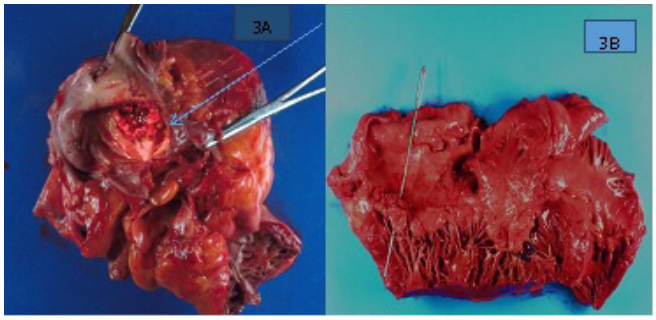

Fig. 3A. *View from LA. Mitral valve endocarditis. Arrow pointing to the atrial aspect of PML with a large irregular reddish tan vegetation and perforation.*

Fig. 3B. *View of the opened left atrium and ventricle at autopsy. Mitral valve endocarditis. The probe passes through a hole in the vegetation that perforated PML The underlying chorda is thickened.*

Discussion

The genus Gemella is comprised of catalase-negative, gram-positive cocci occurring in pairs (commonly with adjacent sides flattened), tetrads, and/or short chains.[15] Six species, namely, *G.haemolysans, G.morbillorum, G.bergeriae, G.sanguinis, G.palaticanis,* and *G.cuniculi,* are currently recognized.[5,15-17] The identification of *Gemella* isolates represents a challenge to clinical laboratories. Manual or commercial phenotypic methods may result in misidentification of Gemella spp. as viridans group streptococci or other related organisms and vice versa.[15,18]

M Collins and co-workers[5] used 16S rRNA gene sequence analysis and an extensive repertoire of biochemical tests to characterize 6 strains of previously undescribed gram-positive, facultatively anaerobic cocci recovered from blood cultures of hospitalized patients (half of whom were diagnosed with subacute bacterial endocarditis), leading to their proposal of a new *Gemella* species, *G.bergeriae*, to which these strains belonged.

Pre-existing damaged heart valves, poor dental hygiene, intravenous drug abuse, and extensive dental procedures have been reported as the main predisposing risk factors for endocarditis due to *G.bergeriae*.[9,19] To date, approximately 45 cases of endocarditis associated with *Gemella spp*. have been reported in the literature [5,7,8,12,20] and among them only 8 were caused by *G.bergeriae*, including 7 adult cases and 1 pediatric case.[5-7,9,10,13] The majority of the patients described in the literature with *G.bergeriae* endocarditis had a good response to antibiotic therapy.[5-7,9,10,12,13] In 2014, Hussain et al.[7] described the first fatal case of *G.bergeriae* infection, in which the patient with a history of rheumatic heart disease developed an embolic stroke as a complication of IE. Despite antibiotic therapy and intensive treatment, the patient expired due to intracerebral and subarachnoid hemorrhage secondary to rupture of a mycotic aneurysm in the right middle cerebral artery.

In 2007, Stroup et al.[11] described another fatal case of IE in which bacteria of the genus *Gemella* were identified without species identification. That patient was diagnosed as having IE with Gram-positive cocci, and on day 2 developed embolic strokes. On day 8, the patient had cardiogenic shock due to perforation of the anterior mitral leaflet and severe mitral regurgitation. This patient underwent valve replacement; on postoperative day 6 the patient expired due to intracranial hemorrhage from the right middle cerebral artery. Blood cultures of this patient on day 3 identified only Gram-positive cocci, and further study of the blood culture and tissue of the mitral valve identified the organism as the genus *Gemella* without determination of the species.

In 2015, Pachirat et al.[9] described the first case of tricuspid valve IE in which *G.bergeriae* was identified. The organism was identified by PCR sequencing of DNA from tissues of the patient's cardiac valve despite negative cultures. The patient was discharged home with a good outcome.

In our case, the patient had a long history of mitral valve prolapse. He had an extensive dental procedure three weeks prior to his clinical presentation. He developed influenza-like symptoms 2 days prior to his admission to the emergency department with lower extremity weakness and pain due to a septic embolus to the abdominal aorta. On the fifth day after admission, the patient expired due to cardiogenic shock. The results of the patient's blood culture were later reported as positive for *G.bergeriae*. Postmortem histology (Fig.4) of the mitral valve and cardiac tissue revealed structures consistent with colonies of lysed bacteria.

