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CLINICAL RESEARCH

Prognostic Factors in Patients with Malignant Pleural Mesothelioma

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Abstract

The aim of the present study was to examine the factors of prognosis in patients with malignant pleural mesothelioma (MPM) after combined and multimodality treatment, including the prognostic significance of preoperative intrapleural perfusion hyperthermo-chemotherapy (IPHC).

Material and Methods: The study included 20 patients (11 men and 9 women) aged from 30 to 70 years (mean age 51.9±8.5 years) who underwent surgical treatment for MPM. The diagnosis of MPM was verified by immunohistochemical data. The patients were divided into two groups. Group 1 included 9 patients who underwent combined treatment that included the extrapleural pneumonectomy (EPP) and 4 courses of adjuvant chemotherapy. Group 2 included 11 patients who received multimodality treatment (IPHC, EPP, and 4 courses of adjuvant chemotherapy). All patients were followed prospectively at three-monthly intervals for the first year and six-monthly thereafter until the last time of contact or death. Statistical analysis was performed by using Kaplan-Meier method and the log-rank test. Cox-regression model was used for multivariate analysis.

Results: Patient's age over 60 years and the sarcomatoid type of the tumor can be regarded as prognostic factors for poor survival in patients with MPM who underwent EPP. Application of IPHC as a part of a multimodality treatment enhances the survivability of MPM patients.

Keywords: *malignant pleural mesothelioma; extrapleural pneumonectomy; intrapleural perfusion hyperthermo-chemotherapy; multimodality treatment.*

Introduction

Malignant pleural mesothelioma (MPM) is a rare disease characterized by an aggressive course and extremely poor prognosis. Tumor involves the visceral, parietal pleura, lung, pericardium, and diaphragm and metastasizes into lymph nodes and other organs. Progressive malignant pleurisy and lesion of lung parenchyma contribute to the development of cardiopulmonary failure in patients with MPM. MPM is often diagnosed in the advanced stages and 70% of patients die within a year [1].

Results of various treatments show a low efficiency. Median survival after first-line chemotherapy is 12.4 months [2]. Multimodality treatment with surgery improves median survival to 20-29 months [3-5]. Multimodality treatment is the aggressive therapy associated with high risk for elderly patients with co-morbidities. The risk may be justified in a case of favorable prognosis. It is advisable to resort to chemotherapy or symptomatic treatment in cases of a poor prognosis with short duration of life. From this point of view adequate information about the prognostic factors affecting survival will help to select patients for multimodality treatment and in the long run increase its efficiency. Little is known about the role of preoperative intrapleural perfusion hyperthermo-chemotherapy (IPHC) in multimodality treatment. In this connection ***the aim*** of the present study was to examine the factors of prognosis in patients with MPM after combined and multimodality treatment, including the prognostic significance of IPHC.

Material and methods

The study included 20 patients (11 men and 9 women) aged from 30 to 70 years (mean age 51.9±8.5 years) who underwent surgical treatment for MPM at the N.N. Alexandrov National Cancer Center of Belarus between January 2006 and December 2013 according to a randomized study on the

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comparative effectiveness of the combined and multimodality treatment of MPM. Written informed consent was obtained from each patient. The diagnosis of MPM was verified by immunohistochemical data. The patients were divided into two groups. Group 1 included 9 patients who underwent combined treatment that included the extrapleural pneumonectomy (EPP) and 4 courses of adjuvant chemotherapy. Group 2 included 11 patients who received multimodality treatment (IPHC, EPP, and 4 courses of adjuvant chemotherapy). One course of adjuvant chemotherapy included cisplatin 90 mg/m2 in 1 day, vinorelbine 30 mg/m2 in 1 and 8 days intravenously. IPHC was carried out in mode 42°C at ThermoChem HT-1000 with cisplatin 120 mg/m^2 and vinorelbine 30 mg/m^2 for 1 hour.

All patients were followed prospectively at three-monthly intervals for the first year and six-monthly thereafter until the last time of contact or death. The follow-up review included clinical examination and assessment of chest and abdominal CT scans. The follow-up status was regularly updated in the database for each patient by a data manager. Statistical analysis was performed by using Kaplan-Meier method [6] and the log-rank test [7]. Cox-regression model was used for multivariate analysis [8]. An algorithm step-by-step "stepwise" was used to identify prognostic factors. Statistical processing of data was carried out using SPSS system version 17.0.1 for Windows.

Sex, age (under 60, and 60 and over), histological type of mesothelioma, hemoglobin, erythrocyte sedimentation rate (ESR), alanine aminotransferase, alkaline phosphatase, aspartate aminotransferase, forced expiratory volume for 1 second, history of the disease, blood loss, radical surgery (RO, R1), and treatment (IHPC or without) were studied as possible prognostic factors. The clinical characteristics of patients are shown in Table 1. Significant differences between two groups were not revealed.

Table 1.

The clinical characteristics of patients with MPM

Features	Treatment	
	Group 1	Group 2
Mean age (y)	51.9±8.9	51.9±9.6
Gender (men/women)	6/3	5/6
Tumor localization (right/left)	5/4	5/6
Histology of MPM (S/E)	1/8	1/10
Lymph nodes (N0/N2)	1/8	2/9
Hemoglobin (≥110/<110g/L)	8/1	8/3
ALT (20 IU/L/>20IU/L)	9/0	9/2
AST (20 IU/L/>20IU/L)	9/0	9/2
ALP (120 IU/L/>120 IU/L)	9/0	10/1
FEV1 (<1,500 mL/≥1,500 mL)	0/9	1/10

S - sarcomatoid type; E - epithelioid type; ALP - Alkaline phosphatase

Results

TNM staging is shown in Table 2. Lymph node metastasis was found in 18(90%) of patients; it was found involving all groups of mediastinal lymph nodes on the affected side. Stage II was diagnosed only in 1 patient. In all remaining cases, the tumor had a greater spread. The histological type of mesothelioma is shown in Table 3. Only one patient in each group had sarcomatoid type of mesothelioma.

Table 2.

Distribution of patients according to TNM staging

TNM	Stage	Number of patients (%)
T2N0M0	II	1 (5)
T3N0M0	III	1 (5)
T2N2M0	III	2(10)
T3N2M0	III	16 (80)
All patients		20 (100)

Table 3.

Histological type of MPM

Histological type	Number of patients (%)
Epithelioid MPM	18 (90)
Sarcomatoid MPM	2(10)
All patients	20 (100)

As is known, IPHC has a systemic and local impact aimed at preventing a relapse of MPM. Assessment of *therapeutic* pathomorphism of tumor according to G.A. Lavnikova [9] was used for the analysis of the cytotoxic effect of IPHC. After IPHC, the second degree of *therapeutic* pathomorphism of tumor was identified. This indicates the effectiveness of IPHC in treatment of MPM.

Overall, the 5-years survival of patients after radical surgery was 23.8±12.7% (Fig. 1). Life expectancy was 29.8 months (95% CI: 16.6–43.0 months); median survival was 18.0 months (95% CI: 3.6–32.3 months).

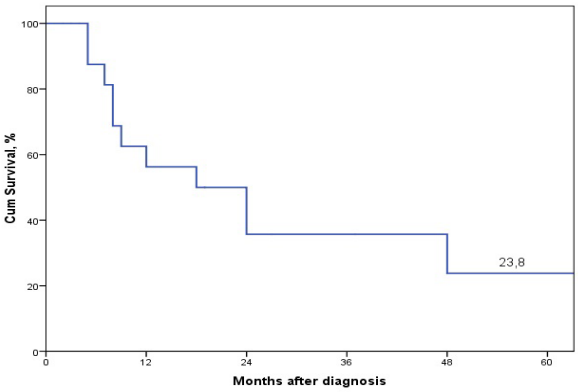

Figure 1. *The 5-year survival of patients with MPM after radical surgery.*

Analysis of the comparative effectiveness of combined and multimodality treatment revealed that 5-year survival was statistically significantly higher when IPHC was used (*P=0.01*, Fig. 2). Age, histopathological subtype and treatment were identified as independent prognostic factors according to

multivariate analysis (Table 4). Poor prognostic factors were age ≥60 years and sarcomatoid type of mesothelioma.

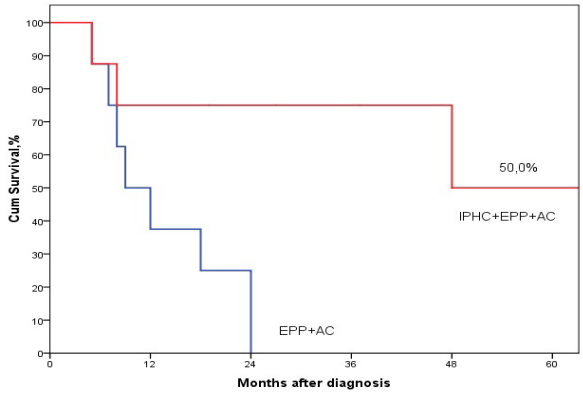

Figure 2. *The 5-year survival of patients with MPM in Groups 1 and 2.*

Table 4.

Prognostic factors in patients with MPM

Variable	Evidence (coding)	Factor β	Level of significance P	Exp(β)
X1	Treatment (combined – 0, multimodality - 1	-2.455	0.022	0.086
X2	Histological type (sarcomatoid - 1, epithelioid - 0)	6.047	0.002	422,910
X3	Age (under 60 y - 0, 60 and over - 1)	2.659	0.002	14,281

The application of IPHC in multimodality treatment was found to be associated with improved survival. According to the resulting model, the hazard ratio (HR) in patients with MPM is defined by the equation:

$$HR=\frac{\exp\left(-2.44\,X_1^*+6.047\,X_2^*+2.659X_3^*\right)}{\exp\left(-2.44\,X_1+6.047X_2+2.659X_3\right)}=\exp[-2.44\bullet(X^*_1-X_1)+6.047(X^*_2-X_2)+2.659(X^*_3-X_3)],$$

where X^*_1, X^*_2, X^*_3 and X_1, X_2, X_3 are values of prognostic variables in 2 patients.

Poor prognosis for survival occurs in patients over 60 years of age with the sarcomatoid type of mesothelioma and without IPHC that corresponds to a set of prognostic variables:

$X^*_1=0$, $X^*_2=1$, and $X^*_3=1$

In a favorable prognosis, the value of the prognostic variables:

$X_1=1$, $X_2=0$, and $X_3=0$

The value of HR for patients with poor and favorable prognosis will be:

HR=exp[-2.455(0-1)+6.047(1-0)+2.659(1-0)]=exp(11.161)=70,333.3

In this way, the conditional probability of death per unit of time will be in 70,333.3 times higher in patients with poor prognosis compared to those with a favorable prognosis. For patients over 60 years of age with epithelioid mesothelioma and treatment without IPHC, the conditional probability of death will be in 166.3 times higher compared to patients under 60 years of age who will receive the multimodality treatment with IPHC:

HR=exp[–2.455(0–1)+6.047(0–0)+2.659(1–0)]=exp(11.146)=166.3

If patients differ only in the treatment used, HR will be:

HR=exp[–2.455(0–1)+6.047(0)+2.659(0)] =exp(2.455)=11.7

This confirms the favorable prognosis for IPHC application for treatment of mesothelioma.

Figure 3 demonstrates the influence of prognostic factors on the survival rates in patients with MPM. In the presence of unfavorable factors all patients died within a year. With favorable prognostic factors, the 5-year survival was 57.1±24.9%. The actual data are in good agreement with the survival estimated according to the Cox model.

Discussion

There is relatively little research on the survival predictors in patients with MPM who have had the surgical and multimodal treatment. D.J. Sugarbaker et al. analyzed 183 patients and concluded that for the non-epithelioid cell type of MPM, positive resection margins and metastatic extrapleural nodes were negative prognostic factors [10].

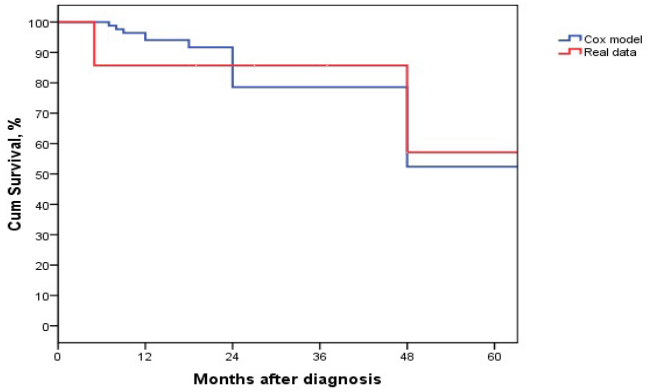

Figure 3. *The estimated survival for patients with MPM according to the Cox model*

R.M. Flores et al. have shown the benefit of multimodality therapy. Other positive factors according to this research were gender (female), left-side tumor, lack of pain syndrome and lack of exposure to asbestos [11].

M. de Perrot et al. examined the results of trimodality therapy with cisplatin-based chemotherapy followed by EPP and adjuvant high-dose (50 to 60 Gy) hemithoracic radiation therapy for MPM. According to this study, the presence of N2 disease was a significant marker of poor outcome, despite completion of the entire trimodality regimen. [12].

According to the largest international database (3101 patients with MPM from 15 centers and 4 continents) developed by the International Association for the Study of Lung Cancer Staging Committee, median survivals by clinical TNM and pathological TNM were similar: stage I, 21 months; stage II, 19 months; stage III, 16 months; and stage IV, 12 months. Median survival by histology: epithelioid 19 months, biphasic 13 months, and sarcomatoid 8 months. By multivariable analyses, significant differences in overall survival were seen for: T4 versus T3 and T3 versus T2 but not T2 versus T1; N0 versus N1 and N2 but not N1 versus N2; stages III and IV versus I but not II versus I; epithelioid histology versus other; age of female versus age of male; and palliative versus curative-intent surgery [13].

A Report from the IASLC Staging Committee analyzed prognostic variables in a surgical population, which are supplementary to previously published CORE variables (stage, histology, sex, age, and type of procedure). Lack of adjuvant therapy, along with the presence of asbestos exposure, weight loss, and chest pain, as well as low hemoglobin, high platelet count, and high white blood count, was found to be associated with a worse prognosis independent of the CORE variables [14].

Our data suggest the prognostic role of MPM histology and age in patients managed with EPP, which is consistent with other studies. In our study, the predictive value of IPHC with MPM has been shown for the first time. The use of IPHC in multimodality treatment enhances the survival rate in MPM patients; this fact confirms the synergetic antitumor effect for the combination of the hyperthermia with chemotherapy against the neoplastic cells [15]. Unfortunately, the small number of patients restricts the possibility for evaluating other prognostic factors. However, our results showed an improvement in survival for MPM patients under 60 years of age, who were managed with IPHC and had the epithelioid tumor type.

Conclusion

Patient's age over 60 years and the sarcomatoid type of the tumor can be regarded as prognostic factors for poor survival in patients with MPM who underwent EPP. Application of IPHC as a part of a multimodality treatment enhances the survivability of MPM patients.