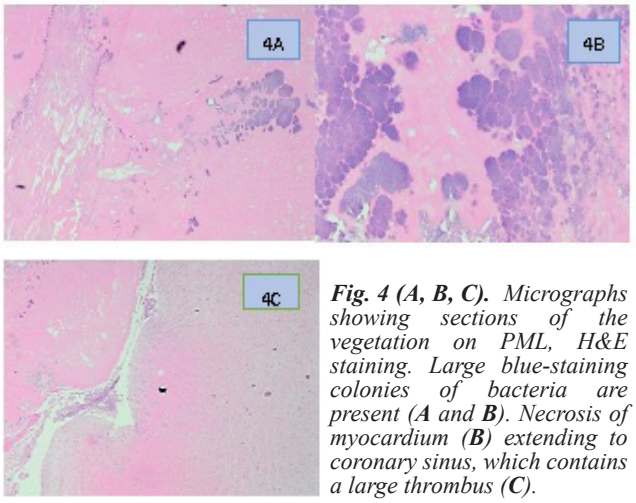

Fig. 4 (A, B, C). *Micrographs showing sections of the vegetation on PML, H&E staining. Large blue-staining colonies of bacteria are present (**A** and **B**). Necrosis of myocardium (**B**) extending to coronary sinus, which contains a large thrombus (**C**).*

Based on the cited literature, it is appropriate to question the general opinion that *Gemella spp*. are harmless microorganisms.[10] *G.bergeriae* endocarditis can have a fulminant course with serious embolic complications.[7] Patients suspected to have *Gemella* endocarditis should be treated immediately with antibiotics due to the potentially aggressive character of this infectious process.[7,10-12] Empiric treatment for *Gemella* endocarditis with penicillin/vancomycin and an aminoglycoside has been recommended[3] to prevent a destructive course and avoid a fatal outcome. Prompt antibiotic treatment is the best way to decrease the risk of embolic complications.[3,7]

Competing interests

The authors declare that they have no competing interests.

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CASE REPORT

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Novel Mutation *chrX:110644366 C>A* of the DCX Gene in 4-year-old Girl with Sporadic Double Cortex Syndrome

Natalia A. Shnayder, PhD, ScD[1]*; Ivan P. Artyukhov, PhD, ScD[1]; Ekaterina V. Egorova[1]; Diana V. Dmitrienko, PhD, ScD[1]; Olga S. Shilkina[1]; Alexander A. Molgachev[2]

[1]V.F. Voino-Yasenetsky Krasnoyarsk State Medical University
[2]S.M. Berezin Medical-Diagnostics Center of the International Institute of Biological Systems
Krasnoyarsk, the Russian Federation

Abstract

Subcortical band heterotopia (SBH), also known as double cortex syndrome (DC), is listed as a "rare disease" by the Genetic and Rare Diseases Information Center of the National Institutes of Health with an incidence of 1 to 200,000 people in the population. The cause of the disease is mutation in the DCX gene (also known as DBCN, XLIS) on chromosome Xq22.3-q23. SBH is an X-linked dominant disorder. Traditionally, genetic testing for SBH has been done in the order of the probability of detection of mutation according to the radiologic features, but the success rate could be variable with this time-consuming approach.

In this study, novel mutation *chrX: 110644366C>A* in the DCX gene was identified in a 4-year-old Russian girl with sporadic SBH. The present report demonstrates that whole exome sequencing may be a useful tool for the identification of previously known and *de novo* mutations in children with SBH as well as malformations of cortical development. **(Int J Biomed. 2017;7(1):67-70.)**

Key Words: Double cortex syndrome • absence seizures • DCX gene • *de novo* mutations

Introduction

Dysfunction of mechanisms regulating the migration of neuronal precursors leads to neuronal heterotopia.[1] Two common neuronal migration disorders, SBH and the X-linked isolated lissencephaly sequence, have been linked to mutations such as missense, nonsense, aberrant splicing, deletion, and insertions in the X-chromosomal gene of doublecortin (DCX).[2]

Case Presentation

A 4-year-old Russian girl was admitted to the Department of Medical Genetics and Clinical Neurophysiology of our Institute with symptomatic epilepsy and developmental delay. She had atypical absences with eye upward rolling (duration: 30-60 sec; frequency: 1-3 times per day), complex focal seizures with disturbance of the balance, swallowing disorder, and hiccough (duration: 1–5 min; frequency: 1-2 times per day; usually immediately after waking from sleep). She was able to sit and walk. She had normal growth but abnormal development. Her speech was limited to a few words. A physical exam was unremarkable. Lab data including CBC, blood biochemical, and urinalysis results were all within normal limits, but the electroencephalography (EEG) revealed generalized poly spike-wave discharges. Family history was negative for febrile seizures and epilepsy. No similar illness with developmental delay was seen in the family.

The girl was the only child of nonconsanguineous parents. She was born from a planned first pregnancy when the mother was 27 years old. There were no occupational hazards or exposure to mutagens and teratogens during pregnancy. During the first trimester of pregnancy, preeclampsia of moderate severity was identified; during the second trimester - oligohydramnios, fetal malnutrition, increased tone of the uterus, threat of pregnancy termination, treatment in a maternity hospital, stitches on the cervix to prolong pregnancy; during the third trimester - oligohydramnios, fetal malnutrition, breech presentation.

A planned delivery was performed by cesarean section with epidural anesthesia at the gestational age of 38 weeks.

***Corresponding author:** Prof. Natalia A. Shnayder, PhD, ScD; Head of the Neurological Center of Epileptology, Neurogenetics and Brain Research of the University Clinic of V.F. Voino-Yasenetsky Krasnoyarsk State Medical University. Krasnoyarsk, the Russian Federation. E-mail: nataliashnayder@gmail.com*

The newborn's body length was 52 cm, weight of 2400 g. The infant was discharged from the hospital a week later. She was subsequently hospitalized to the Krasnoyarsk Perinatal Center. After discharge, she continued to be monitored by a neurologist with regular follow-ups. Rare atypical absence seizures with eyes rolling back debuted at the age of 3 months, but antiepileptic drugs were not prescribed; neither brain MRI nor EEG video monitoring was performed. At the age of 13 months, she had a cluster of complex adversely afebrile seizures with turning of the head and eyes to the left. This episode developed a week after acute respiratory infection with febrile fever. Isolated seizures in this cluster lasted up to 20 seconds, and the cluster up to 20 minutes. First aid was provided by emergency medical care, but the girl was not admitted to hospital. She was seen by a consultant neurologist the following day. A routine EEG in wakefulness was conducted and epileptiform activity was not registered, but antiepileptic therapy was prescribed: Depakine chronosphere (valproic acid) 50 mg in the morning and 50 mg in the evening. Suspected diagnosis was cryptogenic epilepsy (ICD - 10: G40.9). The therapeutic effect was insufficient: short, rare, atypical absence persisted with a frequency up to once per month. However, antiepileptic drugs were canceled after 6 months from start of the treatment. EEG video monitoring was not performed. The patient had a rare atypical absence seizures after discontinuation of treatment. Her parents consulted different neurologists, but antiepileptic drugs were not prescribed.

A brain MRI (Epilepsy Protocol at 1.5 Tesla) revealed SBH for the first time at age 4 years. At this age, complex focal seizures with disturbance of balance, swallowing disorder and hiccough debuted (June 2016). Frequency of seizures increased in August 2016. However, the girl did not take anti-epileptic drugs. The child's parents asked for consultation with a neurologist-epileptologist and neurogeneticist at the Neurological Center of Epileptology, Neurogenetics and Brain Research of the University Clinic of Krasnoyarsk State Medical University named after Prof. V. F. Voino-Yasenetsky. The purpose of consultation was to specify the hereditary nature of the disease in their daughter, select antiepileptic therapy, and specify genetic risk of the disease**.** The girl's parents wanted to have a second child who would be healthy.

DNA was extracted from peripheral blood using in-house operating procedures. Mutations were identified through whole exome sequencing using the next-generation Illumina NextSeq 500 DNA capture method, with an average coverage of at least 70-100x, performed at the Sequencing Facility of GENOMED laboratory (Moscow). After excluding lissencephaly-related genes, novel mutation *chrX:110644366C>A* in the DCX gene was identified. We recommended Sanger sequencing of the patient and her parents to validate this new mutation, not registered in OMIM, and to specify the nature of the mutation (sporadic cases or X-linked dominant). Further Sanger sequencing (GENOMED laboratory, Moscow) validated the variant in the patient but not in both parents, indicating the *de novo* mutation *chrX:110644366C>A* the DCX gene. It was concluded that the results of Sanger sequencing in peripheral blood lymphocytes cannot exclude the germinal and somatic mosaicism. It has been observed that patients with less that 30% mosaicism are clinically unaffected, whereas those with more than 30% of the cells with the mutated allele are symptomatic with SBH.[3] Since the pathogenic mutation has been identified in the family, prenatal testing for pregnancies at increased risk is possible.