Competing interests

The authors declare that they have no competing interests.

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CLINICAL RESEARCH

KRAS Gene Mutations and Gender Differences in Colorectal Cancer

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Abstract

The aim of this study was to investigate the frequency and spectrum of KRAS mutations in men and women with colorectal cancer (CRC), and an impact of KRAS-mutation status on the clinical and morphological features of CRC. The study included 303 patients (168/55.4% women and 135/44.6% men) with CRC T2-4N0-2M0-1. We defined 7 KRAS SNP-mutations (G12D, G12A, G12R, G12C, G12S, G12V and G13D) located within codons 12 and 13 using Bio-Rad real-time thermal cyclers CFX96 and Real-Time-PCR- KRAS-7M Kit. The frequency of KRAS mutations was 35.6% in the CRC patients with a predominant presence of G>A transitions. The KRAS codon 12 and 13 mutations are predictive of poor prognosis The KRAS-mutated CRC has clinical features in view of the gender differences. KRAS-mutation status is a promising predictive biomarker of personalized treatment.

Keywords: *KRAS codon 12 and 13 mutations; colorectal cancer; gender differences.*

Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide [1]. This localization of a malignant tumor is a prime example of the successful application of the fundamental advances in clinical practice [2]. In the last decade, significant improvements have been made in response rates, progression-free survival, and overall survival [3-6]. These significant improvements are mainly a result of the development of new combinations of standard chemotherapy and also new therapeutic agents targeting molecular events involved in colorectal carcinogenesis.

One of the most promising targets is the epidermal growth factor receptor (EGFR), which is activated in colorectal carcinogenesis by the binding of a ligand on the extracellular part of it [7]. With the emergence of two anti-eEGFR-targeted antibodies, cetuximab and panitumumab, the treatment of metastatic colorectal cancer has entered into the era of personalized treatment. However, EGFR, the target of these drugs, which is over expressed in approximately 80% of colorectal carcinomas, failed to predict a therapeutic response when used clinically [8,9]. Therefore, downstream signaling effectors were sought to help predict the efficacy of anti-EGFR treatment.

KRAS is a small G protein that acts as a transducer in the EGFR pathway. KRAS is a membrane-anchored guanosine triphosphate/guanosine diphosphate (GTP/GDP)-binding protein and is widely expressed in most human cells. As a small GTPase (GTP cleaving enzyme), KRAS is involved in intracellular signal transduction and mainly responsible for EGFR-signaling activation [10]. The exchange of the active GTP-bound state and the inactive GDP-bound state is tightly controlled by GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors [11]. Under normal physiological conditions, upstream signals activate wild-type KRAS by promoting the exchange of bound GDP for GTP. This process is transient because of GAP-mediated GTP hydrolysis. However, this process becomes altered when the KRAS gene is mutated. When there is a point mutation in codon 12 [12], the protein is locked in the active state and constitutively transmits to the nucleus mitogenic signals.

Mutant KRAS is found in about 35%-45% of CRCs [13-15], and codon 12 and 13 are two hotspots, which account for about 95% of all mutation types, with approximately 80% occurring in codon 12 and 15% in codon 13. KRAS mutations are almost single nucleotide point mutations as reported, and the most common patterns are G12D, G12A, G12R, G12C, G12S, G12V and G13D. In the codon 12 mutation, p.G12D, pG12V is the most frequent, and in codon 13, the substitution of glycine for aspartate (p.G13D) is the most frequent [16].

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These mutations impair the intrinsic GTPase activity of KRAS and prevent GAPs from promoting GTP hydrolysis by KRAS, therefore causing KRAS proteins to accumulate in the GTP-bound, active form. In this manner, mutant KRAS results in a constitutively active GTP-bound state and the activation of downstream pro-proliferative signaling pathways [17, 18]. Therefore, KRAS mutations play a critical role in human tumorigenesis and are the most prevalent in colorectal cancer. Because *KRAS* is the most frequently mutated factor downstream of the EGFR signaling pathway, it was considered a candidate molecular biomarker for anti-EGFR therapy [19]. At the same time the associations between KRAS-mutation status and the clinical, morphological, and biological characteristics of CRC, as well as gender differences, are unclear [20,21].

The aim of this study was to investigate the frequency and spectrum of KRAS mutations in men and women with CRC, and an impact of KRAS-mutation status on the clinical and morphological features of CRC.

Material and Methods

The study included 303 patients (168/55.4% women and 135/44.6% men) with CRC T2-4N0-2M0-1 treated in the Rostov Cancer Research Institute between 2011and 2014. The age distribution was as follows: the age group under 45 years included 26(8.6%) patients, the 45-to-54 age group included 54(17.8%) patients, the 55-to-64 age group 131(43.2%) patients, and age group over 65 years included 92(30.4%) patients.

The tumor was located in the ampullar part and anal canal of the rectum in 127(41.9%) patients, in the rectosigmoid part of the rectum in 97(32.0%) patients, in the left side of the descending colon in 30(9.9%) patients, and in the right side of the descending colon in 49(16.2%) patients. In all patients, histologically, the tumor was characterized as an adenocarcinoma of varying degrees of differentiation (G2 in 266/87.8% patients, G3 in 29/9.6% patients, and G1 in 8/2.6% patients). We revealed the following T-stages of CRC: T4-stage in 158(52.1%) patients, T3-stage in 80(26.4%), T2-stage in 63(20.8%), and T1-stage in 2(0.7%) patients. All the patients underwent the cytoreductive and radical surgery.

DNA was extracted from paraffin-embedded, formalin-fixed tumor tissue using a QIAamp® DNA FFPE Tissue Kit (QIAGENE, Germany) according to the manufacturer's protocol. The concentration of the extracted DNA was measured by a Qubit 2.0® Fluorometer using Quant-iT ™ dsDNA Kit. The concentration of DNA was normalized to a value of 1 ng/ml.

We defined 7 KRAS SNP-mutations (G12D, G12A, G12R, G12C, G12S, G12V and G13D) located within codons 12 and 13 using Bio-Rad real-time thermal cyclers CFX96 and Real-Time-PCR- KRAS-7M Kit.

Results were statistically processed using the *software* package Statistica 8.0 and the Excel package of Microsoft Excel 2010. We used the Chi-square test to compare observed data. A probability value of $P<0.05$ was considered statistically significant.

Results and Discussion

KRAS codon 12 and 13 mutations were detected in 108(35.6%) patients, which is consistent with other studies [20]. KRAS-mutated CRC was identified in 65(60.2%) women and in 43(39.8%) men (Table 1).

Table 1.

Clinical and morphological features of CRC in patients with KRAS codon 12 and 13 mutations

Features	Women (n=65)	Men (n=43)
Age distribution		
under 45 years	10 (15.3%)	1 (2.3%)*
45-to-54 years	5 (7.7%)	3 (7.0%)
55-to-64 years	25 (38.5%)	23 (53.5%)*
over 65 years	25 (38.5%)	16 (37.2%)
Localization of tumor		
rectum	31 (47.7%)	14 (32.6%)*
rectosigmoid area	18 (27.7%)	14 (32.6%)
the left side of the DC	8 (12.3%)	6 (14.0%)
the right side of the DC	8 (12.3%)	9 (20.8%)
Histology:		
G1 adenocarcinoma	2 (3.0%)	-
G2 adenocarcinoma	56 (86.2%)	40 (93.0%)
G3 adenocarcinoma	7 (10.8%)	3 (7.0%)
T-stage		
2	11 (16.9%)	2 (4.7%)
3	18 (27.7%)	12 (27.9%)
4	36 (55.4%)	28 (65.1%)*
KRAS mutation		
G>A transitions	40 (61.5%)	27 (62.8%)
G>T transversions	13 (20.0%)	8 (18.6%)
G>C transversions	12 (18.5%)	8 (18.6%)

*Note: DC- descending colon; * - P<0.05 between groups.*

Among patients with KRAS-mutated CRC, the age distribution was as follows: the age group under 45 years included 11(10.2%) patients, the 45-to-54 age group included 8(7.4%) patients, the 55-to-64 age group 48(44.4%) patients, and age group over 65 years included 41(38.0%) patients. The tumor was located in the ampullar part and anal canal of the rectum in 45(41.7%) patients, in the rectosigmoid part of the rectum in 32(29.6%) patients, in the left side of the descending colon in 14(13.0%) patients, and in the right side of the descending colon in 17(15.7%) patients. The postoperative histological study found that a moderately differentiated adenocarcinoma was predominant in patients (96/88.9%) with KRAS-mutated CRC. Poorly differentiated adenocarcinoma was found in 10(9.3%) patients and well differentiated adenocarcinoma only in 2(1.8%) patients. In patients with KRAS-mutated CRC, we revealed the following T-stages of CRC: T4-stage in 64(59.3%) patients, T3-stage in 30(27.8%), T2-stage in 13(12.0%), and T1-stage in 1(0.9%) patients.

G>A transitions (G12S, G12D, and G13D mutations) were detected in 67(62.0%) patients. This type of mutation was found in 40(59.7%) women and 27(40.3%) men. The age distribution was as follows: the age group under 45 years included 7(10.4%) patients, the 45-to-54 age group included 6(8.9%) patients, the 55-to-64 age group 31(46.3%) patients,

and age group over 65 years included 23(34.4%) patients. The tumor was located in the ampullar part and anal canal of the rectum in 28(41.8%) patients, in the rectosigmoid part of the rectum in 15(22.4%) patients, in the left side of the descending colon in 9(13.4%) patients, and in the right side of the descending colon in 15(22.4%) patients. Histologically, G>A transitions were associated with a moderately differentiated adenocarcinoma in 56(83.6%) cases. Poorly differentiated adenocarcinoma was found in 9(13.4%) patients and well differentiated adenocarcinoma only in 2(3.0%) patients. In patients with G>A transitions, we revealed the following T-stages of CRC: T4-stage in 37(55.2%) patients, T3-stage in 20(29.9%), and T2-stage in 10(14.9%).

G>T transversions (G12V and G12C mutations) were detected in 21(19.4%) patients. This type of mutation was found in 13(61.9%) women and 8(38.1%) men. The age distribution was as follows: the age group under 45 years included 2(9.5%) patients, the 45-to-54 age group included 2(9.5%) patients, the 55-to-64 age group 8(38.1%) patients, and age group over 65 years included 9(42.9%) patients. The tumor was located in the ampullar part and anal canal of the rectum in 6(28.6%) patients, in the rectosigmoid part of the rectum in 10(47.6%) patients, in the left side of the descending colon in 4(19.0%) patients, and in the right side of the descending colon in 1(4.8%) patients. Histologically, G>T transversions were associated with a moderately differentiated adenocarcinoma in 20(95.2%) cases. Poorly differentiated adenocarcinoma was found in 1(4.8%) patients; well differentiated adenocarcinoma was not found. In patients with G>T transversions, we revealed the following T-stages of CRC: T4-stage in 16(76.2%) patients, T3-stage in 4(19.0%), and T2-stage in 1(4.8%).

G>C transversions (G12A and G12R mutation) were detected in 20(18.6%) patients. This type of mutation was found in 12(60.0%) women and 8(40.0%) men. The age distribution was as follows: the age group under 45 years included 2(10.0%) patients, the 55-to-64 age group 9(45.5%) patients, and age group over 65 years included 9(45.5%) patients. The tumor was located in the ampullar part and anal canal of the rectum in 11(55.0%) patients, in the rectosigmoid part of the rectum in 7(35.0%) patients, in the left side of the descending colon in 1(5.0%) patients, and in the right side of the descending colon in 1(5.0%) patients. Histologically, G>C transversions were associated with a moderately differentiated adenocarcinoma in 19(95.0%) cases. Poorly differentiated adenocarcinoma was found in 1(5.0%) patients; well differentiated adenocarcinoma was not found. In patients with G>C transversions, we revealed the following T-stages of CRC: T4-stage in 12(60.0%) patients, T3-stage in 6(30.0%), T2-stage in 2(10.0%).

KRAS codon 12 and 13 mutations were not detected in 195(64.4%) patient. KRAS-wild-type CRC was found in 103(52.8%) women and 92(47.2%) men. In patients with KRAS-wild-type CRC, the age distribution was as follows: the age group under 45 years included 15(7.7%) patients, the 45-to-54 age group included 46(23.6%) patients, the 55-to-64 age group 83(42.6%) patients, and age group over 65 years included 51(26.1%) patients. The tumor was located in the ampullar part and anal canal of the rectum in 82(42.1%) patients, in the rectosigmoid part of the rectum in 65(33.3%) patients, in the left side of the descending colon in 16(8.2%) patients, and in the right side of the descending colon in 32(16.4%) patients. Histologically, KRAS-wild-type CRC was associated with a moderately differentiated adenocarcinoma in 170(87.2%) cases. Poorly differentiated adenocarcinoma was found in 19(9.7%) patients, well differentiated adenocarcinoma in 6(3.1%) patients. In patients with KRAS-wild-type CRC, we revealed the following T-stages of CRC: T4-stage in 99(50.8%) patients, T3-stage in 45(23.1%), T2-stage in 50(25.7%), and T1-stage in 1(0.4%).

Results of the analysis of clinical and morphological features of CRC based on gender differences are shown in Tables 2 and 3.

Table 2.

Clinical and morphological features of CRC in women with KRAS codon 12 and 13 mutations

Features	G>A transitions (n=40)	G>T transversions (n=13)	G>C transversions (n=12)
Age distribution			
under 45 years	6 (15.0%)	2 (15.4%)	2 (6.6%)
45-to-54 years	3 (7.5%)	2 (15.4%)	-
55-to-64 years	15 (37.5%)	5 (38.5%)	5 (41.7%)
over 65 years	16 (40.0%)	4 (30.7%)	5 (41.7%)
Localization of tumor			
rectum	19 (47.5%	5 (38.5%)	6 (50.0%)
rectosigmoid area	7 (17.5%)	6 (46.1%)	6 (50.0%)
the left side of the DC	6 (15.0%)	2 (15.4%)	-
the right side of the DC	8 (20.0%)	-	-
Histology:			
G1 adenocarcinoma	2 (5.0%)	-	-
G2 adenocarcinoma	31(77.5%)	13 (100%)	12 (100%)
G3 adenocarcinoma	7 (17.5%)	-	-
T-stage			
2	9 (22.5%)	-	2 (16.7%)
3	12 (30.0%)	3 (23.1%)	3 (25.0%)
4	19 (47.5%)	10 (76.9%)	7 (58.3%)

Among the 168 women with CRC, KRAS codon 12 and 13 mutations were detected in 65(38.7%) patients. Most women with KRAS-mutated CRC were over 55 years of age. Among the KRAS codon 12 and 13 mutations in the CRC women, G>A transitions were predominated (40/61.5% of patients). G>T and G>C transversions were detected in 13(20.0%) and 12(18.5%) women, respectively. Among the 168 CRC women, the KRAS-wild-type CRCs were observed in 103(61.3%) patients.