Thus, clinical diagnosis of DCX-related SBH (OMIM: 300067; ICD - 10: Q04.8) and symptomatic focal epilepsy (ICD - 10: G40.2) was updated at 4 years of age in December 2016. Currently, the girl is taking the anti-epileptic drug Depakine chronosphere 100 mg per day. The level of valproic acid in serum is in the middle band (65–85 μg/mL) of the therapeutic range. No adverse drug reactions are noted. EEG video monitoring has not detected focal epileptiform activity. Atypical absences have not been registered. Complex focal seizures with hiccoughs have become very short (3-5 seconds) and rare (1-2 per month). The girl's behavior and speech development have improved. She has become interested in toys, and she likes her mother to read children's books to her.

Discussion

Gleeson et al. (1998) and des Portes et al. (1998) determined that the DCX gene spans over 100 kb of DNA and contains 9 exons with 6 coding exons (OMIM:300121). The structure of the gene is unusual because only 16% of the sequence is coding, and the 3-prime untranslated region, which is contained in 1 exon, is 7.9 kb long.[4] Using in situ hybridization, des Portes et al.[5] observed DCX gene expression in human cerebral cortex at 21 weeks' gestational age. There was strong labeling in the ventricular zone and cortical plate and moderate labeling of the intermediate zone. In the intermediate zone, labeled cells were organized as oriented chains, suggestive of migrating neurons.

To date, 40 various mutations have been identified resulting in nonsense, splice site, and missense mutations throughout the DCX gene,[6] but no clear correlation has been observed between the clinical severity and mutation profiles.[7] The proportion of cases caused by *de novo* mutations of DCX gene is unknown.

DCX-related SBH (OMIM: 300067) is a rare neuronal migration disorder deriving from mutations in DCX gene located on chromosome Xq22.3-q23 [4,8,9] in most patients, and is usually associated with medically intractable epilepsy. About one third of patients with SBH have an association of tonic–clonic and myoclonic seizures with atypical absences and drop attacks.[10-13] However, focal clinical signs such as head deviation or clonic movements of one limb at seizure onset observed in some individuals suggest the diagnosis of focal partial epilepsy.[10-12] Infantile spasms and Lennox–Gastaut syndrome also have been reported.[14] About 60% of the patients have focal lobar or multifocal epileptic abnormalities,[10,11,14] but EEG findings are usually characterized by generalized slow spike-and-wave or polyspike and wave, and multifocal spiking.[11-13] In individuals with SBH, cognitive abilities range from normal to learning disabilities and/or severe intellectual disability. Behavior problems may also be observed. The severity of the clinical symptoms correlates with the degree of the underlying brain malformation.[10-12]

Based on the MRI findings,[18] DCX-related SBH is characterized by symmetric bands of gray matter within the white matter between and parallel to the cortex and the lateral ventricles, which appears as an isointense second cortical structure beneath the cortex (double cortex). The cerebral cortex in SBH may appear normal and/or thickened with or without simplified gyration.[14-16] DCX-related SBH is predominantly located in the frontoparietal lobe and is grade 6 (complete band heterotopia). Grade 5, a more severe malformation that overlaps with classic lissencephaly and band heterotopia, is characterized by SBH in the occipital regions and pachygyria in the frontal regions.[17]

Children diagnosed with a SBH may either have inherited the DCX pathogenic variant from their asymptomatic or only mildly affected mother or have the disorder as the result of a *de novo* DCX pathogenic variant. A detailed family history should be obtained. Special attention should be paid to epilepsy, miscarriages, stillbirths, children who died at a young age without obvious birth defects, and cognitive impairment or developmental delay.[18] Penetrance in females heterozygous for DCX pathogenic variants is greater than 90%; however, heterozygous females with missense and nonsense variants may have no obvious brain malformation or seizures.[19] Approximately 10% of unaffected mothers of children with a DCX pathogenic variant were reported to have somatic mosaicism or germline mosaicism.[18] Somatic mosaicism should, whenever possible, be further explored or confirmed by analysis of DNA from different tissues (e.g., hair roots, buccal swabs).[20] If the DCX pathogenic variant is not identified in the mother, neurologic and/or clinical examination of the mother is warranted. If cerebral MR imaging reveals SBH in the mother, additional maternal tissues should be examined for the DCX pathogenic variant identified in her offspring.[18] Cerebral MRI of the mother can be helpful because some heterozygous females with SBH can be asymptomatic.[18,20,21]

If the pathogenic variant has been identified in the family, carrier testing for at-risk family members and prenatal testing for pregnancies at increased risk are possible.. The optimal time for determination of genetic risk, clarification of carrier status, and discussion of the availability of prenatal testing is before pregnancy. During fetal development, first gyri appear around the 20th week of gestation and a reduced gyration pattern when compared to postnatal images remains physiologic until late gestation. Therefore, in the absence of a positive family history, DCX-related SBH may not be recognized until birth by fetal ultrasonography. However, Zhagn K. et al.[22] identified 11 reports of fetal gray matter heterotopias from 1998 to 2015, involving 43 cases with prenatal diagnoses. Of the total of 44 cases (including one case of the authors), 32 cases that had been confirmed postpartum had prenatal ultrasound and MRI data, which showed a significantly lower detection rates of fetal gray matter heterotopias by prenatal ultrasound than by MRI (43.8% vs 93.8%, $P<0.001$).

Prenatal ultrasound can only detect subependymal heterotopia with characteristic manifestations, and the detection of other types of fetal gray matter heterotopias relies on MRI, which is currently the best option for prenatal diagnosis of fetal gray matter heterotopias, including SBH. [23]

Summary, the diagnosis of *DCX*-related SBH is suspected on MRI findings and confirmed by molecular genetic testing. For patients with suspected SBH, sequence analysis is recommended as the first step in mutation identification. Sequence analysis of the DCX gene has been shown to identify 100% of mutations in families with more than one affected family member. However, mutations in the promoter region, some mutations in the introns, other regulatory element mutations, and large deletions cannot be detected by this analysis.

Conclusion

SBH or double cortex syndrome is a rare neuronal migration disorder, classically present with seizures and intellectual impairment and is seen almost exclusively in females.[24-26] It is an X-linked genetic disorder with DCX gene mutation being the causative factor in most of the cases, [24,27] but the proportion of cases caused by *de novo* mutations of DCX gene is unknown.

We are presenting the first case of the novel mutation DCX-related SBH from the city of Krasnoyarsk (the Russian Federation). As described in our case, a girl with developmental delay and symptomatic epilepsy was treated with antiepileptic medications without any specific diagnosis, and the result was that the parents did not adhere to therapy because they were not given sufficient information. However, by using MRI (Epilepsy Protocol at 1.5 Tesla) and new methods of molecular genetics (whole exome sequencing and Sanger sequencing) on this patient, a specific diagnosis was made, information regarding the management and prognosis was provided to parents, and as a result, her seizures were more effectively controlled. In the case of a female patient with SBH, this usually means two possibilities: a *de novo* mutation or an inherited mutation from a heterozygous asymptomatic mother. In the family presented here a female patient showed typical clinical and imagiological phenotype of SBH. We identified the novel mutation *chrX:110644366C>A* in the DCX gene in the patient, but not in either parent.

The present report demonstrates that whole exome sequencing may be a useful tool for the identification of previously known and *de novo* mutations in children with SBH as well as malformations of cortical development.

The proper use of imaging and molecular genetics modalities leads to a clear diagnosis and a management plan; the use of prenatal testing and genetic counseling offers great benefits on preventing such syndromes in future offspring.

Acknowledgment

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Competing interests

The authors declare that they have no competing interests.

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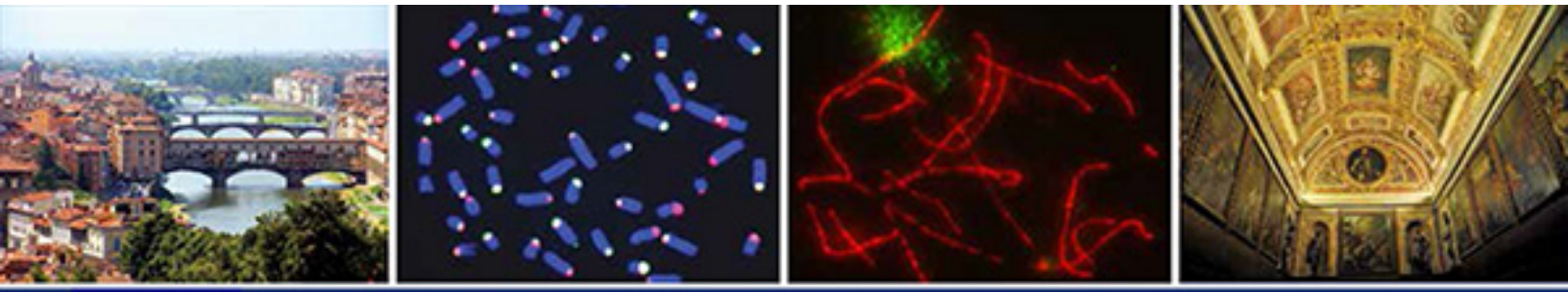

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