Among the 135 men with CRC, KRAS codon 12 and 13 mutations were detected in 43(31.9%) patients. Most men with KRAS-mutated CRC were over 55 years of age. Among the KRAS codon 12 and 13 mutations in the CRC men, G>A transitions were predominated (27/62.8% of patients). G>T and G>C transversions were detected in 8(18.6%) and 8(18.6%) men, respectively. Among the 168 CRC women, the KRAS-wild-type CRCs were observed in 92(68.1%) patients.

Table 3.

Clinical and morphological features of CRC in men with KRAS codon 12 and 13 mutations

Features	G>A transitions (n=27)	G>T transversions (n=8)	G>C transversions (n=8)
Age distribution			
under 45 years	1 (3.7%)	-	-
45-to-54 years	3 (11.1%)	-	-
55-to-64 years	16 (59.3%)	3 (37.5%)	4 (50.0%)
over 65 years	7 (25.9%)	5 (62.5%)	4 (50.0%)
Localization of tumor			
rectum	9 (33.3%)	1 (12.5%)	5 (62.5%)
rectosigmoid area	8 (29.7%)	4 (50.0%)	1 (12.5%)
the left side of the DC	3 (11.1%)	2 (25.0%)	1 (12.5%)
the right side of the DC	7 (25.9%)	1 (12.5%)	1 (12.5%)
Histology:			
G1 adenocarcinoma	-	-	-
G2 adenocarcinoma	25 (92.6%)	7 (87.5%)	7 (87.5%)
G3 adenocarcinoma	2 (7.4%)	1 (12.5%)	1 (12.5%)
T-stage			
2	1 (3.7%)	1 (12.5%)	-
3	8 (29.7%)	1 (12.5%)	3 (37.5%)
4	18 (66.6%)	6 (75.0%)	5 (62.5%)

Our study showed no difference in the frequency of the KRAS codon 12 and 13 mutations in men (43/31.9%) and women (65/38.7%) with CRC. KRAS gene mutations were more common when a tumor was located in the rectum (*P<0.05*). KRAS-mutated CRC was presented as a moderately differentiated adenocarcinoma in 96/88.9% of patients (*P<0.05*) and characterized by a high degree of tumor spread. After surgery, T3-4 states were significantly predominant in the KRAS-mutated CRC than the KRAS-wild-type CRC (87.1% vs. 73.9%, *P<0.05*). These data indicate a worsening prognosis in the CRC patients with KRAS codon 12 and 13 mutations. In our study, among the KRAS codon 12 and 13 mutations, G> A transitions were predominant (66.2%). We revealed some patterns of clinical manifestations depending on the mutation type. G>A transitions compared to other mutations were associated with tumor localization in the right side of the descending colon in a larger number of cases (*P<0.05*). G>T transversions, compared to other mutations, were associated with tumor localization in the ampullar part in a larger number of cases and in the anal canal of rectum in fewer cases (*P<0.05*). In contrast, G>C transversions were associated with a tumor localization in the rectum in 90% of cases (*P<0.05*). G>T transversions compared to other mutations were associated with T4-stage of CRC with the highest frequency (76.2%, *P<0.05*).

Women with KRAS-mutated CRC compared to men were more often in the age group under 45 years (15.3 and 2.3%, respectively) and less in the 55- to- 64 year age group (38.5 and 53.5%, respectively, *P<0.05*). In women in the age group over 65 years compared to men, G>A transitions were more common (40% and 25.9%, respectively); on the contrary, G>T transversions were more rare (30.7 and 62.5%, respectively, *P<0.05*). In women with KRAS-mutated CRC compared to men, tumor was often localized in the ampullar part and the anal canal of the rectum (47.7% and 32.6%, respectively, *P<0.05*) that was associated with an increase in the number of G>A transitions and G>T transversions. In women with G>C transversions compared to men, tumor was detected only in the rectum (100% vs 75% patients, *P<0.05)*. G>T and G>C transversions in men compared to women were more often associated with the localization of tumor in the descending colon (37.5 and 25.0% vs. 15.4% and 0%, respectively, *P<0.05)*. In men with KRAS-mutated CRC, T4-stage of CRC was diagnosed significantly more often in the background of the high frequency of G>A transitions.

Thus, the KRAS codon 12 and 13 mutations are more common in tumors with the rectum localization and T3-4 stages of the disease. These mutations are predictive of poor prognosis. Identified patterns of CRC clinical manifestations according to gender and type of mutations allow to determine the potential biological behavior of CRC based on the assessment of the KRAS-mutation status and create the possibility for personalized approach to therapy and optimize an individual monitoring of patient condition.

Conclusions

- The frequency of KRAS mutations was 35.6% in the CRC patients with a predominant presence of G>A transitions (62%). The KRAS codon 12 and 13 mutations were associated with the tumor localization in the rectum (71.3%) and a moderately differentiated adenocarcinoma (88.9%, *P<0.05*).

- The KRAS codon 12 and 13 mutations are predictive of poor prognosis. The presence of these mutations compared to KRAS-wild-type CRC was significantly more associated with T3-4 stages of CRC.

- The KRAS-mutated CRC has clinical features in view of the gender differences. Women with KRAS-mutated CRC compared to men were more often in the age group under 45 years (15.3 vs. 2.3%, *P<0.05*) and less in the 55-to-64 year age group (38.5 vs. 53.5%, *P<0.05*). In women with G>C transversions compared to men, tumor was detected only in the rectum (100% vs 75% patients, *P<0.05*). G>T and G>C transversions in men compared to women were more often associated with the localization of tumor in the descending colon (37.5 and 25.0% vs. 15.4% and 0%, respectively, *P<0.05*). In men with KRAS-mutated CRC, T4-stage of CRC was diagnosed significantly more often in the background of the high frequency of G>A transitions.

Competing interests

The authors declare that they have no competing interests.

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CLINICAL RESEARCH

Outcomes of the Ross Procedure in the Pediatric Population

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Abstract

The Ross procedure (RP) is considered by many authors as the best option for aortic valve replacement in young children and adolescents. This paper presents the results of the immediate and late postoperative period (more than 5 years) after the RP among pediatric patients.

Keywords: *Ross procedure; pediatric population; immediate results; long term outcome.*

Introduction

Congenital anomalies of the aortic valve account for up to 8% of all congenital heart malformations. In general, the proportion of pathology of the aortic valve is 30–35% of patients with valvular heart disease, ranking second after mitral valve disease. The relatively high incidence of the congenital and acquired diseases of the aortic valve and aortic root necessitates a constant search for the best options for its surgical correction and optimization of the existing methods [1].

The RP, using the native pulmonary autograft as the aortic valve, provides good hemodynamic parameters in children with growth potential and eliminates the need for anticoagulants. However, to date, this method of aortic valve restoration in pediatric patients as a radical procedure is still being debated [2].

Some researchers consider this technology very effective for the correction of aortic valve disease in the whole pediatric population: in young children as well as adolescents [3,4]. However, an analysis of recent literature on the subject reveals that, despite the numerous publications, long-term effects of the RP in pediatric patients, in comparison with those for other types of operations, remain poorly studied. Such studies would enable the effectiveness of this procedure to be assessed in the long term, to clarify the indications for its conduct in patients with different types of aortic defects, and to optimize the selection criteria. In this regard, the study of the immediate and long-term results of the RP in the pediatric population can be a very relevant and meaningful.

The aim of this study was to examine the results of the immediate and late postoperative period (more than 5 years) after the RP among the pediatric patients treated at the Center for Pediatric Cardiac Surgery of the Academician E.N. Meshalkin Novosibirsk Research Institute of Circulation Pathology (NRICP).

Material and Methods

The early and long-term results of the RP were analyzed using the data from 114 pediatric patients who were operated at the Center between 2002 and 2012. The study was approved by Ethics Committee of the NRICP. Written informed consent was obtained from the child's parents.

The age of patients ranged from 12 days to 18 years. The age distribution was as follows: 3(2.6%) patients between 12 days and 1 year, 18(15.8%) patients between 1 and 7 years, 72(63.2%) patients between 7 and 16 years, 21(18.4%) patients between 16 and 18 years. The average body weight of the patientswas 39.17±17.93 kg (between 2.9 and 87 kg).

In accordance with the diagnosis, the distribution of the operations patients received was as follows: isolated aortic valve stenosis (IAVS) in 38(33.3%), aortic valve insufficiency

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(AVI) in 33(28.9%), and mixed lesion (ML) in 56(49.1%). Total aortic root replacement by pulmonary autograft with reimplantation of the coronary arteries in the wall of the pulmonary autograft by type «total root replacement» was performed in 105(88.2%) patients. The subcoronary insertion technique was applied in 14(15.9%) cases. To replace the valve in the pulmonary position we used the pulmonary allografts (homografts) in 24(21.1%) cases and xenograft in 90(78.9%) cases. Among the xenografts, there were "Kemerovo" AV composite (diepoxy-treated xenoconduit) in 40(44.4%) cases, "BioLAB" (glutaraldehyde-treated xenoconduit) in 17(18.9%) cases, and "Contegra" in 12(33.3%) cases. The average diameter of the conduit implanted in the pulmonary position was 23.54mm.

We used hypothermic extracorporeal circulation and Custodiol cardioplegia with external cooling of the heart with crushed ice. The extracorporeal circulation time (ECCT) lasted from 144 to 730 (244.2±42.7) minutes and the time of the aortic occlusion ranged from 90 to 282 (172.2±42.7) minutes.

The postoperative course was evaluated in terms of mortality and complication rates. In the long term, we analyzed the frequency of the reoperations, mortality rate, and the dynamics of the echocardiographic parameters.

Mean follow-up time was 3.9±1.9 years (from 1 to 9 years). The examination included at least two echocardiographic examinations (with transthoracic and transesophageal probes), one catheterization of the cardiac chambers, angiocardiography, and selective aorto-coronary angiography.

Statistical analysis was performed using the statistical software Statistica 7.0 for Windows. We used the chi-square test with the Yates' correction to compare the frequency of the binary trait in two unrelated groups of paired comparisons. For data with normal distribution, inter-group comparisons were performed using Student's t-test. The Mann-Whitney (U Test) was used to compare the differences between the two independent groups (for nonparametric data). We also applied the Cox regression model and the Kaplan-Meier method. The value of $P<0.05$ was considered significant.

Results

The average length a patient's stay in the intensive care unit after surgery was 3.48±2.90 days; the average period of hospitalization was 24.70±10.87 days. The mortality in the early postoperative period was 2.1% and the total complication rate was 51.5%. Among the complications, the most frequent were pericardial effusion (25.6%), cardiac and respiratory failure (7.6%), and cardiac arrhythmias (6.1%). To evaluate the factors affecting the incidence of complications in the hospital period, correlation analysis was performed. The relationship between the frequency of the complications in the hospital period and the child's age ($r=0.18; P=0.24$) and weight ($r=0.22; P=0.12$) was weakly positive. Between the frequency of the complications and LVEF, a statistically significant inverse correlation of moderate intensity was found ($r=-0.42; P=0.021$). The frequency of the complications weakly correlated with RVEDVI ($r=0.29; P=0.52$). The frequency of pericardial effusion was associated with the type of conduit: $r=0.35 (P=0.34)$ for Contegra, $r=0.33 (P=0.007)$ for diepoxy-treated xenoconduit "Kemerovo", and $r=0.42 (P=0.014)$ for glutaraldehyde-treated xenoconduit "Biolab".

The data obtained indicate a number of factors that can be considered as criteria for prediction of outcomes of the RP in children in the postoperative period. The results of multivariate regression analysis confirmed the data of the correlation analysis and led to the conclusion that some factors presented in Table 1 can be identified as the predictors of postoperative complications, such as pericardial effusion and cardiac and respiratory failure.

Table 1.

Risk factors for postoperative complications after the RP in accordance with the data of multivariate regression analysis

	Pericardial effusion		Cardiac and respiratory failure	
Factors	OR (95% CI)	*P*	OR (95% CI)	*P*
Age	1.30 (0.89 – 5.13)	0.042	3.59 (1.28 – 5.36)	0.004
Body weight	1.62 (0.44 – 2.15)	0.012	1.22 (1.39 – 5.65)	0.082
NYHA class	2.14 (1.30 – 3.62)	0.005	1.84 (1.54 – 3.65)	0.052
LV EF	2.78 (0.68 – 4.51)	0.014	1.26 (0.98 – 5.33)	0.054
ECCT	3.48 (1.15 – 5.21)	0.003	1.28 (0.96 – 5.24)	0.039
LVEDVI	0.28 (0.14 – 1.64)	<0.0001	2.14 (0.74 – 3.22)	0.015
RVEDVI	0.14 (0.04 – 1.43)	<0.0001	3.86 (1.49 – 5.63)	0.008
Homograft	1.95 (0.28 – 3.15)	0.033	0.45 (0.19 – 1.76)	<0.0001
«Contegra"	0.86 (0.24 – 2.18)	0.006	0.28 (0.06 – 1.12)	<0.0001
"Kemerovo" xenoconduit)	2.14 (1.78 – 6.27)	0.041	0.32 (0.12 – 1.37)	<0.0001
"BioLab" xenoconduit)	2.28 (1.98 – 6.97)	0.055	0.18 (0.04 – 1.02)	<0.0001

Thus, among the factors associated with the development of cardiac and respiratory failure, LVEDVI, RVEDVI, and age of children were the most significant predictors. Types of conduits were associated with a risk of pericardial effusion developing, the main negative factors being the diepoxy-treated and glutaraldehyde-treated xenoconduits implanted into the right-sided position. Along with these factors, it should also be mentioned that NYHA class, LV EF, and ECCT play a negative role in the development of this complication.

In the postoperative period, in the group from 1 to 9 years of age, 104 patients were examined. The death rate in the late postoperative period was only 1.14%, and the actuarial survival in the long-term period after surgery reached 98.86%.

Based on a catamnestic evaluation of echocardiographic parameters, we did not find signs of autograft valve insufficiency during the entire observation period. Also, there was no significant dilatation of the autograft root. Increasing of the autograft diameter was parallel to somatic growth of the observed patients. There were no significant changes in the value of the systolic pressure gradient across the implanted autograft valve compared with the value at the time of discharge (Table 2).

Table 2.

Systolic pressure gradients and Z- score on the autograft valve

Parameters	Period after surgery		
	1-2 years	3-4 years	≥5 years
LV/Ao gradient	6.1±1.9	6.7±2.7	8.5±3.4
Z- score	1.19±0.10	1.28±0.14	1.32±0.18

The frequency of reoperations in the late postoperative period was 23.7%. At the same time, we would like to emphasize that autograft dysfunction, which required reoperations during the observation period, was not observed; the main reason for reoperations was conduit dysfunction in the pulmonary artery position.

The most frequent cause of pulmonary conduit dysfunction was valve calcification (52% of cases, n=12). Conduit wall calcification was observed less frequently, in 30% of cases (n=7). Calcification at the distal anastomotic level was noted in 17% of the cases (n=4).

For a more complete characterization of the RP efficiency in the long term, a comparative analysis of the frequency of reoperations after the RP and aortic valve replacement during the 5-year follow-up was performed. We found that freedom from reoperation after the RP and aortic valve replacement was 97% and 94%, respectively (Fig.1); a Cox-Mantel test revealed no significant intergroup differences.

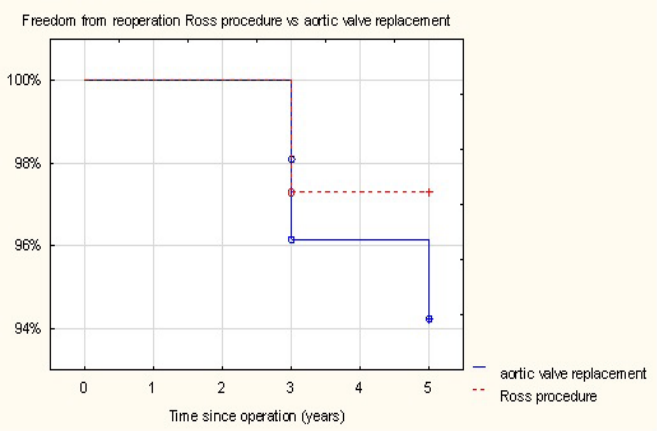

Figure 1. *Freedom from reoperation after the RP and aortic valve replacement*

Correlation analysis revealed a number of statistically significant relationships between the studied parameters and the freedom from reoperations in the long term. Thus, the frequency of reoperations was moderately positively associated with age (*r=0.38; P=0.004*) and male gender (*r=0.31; P=0.78*). At the same time, we discovered a moderate negative correlation between this parameter and the EF of both ventricles (RV - *r =- 0.35; P=0.011*; LV - *r=-0.40; P=0.036*). We also found a moderate positive correlation between the frequency of reoperations and the RV/LA gradients (*r=0.39, P=0.009* for mean value and *r=0.42, P=0.040* for peak value) and the ratio of the Ao/LA ring diameters (*r=0.45; P=0.001)*. The relationship with ECCT was less pronounced (*r=0.28; P=0.028*).

The frequency of reoperations depended on the type of conduits. Negative correlations were found with Contegra (*r=-0.38; P=0.001*) and homograft (*r=-0.26; P=0.026*); positive correlations were found with the diepoxy-treated (*r=0.31; P=0.47*) and glutaraldehyde-treated (*r=0.48; P=0.02*) xenoconduits. Unfortunately, the relatively small sample size limited a more detailed analysis.

The Cox regression model and the *Kaplan-Meier method* allowed us to establish the number of risk factors for reoperations due to conduit dysfunction in the right-sided positions after the RP. As can be seen from the obtained data, significant factors in the late postoperative period are the RV/LA gradients (*P=0.012*) (peak and mean values), RVEF (*P=0.035)*, and to a lesser degree, the ratio of the Ao/LA ring diameters. CPBT (*P=0.042*), which characterizes the technical complexity of the operation, can also be a predictor of reoperations in the long term (more stitches for bleeding, malposition conduit et al.).

As noted previously, the risk of operations was also related to the type of conduit used. It was found that the probability of dysfunction was greater with use of xenografts (Fig.2).

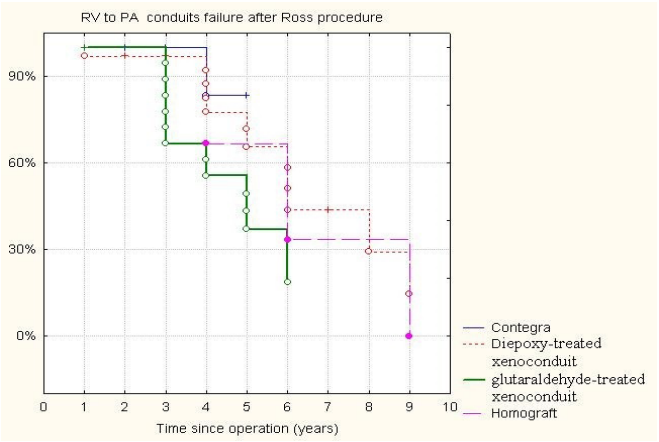

Figure 2. *Conduit dysfunction in the* pulmonary position *after the RP*

Discussion

In our study, the frequency of deaths in the early and long-term periods after the RP was 6.14% and 1.14%, respectively; actuarial survival in the long term was 98.86%. These data are consistent with the results obtained in other studies, where mortality in the pediatric group after the RP during 6.1 years of follow-up was 2.6%, and the 5- and 10-year survival rates were at 93.9% and 90.4%, respectively. If we compare these data with the frequency of deaths in open commissurotomy, the advantages of the RP become apparent. According to the data presented by various authors for open commissurotomy, the mortality ranges from 3 to 25%, averaging 12%, i.e., several times higher than for the RP [5-7].

The complication rate in our study was higher than the rate according to the data in the literature. For example, the study of T. Karamlou et al. showed that the complications

in the postoperative period were diagnosed in only 10 of 53 patients, whereas in this study the overall complication rate reached 51.5% [8]; however, differences in the structure of the complications were minor. In a study by T. Karamlou et al. and our study, pericarditis and cardiac arrhythmias were the leading complications, but T. Karamlou et al. also noted a relatively high incidence of bleeding in the postoperative period, whereas in our study, the pericardial effusion and cardiac/respiratory failure were observed more frequently.

In our study, the clinical efficacy of the RP was also demonstrated by the dynamics of hemodynamic parameters. Thus, in the early postoperative period, there was a trend toward a reduction of the left ventricle cavity, an increase in its contractility and a decrease in its stroke volume, and a decrease in systolic pressure gradient between the left ventricle and the ascending aorta in patients with IAVS. In addition, we have registered the absence of the hemodynamically significant systolic pressure gradients and regurgitation on the substituted valves in the left- and right-sided positions.

However, repeated operations over several years are almost inevitable. According to our data, the frequency of reoperation was 23.7%; the main indication for reoperation was conduit dysfunction in the pulmonary position, whereas cases of autograft dysfunction were not recorded. The authors of other studies also indicated that the autograft dysfunction was rare. According to the International Registry of the RP, the frequency of such complications is less than 1%. [6]. Comparison of the freedom from reoperations after the Ross procedure and other types of surgical interventions on the aortic valve revealed no significant difference. At the same time, according to other studies, in a 10-year follow-up reoperations were required in 26% of patients after aortic valve replacement and 28% of patients after an open commissurotomy [8,9]. When comparing the results of our study with the above-mentioned data, their longer duration of the follow-up must be taken into account; at the same time, the identified trends are very revealing because they were based on an analysis of a large single-center study.

In summary, it can be stated that the RP has a number of advantages for pediatric patients over other types of surgical interventions on the aortic valve. This procedure provides normalization of hemodynamic parameters, provides the growth potential for autograft proportionally to the child's growth, and (very important) demonstrates a high level of safety for the patient and positive long-term results. These findings are in agreement with the published data [10,11]. The listed advantages of the Ross procedure, despite its technical complexity, indicate that it is the best type of surgical intervention in aortic valve replacement in children. Risk factors for postoperative complications and reoperations, which were identified in our study, will clarify the selection criteria for the operation, as well as aid in developing new preparation schemes for surgery and protocols for the postoperative period, which will further improve the safety and efficacy of the RP.

Competing interests

The authors declare that they have no competing interests.

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CLINICAL RESEARCH

Surgical Treatment of Patients with Bilateral Atherosclerotic Lesions of Carotid Arteries

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Abstract

The objective of study was to improve the results of surgical treatment of patients with bilateral atherosclerotic lesions of the carotid arteries.

Material and Methods: The study included 180 patients between the ages of 42 and 82 (mean age 59±5.6 yrs) who underwent surgical treatment for bilateral atherosclerotic lesions of the carotid arteries during 2008-2014. Depending on the surgical treatment tactic, a total of 180 patients were divided into two groups. Group 1 included 60 (33.3%) patients who *underwent s*taged bilateral carotid endarterectomy (CEA). Group 2 included 120 (66.7%) patients, in whom the first stage of CEA was performed on the side of primary importance.

Results: The differentiated approach for identification the side for the first stage CEA is very important. Our experience shows that the intervals between CEA on both sides should not exceed 3 months and should be no less than 3 weeks. This tactic reduces not only mortality and complications, but also significantly improves the patient life quality by promoting rapid regression of neurological deficit.

Keywords: *bilateral atherosclerotic lesions; carotid arteries; staged bilateral carotid endarterectomy.*

Introduction

Atherosclerotic lesions of the brachiocephalic arteries are one of the main reasons of acute and transient brain circulation disorders [1-6]. Bilateral atherosclerotic lesions of carotid arteries are more usual then isolated lesions [7-8]. The most reliable and effective method for the prevention of stroke that is caused by atherosclerosis of carotid arteries is carotid endarterectomy (CEA). It is important to study the cerebral hemodynamics before planning intervention [9-11]. According to some authors, it is necessary to focus not only on the degree of stenosis but mainly on the embologenic properties of atherosclerotic plaque (ASP) [12-14].

Although, some questions have not been answered yet, namely criteria to be used for determining surgical tactics, the stages, and the sequence of the operations and the intervals between them? It should be borne in mind that a one-sided approach that considers the hemodynamic importance of the lesions only or the carotid pool with the prevalent carotid symptoms, is hopeless and fraught with serious consequences [15]. In connection with this, it is necessary to have a specific tactic in choosing a side with primary importance for CEA performance.

Taking into account the above-mentioned considerations, the aim of our study was to improve the results of the surgical treatment for patients with bilateral atherosclerotic lesions of the carotid arteries by determining the optimal time frame and the stages of the carotid reconstruction.

Material and Methods

The study included 180 patients between the ages of 42 and 82 (mean age 59±5.6 yrs) who underwent surgical treatment for bilateral atherosclerotic lesions of the carotid arteries during 2008–2014. Written informed consent was obtained from each patient. Depending on the surgical treatment tactic, a total of 180 patients were divided into two groups. Group 1 included 60/33.3% patients who underwent

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staged bilateral CEA. Group 2 included 120/66.7% patients, in whom the first stage of CEA was performed on the side of a primary importance. Patients of both groups did not differ significantly in mean age and sex.

A duplex scan (DS) was performed in all patients, using Samsung Medison SonoAce X6 (South Korea, 2008). The diameter of common carotid artery (CCA), the internal carotid artery (ICA), and external carotid artery (ECA), as well as CCA intima-media thickness (IMT) cerebral blood flow velocity (CBFV), the pulsatility index (PI) and resistance index (RI) were measured.

The ECST method of measuring carotid stenosis was applied [2]. All patients underwent duplex imaging investigation with identification of the percentage of stenosis, velocity and direction of the blood flow, and the structure of plaques. For a description of ASP, the Gray-Weale classification was used [16]. The volume velocity in the brachiocephalic arteries was measured at extracranial and intracranial levels. The volume velocity in CCA, ICA, ECA, and the vertebral artery was measured three times on each side, and then the average volume velocity was calculated on the left and right side of each patient. Hemodynamics in the intracranial arteries was analyzed using the analogical method for calculating average volume velocity. Performing transcranial duplex imaging, we paid attention to the blood flow direction and velocity in the MCA; compression tests for estimating the communicant arteries were also carried out. CT scans of the brain were performed in all patients to estimate the nature and localization of the brain lesions.

All patients were examined by a neurologist before and after intervention, during both the short and *l*ong postoperative periods. For quantifying neurological deficit in patients with ischemic stroke or transient ischemic attack (TIA), the Hachinski Ischemic Scale scale was used [17].

Statistical analysis was performed using the statistical software «Statistica». Data significance was assessed by the Student's t-test. Statistical significance was determined at $P<0.05$.

Results and Discussion

According to the A.V. Pokrovskiĭ classification, the patients of both groups were divided into stages of chronic cerebrovascular insufficiency (CCVI) (Table 1). In both groups, the stroke patients dominated.

Table 1.

Stages of chronic cerebrovascular insufficiency

Stages of CCVI	Number of the patients		Total %
	Group 1 (n=60)	Group 2 (n=120)	
Asymptomatic CCVI	3 (5.0%)	7 (5.8%)	5.6
TIA	12 (20.0%)	23 (19.2%)	19.4
Discirculatory encephalopathy	20 (33.3%)	42 (35.0%)	34.4
Consequences of ischemic stroke	25 (41.7 %)	48 (40.0%)	40.6
Total	180		100

According to the type of carotid reconstructions performed, patients of both groups were similar; in both groups the technical procedures of the operations were identical (Table 2). In total of 296 CEA were performed.

Table 2.

Types of of carotid reconstructions

Name of operation	Quantity of procedures				
	Group 1 (n=117)		Group 2 (n=200)		Total (n=317)
	1 stage	2 stage	1 stage	2 stage	
CEA with patch	30	33	41	59	166
Eversion CEA	21	24	44	41	130
ICA bypass grafting	1	-	-	-	1
ECA plasty	8	-	15		23

Group 1 patients underwent staged bilateral CEA. In patients with ischemic stroke, in determining the side for the carotid reconstruction, the preference was given to the appropriate carotid pool; the degree of stenosis played a secondary role. Twenty-two (36.7%) patients underwent CEA on the side of the ischemic episode. In 23 (38.3%) cases, the patients with discirculatory encephalopathy and asymptomatic patients underwent CEA on the side with a higher degree of stenosis. Most patients with TIA at the first stage intervention underwent CEA on the side of the ischemic episode regardless of the degree of stenosis on the contralateral side. Among patients in this group, unstable ASP was detected in 12 (20.0%) cases. The patients (8/13.3%) with ICA occlusion at the first stage intervention underwent CEA on the side of the ischemic episode regardless of the degree of stenosis on the contralateral side. Three (5.0%) patients with ICA occlusion underwent CEA on the contralateral side because of ischemic stroke in that carotid pool. The time frame of carotid reconstruction on the contralateral side varied from 2 weeks to 2 years (average period 5.2 months). Thirty-two (53.3%) patients of Group 1 underwent CEA within 6 months.

The analysis revealed a pronounced association between the clinical effect of the operation and the initial level of the neurological deficit, as well as time frame from stroke and time frame of the second stage of carotid reconstruction. The first stage of CEA was performed in 14/56.0% patients with a mild degree of neurological deficit, in 6/24.0% patients with a moderate degree of neurological deficit, and in 5/20.0% patients with a severe degree of neurological deficit.

Absolute increase in scores among different levels of neurological deficit was not the same for all patients. The highest rate of neurological deficit involution was observed in patients with a mild to moderate degree of neurological deficit, and who were operated on within 6 months of a stroke, and also in patients who underwent the procedure on the contralateral side within 2 or 3 months. According to the Hachinski Ischemic Scale, an increase of points in patients with mild, moderate, and severe degrees of neurological deficit was 29.2, 17.1, and 4.4, respectively.

In Group 1, the total complication rate was 5.0%. Ischemic stroke occurred in one (1.7%) patient in the carotid

artery operation pool after 30 days from the first stage of CEA. Neurological deficit totally disappeared during 15 days. On the contralateral side, SEA was performed after 2 months, and the patient was discharged in satisfactory condition. Perioperative ischemic stroke occurred in one patient with occluded ICA on the contralateral side. In one (1.7%) patient with a local occlusion of the left ICA and a complete occlusion of the right ICA, staged bilateral CEA was performed with an interval of 2 weeks because of frequent TIA on the contralateral side. This patient underwent hemorrhagic stroke after the 2nd stage of CEA. We suppose that this stroke resulted from irregular taking of hypotensive drugs and the development of the cerebral hyperperfusion syndrome after discharge from the hospital.

In Group 2 patients, the differentiated approach was performed to identify the side of primary importance in determining the indications for reconstruction. The severity of of the atherosclerotic lesions in the carotid pool was taken into account. Particular attention was paid to total stenosis of the carotid arteries and the volume velocity in the extra- and intracranial cerebral arteries while taking into account the adapted flow redistribution and the brain's tolerance to ischemia. The structure, surface and spread of the plaque according to the data of DS and MSCTA were also considered. The remoteness of the ischemic episode, and the severity and speed of the regress of neurological deficit were taken into account in patients with ischemic stroke in anamnesis. Thus, the preference was given to the side of the occlusion for the first stage of CEA in patients with occlusion of ICA and the opposite critical stenosis.

For an objective assessment of the carotid pool, we developed and applied the estimated scale of the severity of the carotid artery pool lesions (Table 3). Every lesion parameter was estimated in points. The first stage of CEA was performed on the side with the highest number of points that corresponded to the greatest lesion of the carotid artery pool. Two hundred CEAs were performed in Group 2 patients, and each case was considered independently. The timing for performing CEA on the contralateral side varied from 2 to 3 months.

The clinical effect of surgery was significantly increased by the identification of the most affected carotid pool and the choice of the optimal time frame for CEA on the contralateral side. Also in 22/18.3% patients with low brain tolerance to ischemia and severe lesions of carotid arteries on both sides and in patients with stroke we used an intra-arterial temporary shunt. In the cases of temporary shunting, a mean time of ischemia was 7±2 min, which was necessary to perform CEA, insert the shunt, and complete anastomosis.

At the first stage of CEA, 20/41.6% patients had a mild degree of neurological deficit; 22/45.8% patients, a moderate degree; and 6/12.5% patients, a severe degree. A full regress of the neurologic symptoms took place in 16/33.3% patients after CEA had been performed on the opposite side, and 3/6.2% patients passed from a moderate degree to a mild degree of neurological deficit.

In Group 2, the total complication rate was 1.7%. Ischemic stroke occurred in one patient after 5 months from the second stage of CEA. We believe that ischemic stroke was connected with discontinuation of hypotensive drugs and antiplatelats. It is necessary to underline that such complications as stroke and mortality were not detected in 30 days after 2nd stage CEA in this group of the patients.

Table 3.

The estimated scale of the severity of the carotid artery pool lesions

Variables			
On the right	Points	On the left	Points
1.Degree of stenosis		1.Degree of stenosis	
55 - 70%	1	55 - 70%	1
70 - 99%	2	70 - 99%	2
Occlusion	3	Occlusion	3
2. Stroke (terms) and TIA		2. Stroke (terms) and TIA	
TIA	3	TIA	3
< 6 months	2	< 6 months	2
> 6 months	1	> 6 months	1
3. Neurologic deficit		3. Neurologic deficit	
Permanent	1	Permanent	1
Hidden	2	Hidden	2
Progressive	3	Progressive	3
4. Number of the strokes (in anamnesis)		4. Number of the strokes (in anamnesis):	
1	1	1	1
2	2	2	2
>2	3	>2	3
5. Brain CT (sizes of the focus):		5. Brain CT (sizes of the focus):	
<3 cm	3	<3 cm	3
<6 cm	2	<6 cm	2
>6 cm	1	>6 cm	1
6. ASP features:		6. ASP features:	
Stabile	1	Stabile	1
Occlusion	2	Occlusion	2
Embologenous	3	Embologenous	3
7. Deficit of total cerebral blood flow		7. Deficit of total cerebral blood flow	
1 degree	1	1 degree	1
2 degree	2	2 degree	2
3 degree	3	3 degree	3
8. Compensation in the MCA during the clamped trials		8. Compensation in the MCA during the clamped trials	
Bad	1	Bad	1
Satisfactory	2	Satisfactory	2
Good	3	Good	3

The identification of the most important side for CEA with detection of the severity of the carotid artery pool lesion helped to reduce the complications connected with the stroke in 6 months after 2nd stage CEA from 5% to 1.7% (Table 4).

Table 4.

Character of complications

Complications	Group 1	Group 2	Total
Stroke on the atipsilateral side	2 (3.3%)	1 (0.83%)	3 (1.7%)
Stroke on the contralateral side	1 (1.7%)	1 (0.83%)	1 (0.5%)
"Stroke+mortality"	1 (1.7%)	-	1 (0.5%)
Total	3(5.0%)	2 (1.7%)	4 (2.2%)

The differentiated approach for identifying the side for the first stage CEA is very important. Our experience

shows that the intervals between CEA on both sides should not exceed 3 months and should be no less than 3 weeks. This tactic not only reduces mortality and complications, but also significantly improves the patient's quality of life by promoting rapid regression of ND. Determining the stages and timing of carotid reconstruction is very important. Choosing the side with more pronounced stenosis for the first stage of EAS contributed to the reduction of complications associated with stroke from 5.0% to 1.7%.

Competing interests

The authors declare that they have no competing interests.

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EXPERIMENTAL RESEARCH

Expression of Endoplasmic Reticulum Stress Related Genes in Blood Cells of Obese Boys with and without Insulin Resistance

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Abstract

Objective: to study the changes in the expression level of the subset of genes, encoding for important cell growth factors and enzymes, which play an important role in the control of cellular growth and apoptosis, in blood cells of obese boys with and without insulin resistance for evaluation of its possible significance in the development of obesity and impaired insulin sensitivity.

Material and Methods: We studied the expression of genes, which are responsible for control of cell growth and survival, in blood cells of obese boys with normal and impaired insulin sensitivity as compared to normal (control) individuals.

Results: It was shown that the expression level of *PLAGL, CYR61, ITGA5*, and *TFPI2* genes is increased, but the *GADD45A* gene is decreased in blood cells of obese children with normal insulin sensitivity as compared to the control group. Insulin resistance in obese boys leads to the up-regulation of *PPP1R15A* and *PLAGL*1 gene expressions as well as to the down-regulation of *TFPI2, GADD45A, ALDH1A2, CYR61*, and *HSPA6* genes in blood cells as compared to obese patients with normal insulin sensitivity.

Conclusion: Results of this study provide evidence that obesity affects the expression of the subset of genes related to the control of cell growth and survival in blood cells and that impaired insulin sensitivity in obesity is associated with changes in the expression level of *PLAGL, CYR61, GADD45A, PPP1R15A, TFPI2, ALDH1A2,* and *HSPA6* genes, which possibly contributes to the development of obesity and its metabolic complications, including insulin resistance.

Keywords*: mRNA expression; CYR61; PLAGL; ITGA5; PPP1R15A; GADD45A; TFPI2; blood; obesity; insulin resistance.*

Introduction

The development of obesity and its metabolic complications, one of the most profound public health problems, is associated with dysregulation of various intrinsic mechanisms, which control the most basic metabolic processes, as well as changes in numerous gene expressions, which contribute to the development of obesity as well as its metabolic complications and possibly reflect some changes in fat and other tissues. Obesity and its metabolic complications are associated with dysregulation of numerous intrinsic mechanisms, which control most of the key metabolic processes, including cellular growth, apoptosis, and insulin sensitivity [1-5]. Moreover, obesity and metabolic syndrome result from interactions between genes and environmental factors and are associated with changes in gene expressions of the regulatory network in adipose tissue as well as in various organs and tissues, including blood cells [5-9]. Adipose tissue growth is a center of obesity, is tightly associated with apoptosis and the cell proliferation processes, and is controlled by different interconnected regulatory factors and enzymes [1,6,8]. Special interest should be given to key regulatory factors and enzymes, which control cell growth and survival, especially *GADD45A, PLAGL1, CYR61, PPP1R15A, TFPI2, HSPA6,* and *ITGA5* [10-17].

The cysteine-rich angiogenic inducer 61 (*CYR61*), also known as CCN family member 1 and insulin-like growth factor-binding protein 10 (*IGFBP10*), is a secreted, cysteine-rich, heparin-binding protein encoded by a growth factor-inducible, immediate-early gene and acting as an extracellular, matrix-associated signaling molecule, which promotes matrix remodeling, cell proliferation, cell migration and adhesion by up-regulating the expression of a number of genes [10-12].

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Moreover, the expression of the *CYR61* gene is regulated by TP53, miRNA-22 and miRNA-100 as well as through activation of the PI3K/Akt signaling [10,18,19]. It is interesting to note that degradome products of this matricellular protein may modulate the pathological angiogenesis in the retina [20]. The *PLAGL1* (pleiomorphic adenoma gene-like 1) gene encodes a C2H2 zinc finger protein, which is a transcriptional regulator with anti-proliferative properties [21,22].

The *ITGA5* (integrin, alpha 5) gene encodes an alpha polypeptide of fibronectin receptor, which interacts with different extracellular matrix proteins, including *CYR61* and vascular endothelial growth factor receptor 2, and participates in cell-surface mediated signaling. Integrins can also activate protein kinases involved in the regulation of cell growth, division, survival, differentiation, migration and apoptosis [13,23]. There are data that *TFPI2* (tissue factor pathway inhibitor 2) can also participate in the control of tumor growth preferentially through inhibition of a variety of serine proteases as well as through the regulation of plasmin-mediated matrix remodeling [14,24,25]. The growth arrest and DNA-damage-inducible, alpha (*GADD45A*) gene plays an important role in cell cycle control as a regulator of some protein kinases and responds to environmental stresses [15,26]. Furthermore, *GADD45A* is associated with growth arrest and apoptosis and has both tumor suppressor and tumor promoter functions, depending on the tissue/cell type and transforming event [15,26]. *PPP1R15A* (protein phosphatase 1, regulatory subunit 15A) is a regulatory subunit of serine/threonine-protein phosphatase PP1, which dephosphorylates the translation initiation factor eIF-2A/EIF2S1, thereby reversing the shut-off of protein synthesis initiated by stress-inducible kinases and facilitating recovery of cells from stress, as well as down-regulating the TGF-beta signaling pathway, and may promote apoptosis by inducing TP53 phosphorylation [16,27]. The aldehyde dehydrogenase 1 family member A2 (*ALDH1A2*) catalyzes the PPARγ-directed synthesis of retinoic acid, the active derivative of vitamin A, which is a hormonal signaling molecule that functions in developing and adult tissues and has a relation to suppression of tumor growth [17,28]. The heat shock 70kDa protein-6 is a stress responsible protein, which mediates the folding of newly translated polypeptides and is associated with childhood leukemia [29].

The endoplasmic reticulum stress is also recognized as an important determinant of obesity, insulin resistance, and impaired glucose tolerance and contributes to the expression profile of many regulatory genes, including *GADD45A, PLAGL1, CYR61, PPP1R15A, TFPI2, HSPA6,* and *ITGA5,* resulting in peripheral insulin resistance and other obesity complications [1,5,30-32], although detailed molecular mechanisms cannot be ruled out.

It is possible that identification of real mechanisms of metabolic abnormalities in obesity, as well as its complications at molecular and cellular levels, helps to better understand why obesity develops and why only some obese individuals develop secondary metabolic disorders [33]. However, a detailed molecular mechanism of the involvement of different genes of the regulatory network in the development of obesity and its complications is not clear yet and remains to be determined.

The main goal of this study was to clarify the role of the subset of gene expressions, encoding for important cell growth factors and enzymes, which play an important role in the control of cellular growth and apoptosis, in blood cells of obese boys with and without insulin resistance for evaluation of its possible significance in the development of obesity and impaired insulin sensitivity.

Materials and Methods

Fifteen boys participated in this study. They were divided into three equal groups (5 subjects in each group): normal individuals as control, obese patients with insulin resistance, and obese patients without insulin resistance. Written informed consent was obtained from all participants. The study was approved by the local research ethics committees of Institute of Children and Adolescent Health Care of the National Academy of Medical Science of Ukraine.

Clinical characteristics of the study participants are shown in Table 1. The normal (control) participants were individuals with mean age 14±0.7 years and mean body mass index (BMI) 18.7±0.12 kg/m^2. The obese participants, both with normal insulin sensitivity and with insulin resistance, had a mean age of 14±0.6 and 14±0.4 years, respectively, and mean BMI 31.0±0.40 and 34.2±2.39 kg/m^2, respectively.

Table 1.

Clinical characteristics of the study participants

Variable	Control	Obesity	Obesity + IR
Age at visit (years)	14±0.73	14±0.6	14±0.38
BMI (kg/m^2)	18.7±0.12	31±0.40 *	34.2±2.39 *
HOMA-IR	2.36±0.17	2.70±0.28	8.70±1.41*^
FI (mIU/mL)	13.0±0.95	14.1±1.35	43.4±6.70 *^

*IR – insulin resistance; HOMA-IR - insulin resistance index; FI - fasting insulin; * - P< 0.05 versus the control group; ^ - P<0.05 versus the obese group.*

Thus, BMI, which is a main criterion of obesity, in these last two groups of patients was significantly higher (+66 and +83 %, respectively; P<0.05 in both cases) as compared to the control individuals (Table 1). Moreover, no significant changes were found in insulin resistance index in obese individuals as compared to the control group. In obese patients with impaired insulin sensitivity, versus both the control boys and the obese subjects with normal insulin sensitivity, the insulin resistance index was significantly increased (3.7 and 3.2 fold, respectively; P<0.05 in both cases). Similar results were observed in the fasting insulin levels: no significant changes in obese individuals and strong increase in obese children with insulin resistance (3.3 fold; P<0.05) as compared to the control group.

RNA isolation

Trisol reagent (Invitrogen, USA) was used for RNA extraction from blood of normal (control) and obese individuals with or without insulin resistance.

Reverse transcription and quantitative real-time polymerase chain reaction analysis

The expression levels of genes related to regulation of cell growth and glucose homeostasis (*PLAGL, CYR61, GADD45A, GADD34(PPP1R15A), ITGA5, TFPI2, ALDH1A2,* and *HSPA6*) were measured in blood cells by a real-time quantitative PCR of complementary DNA (cDNA). QuaniTect Reverse Transcription Kit (QIAGEN, Germany) was used for cDNA synthesis. The 7900 HT Fast Real-Time PCR System (Applied Biosystems), Absolute QPCR SYBRGreen Mix (Thermo Scientific, UK) and pair of primers specific for each studied gene (Sigma/Aldrich, USA) were used for quantitative PCR (Table 2).

The expression of beta-actin mRNA was used as control of analyzed RNA quantity. The amplified DNA fragments were analyzed on a 2% agarose gel and visualized by 5x-Sight DNA Stain (EUROMEDEA). An analysis of quantitative PCR was performed using special computer program "Differential expression calculator". Statistical analysis was performed as described previously [34]. All values are expressed as mean ± SEM from five independent experiments; P<0.05 was considered as significant difference.

Results and Discussion

We studied the expression of the subset of genes (*CYR61, PLAGL, GADD45A, GADD34 (PPP1R15A), ITGA5, TFPI2, ALDH1A2,* and *HSPA6*), which control cell proliferation and apoptosis, in blood cells of the three groups for evaluation of its possible significance to the development of obesity and insulin resistance. As shown in Fig.1, the levels of *PLAGL1* and *CYR61* mRNA expression are increased (+25 and +69 %, respectively; *P<0.05* and *P<0.01*, respectively) in blood cells of obese boys with normal insulin sensitivity as compared to the control group.

The development of insulin resistance in obese boys is associated with additional up-regulation of *PLAGL1* mRNA expression (+34%; *P<0.05*) in blood cells as compared to the group with obesity and normal insulin sensitivity. At the same time, the expression level of *CYR61* mRNA is decreased (-17%; *P<0.05*) in blood cells of obese children with impaired insulin sensitivity versus the group of obese boys without insulin resistance, but it is significantly higher relative to the control group (+41%; *P<0.05*; Fig. 1).

These data clearly demonstrate that obesity leads to significant dysregulation of *PLAGL1* and *CYR61* genes in blood cells, being more evident for the *CYR61* gene, and that this dysregulation of *PLAGL1* and *CYR61* genes possibly contributes to the development of enhanced cell proliferation, obesity, and insulin resistance. Moreover, we have also shown that development of insulin resistance in obese individuals is associated with down-regulation of *CYR61* and up-regulation of *PLAGL1* gene expressions. These results correlate with data

Table 2.
Characteristics of the primers used for quantitative real-time PCR

Gene symbol	Gene name	Primer's sequence	Nucleotide numbers in sequence	GenBank accession number
PLAGL1	pleiomorphic adenoma gene-like 1	F: 5'- gggaccattgaagaattcca R: 5'- acactcctcacacccaaagg	436–455 716–697	NM_002656
CYR61 (IGFBP10)	cysteine-rich, angiogenic inducer, 61 (insulin-like growth factor binding protein 10)	F: 5'- ctccctgtttttggaatgga R: 5'- tggtcttgctgcatttcttg	852–871 1092–1073	NM_001554
PPP1R15A (GADD34)	protein phosphatase 1, regulatory subunit 15A (growth arrest and DNA-damage-inducible 34)	F: 5'- gaatcaagccacggaggata R: 5'- cagggaggacactcagcttc	953–972 1261–1242	NM_014330
GADD45A (DDIT1)	growth arrest and DNA-damage-inducible 45 alpha (DNA damage-inducible transcript-1)	F: 5'- acgaggacgacgacagagat R: 5'- tcccggcaaaaacaaataag	503–522 764–745	NM_001924
ITGA5	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	F: 5'- gtggtgctgtctacctctgt R: 5'- tcagtggctccttctctgtg	346–365 576–2557	NM_002205
TFPI2	tissue factor pathway inhibitor 2	F: 5'- gggccctacttctccgttac R: 5'- cacactggtcgtccacactc	212–231 394–375	NM_006528
ALDH1A2 (RALDH2)	aldehyde dehydrogenase 1 family, member A2 (retinal dehydrogenase 2)	F: 5'- c agcccacagtgttttccaac R: 5'- ctgggcatttaaggcattgt	1472–1491 1713–1694	NM_003888
HSPA6	heat shock 70kDa protein 6	F: 5'- ccaagcagacccagactttc R: 5'- gccttacctgtgctcctgtc	1684–1703 1912–1893	NM_002155
ACTB	beta-actin	F: 5'- ggacttcgagcaagagatgg R: 5'- agcactgtgttggcgtacag	747–766 980–961	NM_001101

of other authors that down-regulation of *CYR61* is associated with inhibition of tumor cell proliferation [10,12] and that *PLAGL1* may have a role in tumorigenesis of von Hippel-Lindau-associated central nervous system hemangioblastoma [22].

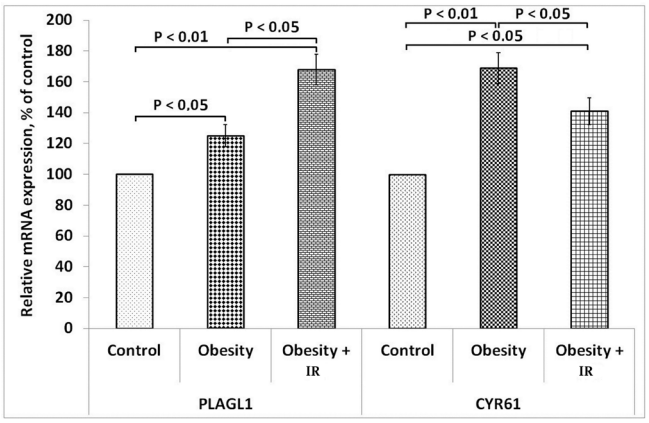

Fig.1. Relative mRNA expression of PLAGL1 and CYR61 in blood cells of normal boys (Control) and obese individuals with normal insulin sensitivity (Obesity) and obese patients with insulin resistance (Obesity + IR). Levels of PLAGL1 and CYR61 mRNA were normalized to the beta-actin mRNA and are represented *as mean ± SEM and* expressed *as a percent of the control (100 %).*

Investigation of the expression level of the growth arrest and DNA-damage-inducible 34 (*GADD34)* gene, also known as regulatory subunit 15A of protein phosphatase 1 (*PPP1R15A)*, in blood cells of obese boys who have normal insulin sensitivity has revealed that obesity does not change significantly the expression of this gene when compared to the control group, but insulin resistance leads to an up-regulation of *GADD34* mRNA expression (+21%; *P<0.05*; Fig. 2) as compared to the group of obese boys with normal insulin sensitivity.

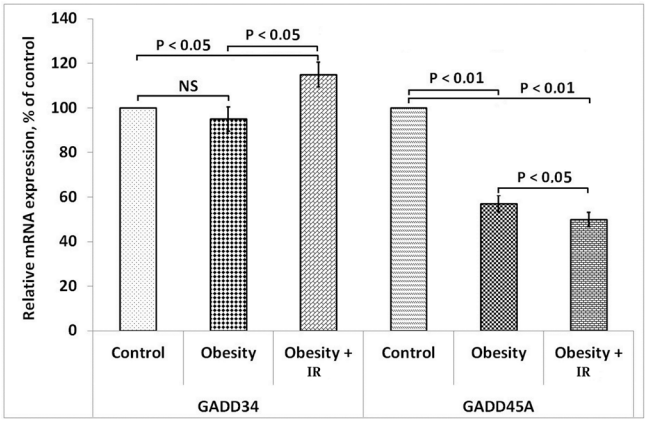

Fig. 2. Relative mRNA expression of GADD34 and GADD45A in blood cells of normal boys (Control) and obese individuals with normal insulin sensitivity (Obesity) and obese patients with insulin resistance (Obesity + IR). Levels of GADD34 and GADD45A mRNA were normalized to the beta-actin mRNA and are represented *as mean ± SEM and* expressed *as a percent of the control (100 %).*

At the same time, another member of the DNA-damage-inducible protein family, DNA-damage-inducible proteins 45 (*GADD45A*), also known as DNA damage-inducible transcript-1 (*DDIT1)*, is significantly decreased in obesity without insulin resistance (almost two fold; *P<0.05*; Fig. 2). Furthermore, development of an insulin resistance induces additional down-regulation of *GADD45A* mRNA expression (-12%; P<0.05). Thus, the expression of both DNA-damage-inducible proteins is decreased in obesity with normal as well as with impaired insulin sensitivity. These results completely correlate with the anti-proliferative properties of both *PPP1R15A* and *GADD45A* [15, 26, 27].

As shown in Fig.3, the expression level of both *ITGA5* and *TFPI2* mRNA is increased (+24 and +18 %, respectively; *P<0.05*) in blood cells of obese boys with normal insulin sensitivity, as compared to control children, but development of insulin resistance in obese individuals is associated with the down-regulation of *TFPI2* mRNA expression (-19%; *P<0.05*) in blood cells, as compared to the group with obesity and normal insulin sensitivity, up to the level in the control boys.

At the same time, the expression level of *ITGA5* mRNA does not change significantly in blood cells of obese children with impaired insulin sensitivity versus the group of obese boys without insulin resistance, and it is significantly higher relative to the control group (+29%; *P<0.05*; Fig. 3).

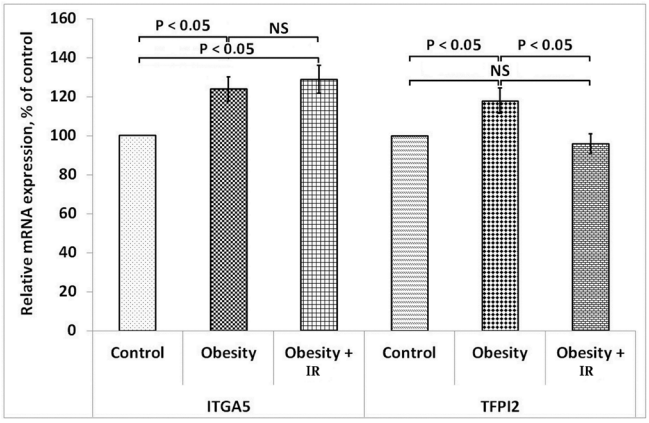

Fig.3. Relative mRNA expression of ITGA5 and TFPI2 in blood cells of normal boys (Control) and obese individuals with normal insulin sensitivity (Obesity) and obese patients with insulin resistance (Obesity + IR). Levels of ITGA5 and TFPI2 mRNA were normalized to the beta-actin mRNA and are represented *as mean ± SEM and* expressed *as a percent of the control (100 %).*

The increased level of *ITGA5* and *TFPI2* mRNA expression agrees with the biological significance of proteins encoded by these genes, because an alpha polypeptide of fibronectin receptor as well as *TFPI2* interacts with different extracellular matrix proteins, including *CYR61* and vascular endothelial growth factor receptor 2, and participates in cell-surface-mediated signaling and is involved in the regulation of cell growth, division, survival, differentiation, migration and apoptosis through activation of some protein kinases [13, 14, 23-25].

We next tested whether obesity also affects the expression of member A2 of the aldehyde dehydrogenase 1 family (*ALDH1A2*), which is a retinal dehydrogenase 2, as well as heat shock 70kDa protein-6 (*HSPA6*) mRNA in blood cells of obese children with normal and impaired insulin sensitivity (Fig. 4).

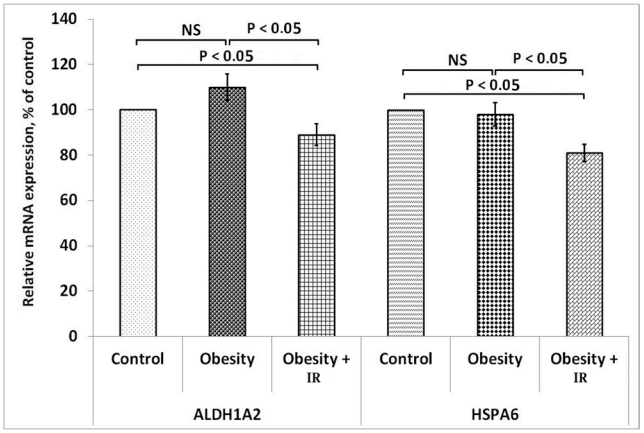

Fig.4. Relative mRNA expression of ALDH1A2 and HSPA6 in blood cells of normal boys (Control) and obese individuals with normal insulin sensitivity (Obesity) and obese patients with insulin resistance (Obesity + IR). Levels of ALDH1A2 and HSPA6 mRNA were normalized to the beta-actin mRNA and are represented *as mean ± SEM and* expressed *as a percent of the control (100 %).*

As shown in Fig.4, no significant changes were observed in the expression level of both *ALDH1A2* and *HSPA6* mRNA in blood cells of obese boys with normal insulin sensitivity, as compared to control children, but development of insulin resistance in obese individuals is associated with down-regulation of both mRNA expressions (-19 and -17%, respectively; *P<0.05*) in blood cells versus the group with obesity and normal insulin sensitivity.

The decreased level of *ALDH1A2* and *HSPA6* gene expressions correlates to data that *ALDH1A2* may control the synthesis of retinoic acid, a hormonal signaling molecule that has a relation to suppression of tumor growth, and that the expression of heat shock protein A6, as a stress-responsible protein, responds to oxidized low density lipoprotein immune complexes [17, 28, 35].

Thus, results of this study provide evidence that obesity affects the expression of the subset of genes related to cellular growth and apoptosis in blood cells and that insulin resistance in obese children is associated with changes in the expression level of *CYR61, PLAGL, GADD45A, PPP1R15A, TFPI2, ALDH1A2,* and *HSPA6* genes, which possibly contribute to the development of obesity and its complications, and reflect some changes in other tissues, including fat tissue.

Conclusions

Obesity (with normal insulin sensitivity) enhances the expression of *CYR61, ITGA5,* and *TFPI2* genes, which control cell growth, and decreases the expression of the *GADD45A* gene, which controls apoptosis, in blood cells, versus the control group. Insulin resistance in obese boys leads to up-regulation of *PLAGL1* and *PPP1R15A* gene expressions and to down-regulation of *CYR61, GADD45A, ALDH1A2, TFPI2,* and *HSPA6* genes in the blood cells versus obese patients with normal insulin sensitivity.

This study has demonstrated that obesity affects the expression of the subset of genes related to the control of cell growth and survival in the blood and that insulin resistance in obesity is associated with changes in the expression level of *CYR61, PLAGL, GADD45A, PPP1R15A, TFPI2, ALDH1A2,* and *HSPA6* genes, which contribute to the development of obesity and its metabolic complications and possibly reflect some changes in other tissues.

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EXPERIMENTAL RESEARCH

Studies of Frequency–Dependent Changes under Modulated Ultrasound Exposure on Cells in Suspension

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Abstract

Characteristics of the modulated ultrasound effect (range of modulation frequencies 10Hz–1000Hz intensity 0.2 W/cm^2) on *white blood cells (*WBCs) from different animals were studied. The quantitative ratio of WBCs was the most strongly altered by ultrasonic modulation frequency 1000 Hz. This frequency led to degenerative changes of cells. The presence of non-typeable or destroyed cells in smears was indicated. The results obtained demonstrated the possibility of directed impact on different WBC forms.

Keywords*: ultrasound; modulation; white blood cells.*

Introduction

Studies conducted from the 1960s to the 1990s showed that amplitude-modulated ultrasound (US) used on biological tissue is more efficient than continuous ultrasound [1-4]. This position was confirmed by revealed features of continuous and modulated US influence on testae and blood of different species of animals [5].

The purpose of this study was to detect possible changes in the functional state of WBCs of different species of animals exposed to modulated ultrasound of therapeutic intensity.

Materials and Methods

In our study, no laboratory animals were harmed. Purposes, methodology and principles of our operations didn't include experiments on living beings. Tests *in vivo* as well as tests on other animal and human organisms are impractical as long as no satisfactory explanations of phenomena mentioned are found.

The experimental work was carried out at the Department of Information Technology, Mathematics and Physics of Moscow State Academy of Veterinary Medicine and Biotechnology. All animals were adult and healthy.

Groups of animals: 11 cats aged from 2 to 3 years (6 males and 5 females) and 18 horses (an equal number of males and females) aged from 5 to 8 years. Animals' blood was exposed to US with a space-averaged, time-averaged (SATA) intensity of 0.2W/cm^2 during a period from 15 seconds to 5 minutes, with modulation frequency of 10 Hz and 1000 Hz and the carrier frequency of 880k Hz, according to the previously proven technique [6]. US therapy medical devices were: UST–1–01F, UST–5 and UST–1.02S, combined with thermostat U7^c. We applied pulse mode – 10, amplitude modulation pulse ratio – 2, modulator model– GZ–112. We consider it inhumane to receive regularly a large amount of blood from small animals for the extended biophysical series of experiments for reliable statistics. Therefore, the sonication technique has been specifically designed and tested in samples with the minimum volume. We adjusted the US exposure for each blood volume in order to receive comparable results. Blood samples of 1.5 ml (cat) and 10 ml (horse) were sonicated under absolutely identical conditions (oscillator square, cooling fluid, circulative rate). The therapeutic blood cell sonication was carried out in a temperature-controlled cuvette. Its walls were made of US conductive plastic. A coolant, distilled water, circulated continuously (so-called "flow-through cooling").

Blood smears and cytochemical methods

US effects on *WBCs* were observed under a light microscope. Blood of the same animal, untreated (intact

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specimens) served as a control on determining the US effect on WBCs. Blood smears (control and after the US exposure) were examined under immersion in a transmitted light microscope «Mikmed-5» (optical objective – 100^x/1.25; ocular lens–10^x/18).

Smears were made and stained according to the DIFF-QUICK technique. Smears were fixed in absolute methanol 15s, heated in dye solutions for 10 seconds, washed with buffered water, dried and examined under the microscope. WBC count was led via "Meander" line 3–5 along the edges of fields of view smear, 3–5 fields of view at right angles to the middle of the stroke, then 3–5 fields parallel to the edge of the stroke and again at right angles to the edge of the smear. Each sample was examined at least 10 times. We continued our account until 100 whole cells had been estimated [7]. Statistical results processing was performed with the use of "Statistica 6.0" program. Differences were considered significant at $P<0.05$.

Results

Only extreme ranges of frequency modulation were tested in order to compare and to determine the spectrum of their biological activity in this paper. A detailed comparison of the features of modulated ultrasonic interaction (modulation mode is from 10 to 1000 Hz) with blood cells was performed earlier [5]. Preliminarily, we can say that short–term US exposure had multidirectional effects on the WBCs of different animal species. The relative *changes* in the *leukogram* were determined after the differential WBC count. The ratio of cells tested to the respective sample control (ctrl) cells was calculated (Table 1.) Some species' features of this interaction were revealed. For example, a 15–second US exposure with modulation of 10 Hz on cat's blood led to changes in the percentage ratio of cells: the absolute and relative amount of lymphocytes increased 1.5–2 times; the amount of eosinophil and basophil increased 2.5–3 times; the amount of monocytes increased 4 times; band neutrophils, 1.5–2 times, while the amount of segmented granulocytes was reduced ($P<0.05$). Further increase of exposure time to more than 30 sec enhanced the effect. We observed cell lysis (Photo: Fig.1) and damage of cytoplasmic membrane (CPM).

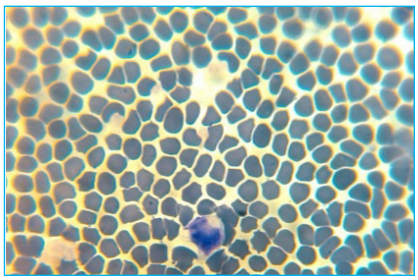

Fig. 1. *Cat's blood. Modulation of 10 Hz for 30 sec. Lymphocyte lysis.*

Eosinophils and basophils of a horse were not so "sensitive" to short-term exposure: their amount did not change significantly. Monocytes increased 4–5 times compared to the control, $P<0.05$.

Table 1. Effect of the modulated ultrasound with intensity of 0.2 W/cm²on WBCs

Frequency and Time	Eosinophils	Basophils	Monocytes	Lymphocytes	Neutrophils	
					band	segmented
10 Hz - 15s	> 2.5 ctrl	> ctrl	≥4ctrl	2ctrl	2ctrl	≤ ctrl
10 Hz - 30s Degenerative changes in all WBCs	< ctrl	= ctrl	2 ctrl	3ctrl; lysis	3ctrl; groups of cells	ctrl/2
10 Hz - 15s	= ctrl	= ctrl	1.1ctrl	= ctrl	ctrl/1.1	>1.1ctrl
10 Hz - 30s	=ctrl	=ctrl	5ctrl	ctrl/1.4	<ctrl/1.5	>1.4ctrl; groups of cells; degenerative changes
10 Hz 60s	5 ctrl–13 ctrl for groups of cells	= ctrl	6ctrl	ctrl/2	ctrl/3	>1.4 ctrl; groups of cells; degenerative changes
10 Hz - 2 min	=ctrl	4ctrl	6ctrl	ctrl/2	<ctrl/3	ctrl/1.5; degenerative changes
10 Hz – 3 min. Degenerative changes in all WBCs excl lymphocytes	<ctrl	<ctrl	<ctrl	ctrl/1.5	—	ctrl/3.4
10 Hz - 5 min Only 40–45 cells/ smear	<ctrl	4ctrl	3ctrl	ctrl/3	—	—
1000 Hz - 15s Beginning of cell aggregation	=ctrl	>2ctrl	ctrl	> 2ctrl	>2ctrl	=ctrl
1000Hz - 30s Cell aggregation and damage	<ctrl	=ctrl	>3ctrl	>2ctrl	>5ctrl	ctrl/2
1000 Hz - 30s	=ctrl	5ctrl	6ctrl	ctrl/1.4	=ctrl	ctrl/1.3
1000 Hz - 60s. 10% of cells with the damaged CPM	<ctrl	2ctrl	5ctrl	<ctrl	ctrl/3	>2ctrl

Note: – Cats. – Horses.

The percentage composition of other formed elements dramatically changed, with the reversed sign, compared with the same effect in cats: the amount of lymphocytes decreased and the amount of segmented neutrophils increased the same—1.4 times against the control, $P<0.05$. Microscopy data showed degenerative changes of segmented neutrophils. Consistent increase in exposure time up to 3 minutes enhanced the effect. Only 40–45 cells were revealed after five–minute exposure (Table 1.): eosinophils, basophils, monocytes and a small number of lymphocytes.

It should be noted that degenerative changes were evidenced in all WBCs of both types of animals (except lymphocytes) (Photo: Fig. 2-5).

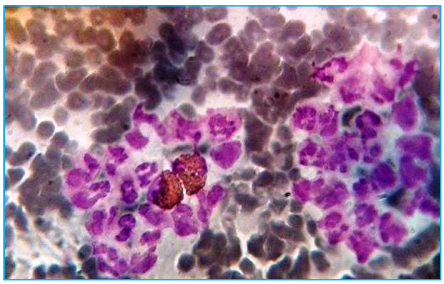

Fig.2. *Horse's blood. WBC aggregation at US intensity of 0.2 W/cm². Modulation of 10 Hz for 60 sec. The ratio of neutrophils and other types of WBCs >80%.*

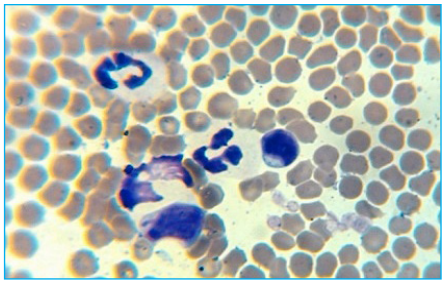

Fig.3. *Horse's blood. Modulation of 10 Hz for 3 min. The segment is seen at the "11 o'clock"-position. Group: average sized lymphocyte, segmented neutrophil, monocyte? (butterfly), degenerative leukocyte with destruction, probably, it is the deformed lymphocyte or basophil without cytoplasm (identification is impossible).*

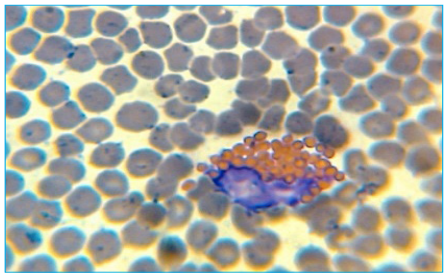

Fig.4. *Horse's blood. Modulation of 1000 Hz for 60 sec. Eosinophil. Cell wall destruction, deformation (lysis) of the nucleus. Eosinophilic granules (bottom left) came out of the cell, but were not destructed; nucleus has been torn.*

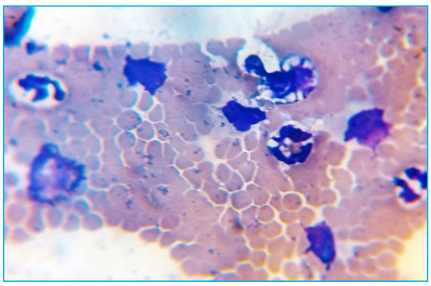

Fig.5. *Horse's blood. Modulation of 1000 Hz for 60 sec. Lysis of the cell membrane and nucleus and, may be, an alteration in surface tension of WBCs of various types. Upper row: segmented neutrophils, lymphocyte, "vacuole" = 2 cells, connected by nuclei.*

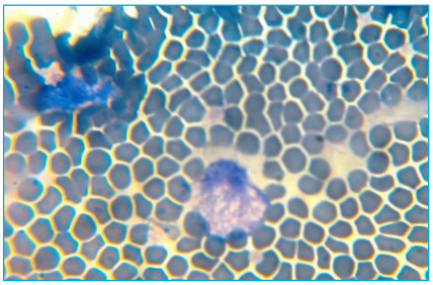

Fig.6. *Cat's blood. Modulation of 1000 Hz for 40 sec. Lysis of the WBC.*

Effect of modulated US exposure with a frequency of 1000 Hz

US waves have the same effect on the eosinophils and basophils of cats and horses: the amount of eosinophil does not change, but the amount of basophil gradually increases (from 2 to 5 times) with increasing of exposure time (15–30 seconds) compared to the control. The percentage of lymphocytes and band neutrophils in the leukogram of cats increased 2–5 times; the percentage of segmented neutrophils was reduced 2 times, $P<0.05$. Further increase in exposure time led to cell lysis (Photo: Fig.6) and destruction of the both CPMs and the nuclei of WBCs. The effect on WBCs of a horse was different again. The amount of segmented neutrophils grew (1.3–2 times, $P<0.05$), while the amount of lymphocytes and band neutrophils decreased; 10% of the typeable cells had the destructed CPM (Photos: Fig.4, 5).

Discussion

An analogical consistent pattern—cell aggregation, their partial death, left shift in leukogram, i.e. increase in the amount of young neutrophil in the leukocyte formula (as now we've obtained in cats' blood samples), monocytosis and slight eosinophilia—we also observed under the action of other US intensity 1.0 W/cm² with modulation frequency of 1000 Hz in preliminary studies. The phenomenon of WBC aggregation can be explained by loss of the surface charge of cells [5]. The data obtained from the literature on the effect of continuous and modulated acoustic waves on the cell suspensions were scarce and not very informative. Therefore, our further study of the possibility of non-invasive management of cell

functional state with the help of ultrasonic waves was relevant and theoretically meaningful.

It was shown earlier that low-intensity US 0.05–0.1 W/cm^2, during the exposure time of 1–3 minutes, had almost no effect on the subsequent growth and development of bacterial culture *A. fischeri* strain 6. The impact of 0.4 W/cm^2 had a stimulating effect on bioluminescence and the rate of growth of those bacteria. At intensities over 0.6 W/cm^2 there was an irreversible inhibition of bioluminescence and the number of viable cells progressively decreased [8]. The effect of US on the proliferative rate of transplantable cell culture MDBK was also investigated. The maximum stimulating effect was identified under intensity of 0.05 W/cm^2, exposure 10 sec [9]. Higher intensity of irradiation (0.1 W/cm^2, continue waves) resulted in a significant reduction in cell growth and complete cell destruction (over 0.2 W/cm^2). The morphology of cells changed significantly. Plasmolemma was destroyed and the nuclear membrane was ruptured due to the occurrence of microflows inside cells that accompanied the US cavitations in biological media [10]. According to our theory, cell changes in blood of cats and horses were caused by the same factors, but this requires much further investigation and evidence.

Our next objects were WBCs. The influence of US waves and the output of practically important metabolites were studied. A 3-minute US exposure stimulated the release of interferon [11]. There was no cell disruption after treatment with US intensity from 0.01 to 0.05 W/cm^2, the intensity exposure of 0.1W/cm^2 resulted in decreased viability of WBCs (-10.6%). After treatment with US intensity 0.2-2.0 W/cm^2, the number of viable cells decreased even more significantly. Higher US intensity caused a sharp reduction in the viability of WBCs in the suspension due to the threshold of cavitations which, depends on the cell concentration in the bulk medium, and in diluted suspensions coincides with the threshold of cavitations in water ~ 0.3 W/cm^2. After US influence, the morphology changes in WBCs and their aggregation were found, identified and studied. It was determined that the number of agglomerates formed during a 5-minute US exposure and increased with US intensity [12]. Our last results (modulated US, intensity 0.2 W/cm^2) confirmed the 'dose-effect' pattern: cell viability decreased with exposure increase. Photos (Fig. 1–6) show the possible cause of this phenomenon – partial or total CPM damage.

These results made it possible to test the acoustic waves targeting exposure effect on the functional state of WBCs. Experiments on blood of healthy animals *in vitro* showed stable changes in the state of granulocytes (basophils, eosinophils, neutrophils) and agranulocytes (lymphocytes, monocytes) . The effect depends not only on ultrasound exposure, but also on the inherent characteristics of the species' blood. The mechanism of the biological activity of physical agents is caused by the external field forced vibrations of ions near the surface of the CPM, fluctuations in the channels, and the ability of accelerated ions to penetrate the membrane pores and specialized ion channels due to the active transport systems [13]. It was shown that the modulated UHF, EMR or US effect at some frequencies can cause a change in enzymatic activity towards both activation and inhibition [14,15].

Conclusions

In brief, we can point out some conclusions:

• Experiments on blood of healthy animals *in vitro* have shown stable changes in the state of granulocytes and agranulocytes; these experiments have also shown that the effect depends on US exposure, modulation frequency, and on the inherent characteristics of the species' blood.

• Pulse amplitude modulation allowed performance of equal energy exposure on cells and to determine biologically active frequencies.

• Biologically active modulation frequencies – 10 Hz and 1000 Hz, leading to evidential changes in leukograms of animals, were determined.

• 15–second US exposure with modulation of 10 Hz on cat's blood led to the absolute and relative increase in the amount of lymphocytes, eosinophils, basophils, monocytes, and band neutrophils.

• 15–second US exposure with modulation of 10 Hz on horse's blood led to an increase in the amount of monocytes and segmented neutrophils while the amount of lymphocytes decreased.

• Modulated US with a frequency of 1000 Hz had the same effect on eosinophils (without change) and basophils (gradually increased with increasing of exposure time) of cats and horses. Percentage of lymphocytes and band neutrophils in leukogram of cats increased; the percentage of segmented neutrophils was reduced. In the horse blood the amount of segmented neutrophils grew, while the amount of lymphocytes and band neutrophils decreased.

• Further increase in exposure time led to cell lysis and destruction of the both CPMs and the nuclei of WBCs. Cell viability decreased with exposure increase: 'dose-effect' pattern.

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DENTISTRY

Anthropometrical Parameters of the Orthognathic Bite in People of Uzbek Nationality

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Abstract

The aim of our work was to study anthropometric characteristics of jaws for Uzbek people with an orthognathic bite.

Material and Methods: The study included 42 ethnic Uzbeks (20 women and 22 men) aged from 17 to 25 years with a developed orthognatic bite; the control group consisted of the 25 age- and sex-matched Caucasians and Southern Altaians (mongoloids). The object of the research was 86 dental casts of upper and lower jaws of young Uzbek volunteers of both genders. Measurements were carried out on the plaster casts in sagittal and transversal directions using anthropometric measurement methods.

Results: The total width of 4 upper incisors in Uzbek females and males was less than that of South Altaians; the width of upper and lower dentitions at the level of the first premolars, second premolars and first molars was significantly more than that of Caucasians.

Conclusion: The ascertained features of the tooth size, the dentition size and form should be taken into account when the orthodontic arches are being chosen for treatment of dental and maxillary anomalies.

Keywords: *orthognathic bite; anthropometric reference points; Pont's index; plaster casts of jaws.*

Introduction

Diagnostic dental casts represent the clinical condition of the oral cavity, and dimensions made on them facilitate determination of particularities of an existing anomaly or deformation. These casts are necessary for making a diagnosis and plan of treatment and also for determining the most appropriate type of orthodontic appliance [1]. When losing teeth, the re-creation of bite becomes problematic because parameters of the rows of teeth in correlation with proportions of the head, face and jaws in different ethnic groups are not determined in contemporary anthropomorphology and dentistry. Therefore, bite evaluation according to the nature of how tooth rows close does not meet modern demands and the potential of the science [2-10].

Determination of the dimensions of tooth rows of the orthognathic bite in the proportionally hierarchical network of face and head, performed on a naturally volumetrical arrangement of structures in a live person, is not only for ethnic anthropology and clinic anatomy but also for cosmetology, oral surgery, orthodontics, and prosthodontics, especially when reconstruction of teeth, tooth rows and tainted bite take place, as well as in forensic medicine for personality identification. Determination of parameters of the orthognathic bite in people of Uzbek nationality, and identification of a correlation with a common shape of the head and face, tooth rows and teeth, becomes the actual problem [11-14].

Objective of our work was to study anthropometric characteristics of jaws for Uzbek people with an orthognathic bite.

Material and Methods

The study included 42 ethnic Uzbeks (20 women and 22 men) aged from 17 to 25 years with a developed orthognatic bite. Written informed consent was obtained from each patient. The control group consisted of the 25 age- and sex-matched Caucasians and Southern Altaians (mongoloids) according to O.D. Baydik [7]. The study was approved by the Tashkent State Dental Institute Ethics Committee.

The orthognathic bite was determined by the following criteria:

1. Upper lateral teeth cover the lower ones on the depth

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of the longitudinal fissure and in the frontal part, upper incisors cover lower incisors by 1/3 of the length of the crown;

2. Each tooth has two antagonists except for the lower central incisors and upper third molars;

3. The centerline between the central incisors of the upper and lower jaws coincides;

4. The length of the crowns decreases from central incisors to molars, except for canines;

5. A multiple fissure-papulose contact;

6. Angle's class I occlusion (or neutroclusion) on the right and left.

The object of the research was 86 dental casts of upper and lower jaws of young Uzbek volunteers of both genders. Measurements were carried out on the plaster casts in sagittal and transversal directions using anthropometric measurement methods.

All subjects tested were born and lived in the territory of the Republic of Uzbekistan; all of them grew up and were formed in the same geo climatic conditions, namely in an acutely continental region. We determined ethnic heritage by surveying and identifying representatives' genealogy for four generations: 1).The proband; 2).The proband's mother and father; 3).The proband's grandmother and grandfather from the mother's and father's line; 4).The proband's great grandmother and great grandfather from the mother's and father's line. Research included the probands whose ancestors belonged to the same ethnic group.

Observable groups included people selected according to the following criteria: normally functioning tooth-jaw-facial system and masticatory efficiency and the decayed, missing, and filled teeth (DMFT) index. Absence of any tooth was not acceptable. All examined did not have any morphological and functional deviations from accepted standards and were characterized by absence of clinical pathologies. None of them had previously received orthodontic treatment. As for social status, all of them were students of different institutes and universities of Tashkent city.

Position of teeth and the dimension of tooth rows

Tooth position was determined in three mutually perpendicular directions. In the transverse direction, the Pont's method was used to determine the individual norm of the width of tooth rows [15]. A. Pont established a correlation between the sum of the width of four upper incisor crowns and the width of tooth rows in the area of premolars and molars. To determine this correlation, we measured the width of crowns of the upper incisors and the distance between Pont's checkpoints on the chewing surface of the first premolars and molars. A.Pont discovered premolar and molar indices. He concluded that in the ideal dental arch (in the French population) the ratio of combined incisor width to transverse arch width was .80 in the premolar area (Premolar index=80) and .64 in the molar area (Molar index=64). In the orthognathic bite, the width of upper and lower tooth rows is the same because checkpoints on the upper teeth in a central occlusion match checkpoints on the lower teeth.

H. Linder and G. Harth [16] made corrections in index numbers by using Pont's method. According to these authors, the premolar index is equal to 85, and the molar to 65. In practice, doctors can use these numbers for to measure tooth rows in the period of mixed and permanent dentition.

Dimensions in the sagittal direction were conducted by G. Korkhaus's method [17]. G. Korkhaus complemented Pont's method by assuming that the length of the frontal part of upper tooth row could be determined in relation to the sum of the width of crowns of upper incisors. To determine existing length, we measured the distance from the midpoint between the central incisors from the vestibular surface of their crown along the midline of the jaw to its intersection with the line connecting Pont's point on the first premolars. The length of the frontal part of the lower tooth row was calculated by subtracting two millimeters from the quantity of the frontal part of the upper tooth row (the thickness of the incisal edge of upper central incisors).

Received results were processed by the method of variation statistics on computer IBM PC Pentium-IV with use of Microsoft Excel programs for Windows 2003. All values are presented as mean ± SEM. For data with normal distribution, inter-group comparisons were performed using Student's t-test. A value of $P<0.05$ was considered statistically significant.

Results and Discussion

We were the first to carry out biometric measurements of teeth on jaw casts of Uzbek people for identification of racial and ethnic differences. The total width of 4 upper incisors in Uzbek females and males was less than that of South Altaians (Table 1).

According to the data of O.D. Buydik [7], Mongoloids have an inclination to macrodontia. Macrodontia is considered as pathogenetic factor of dentition anomalies in orthodontics. M. Abu-Hussein and A. Sarafianou [10] consider macrodontia as an ethnic variant of normality. It is interesting that apart from a significantly low sum of the 4 upper incisors' width in Uzbek males and females, the width of upper and lower dentitions at the level of the first premolars, second premolars and first molars was significantly more than that of Caucasians. The length of frontal segments of the upper and lower dental arches was also more than that of Caucasians; additionally, the premolar and molar Pont's indexes were less than those of South Altaians and Caucasians (Table 1).

Studying the jaws, dentitions and teeth and determining the connection among them allows us to detect more reliable features of bite, which can help to develop jaw analogue casts and working casts for restoration of dentitions, taking into account the racial and ethnic features of the patient.

On the basis of our analysis we arrived at the conclusion that indexes for Caucasian dentitions developed by Pont and Linder-Hart may be causes of mistakes in diagnostics of dentition anomalies of people of other races and ethnic groups. The ascertained features of the tooth size, the dentition size and form should be taken into account when the orthodontic arches are being chosen for treatment of dental and maxillary anomalies.

Table 1.

Anthropometric parameters of the upper and lower dentitions in Uzbeks, Caucasians and Southern Altaians

Tooth row	Parameters	Southern Altaians n=79	Caucasians n=40	Uzbeks n=42
Upper	Total width of four upper incisors, mm	31.33±0.25	29.75±0.36	29.88±0,29*
	Width of tooth row at the level 6|6, mm	49.59±0.38	46.46±0.55	50.49±0.74^
	Width of tooth row at the level 4|4, mm	37.47±0.29	35.30±0.45	38.63±0.62
	Pont's molar index, %	63.24±0.74	64.05±0.52	60.33±1.11*^
	Pont's premolar index, %	83.69±0.93	84.90±0.62	77.05±1.06*^
	Length of the frontal part of tooth row, mm	16.72±0.23	12.39±0.17	16.1±0.22^
Lower	Total width of four lower incisors, mm	22.25±0.55	22.41±0.24	21.7±0.18
	Width of tooth row at the level 6|6, mm	48.31±0.59	46.28±0.67	51.3±0.75^
	Width of tooth row at the level 4|4, mm	36.30±0.34	34.06±0.48	37.4±0.66^
	Pont's molar index, %	47.88±0.92	48.45±.077	42.75±0.61*^
	Pont's premolar index, %	63.26±1.22	65.89±1.14	58.7±0.71*^
	Length of the frontal part of tooth row, mm	16.59±0.34	11.81±0.24	14.1±0.22*^

P<0.05 vs Southern Altaians; ^ - P<0.05 vs Caucasians .

Competing interests

The authors declare that they have no competing interests.

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DENTISTRY

The Role of Biomimetic Incubation of Sandblasted Titanium Implants in the Process of Osseointegration: An Experimental Study in Dogs

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Abstract

The aim of the present study was to examine the surface characteristics and values of removal torque of an implant surface subjected to sandblasting with 125 µm Al_2O_3 particles with a following immersion in biomimetic fluid and to compare that surface with a machined implant surface.

Study protocol: Forty-eight conical implants were initially made of second-grade titanium alloy. The diameter of implants was 4 mm at the head and 2.6 at the apex, all implants were of 8 mm length and of large variable thread design. Half of them were subjected to sand blasting and immersion in biomimetic fluid at 37 °C for four weeks with daily replenishment of solution until the moment of placement; another 24 implants were left with untreated machined surface. Three-dimensional roughness values were obtained with the help of confocal laser scanning microscope.

Forty-eight implants were implanted in 12 dogs. Twenty-four implants were retrieved after a 6-week healing period following installation, and the remaining 24 were removed upon the completion of 16 weeks, using a torque calibrator ((BTG150CN-S TOHNICHI) with a 20 cN·m - 150 cN·m scale of force registration was applied for the measurements of the removal torque.

Results: The mean 3-dimensional roughness value of biomimetically treated implant surfaces was 1.34±0.24 µm and the mean roughness value measured for the machined surfaces was 0.33±0.04 µm ($P<0.05$). As to the average parameters of maximum peak-trough distance, these were equal to 2.85 for machined and 24.25 for incubated sandblasted implants. Machined implants demonstrated 49.5±10.3 removal torque values after the 6-week healing period. But for the immersed sandblasted implants the same parameter was equal to 72.7±15.98 cN·m. During a 16-week recovery period, these values increased up to 77.5±15.16 cN·m and 89.7±11.83 cN·m for machined and biomimetically treated sandblasted implants, respectively, $P<0.05$.

Conclusion: The present study demonstrated the rapid recovery time for biomimetically incubated sandblasted dental implants in comparison to machined surface implants based on findings of early (6 weeks healing period) removal tests. Although there was established only a 13.4% difference in values of removal torque after a 16-week healing period (instead of 32% after 6 weeks of recovery) between two groups of implants which could be associated with delayed bone integration.

Keywords: *dental implants; surface treatment; biomimetic fluid; roughness; removal torque.*

Introduction

Currently, the use of threaded titanium implants with different types of osseoconducive rough surfaces is considered a conservative treatment modality for partially and fully edentulous patients [1]. Clinical experience of successful application of machined dental implants with a smooth surface texture has a history of about 50 years [2]. However, the shortcomings of the first generation of implants associated with a long recovery period, the demands of patients who want to have their teeth sooner, and achievements of scientific research in this field have made it possible to introduce a dental product of higher quality which could meet the needs of doctors and patients.

The surface chemical composition of titanium implants is among the most important quality characteristics. A second-generation manufacturing process of dental implants means an application of physical and chemical factors, such as temperature, machining, sand blasting, anodization, sputtering, coating, acid etching, laser treatment, and sterilization. All of

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these are a source of ion, metal, lubricant and other kinds of contamination, which usually have a negative influence on successful osseointegration. That is why careful control of the chemical composition of the titanium implant surface in the manufacture of high quality dental products is of paramount concern [3-5].

One of the methods of titanium surface treatment which could avoid the consequences associated with the presence of chemical impurities in dental implants was suggested by Kokubo and co-workers and was called the biomimetic treatment [6,7]. Biomimetic deposition of microelements onto surfaces of titanium implant materials is a time consuming technique in the manufacture of implants. This method may take several weeks but allows hydroxyapatite and other calcium phosphate molecules to be deposited on the surfaces with complex geometry in a simulated body fluid solution under physiological conditions of temperature and pH [8-11]. As to the methods of examination, one of the most valuable quality tests of bone-to-implant integration cited frequently in the scientific literature is the determination of removal torque. Usually this type of biomechanical investigation is carried out in the course of an experimental animal study. Rabbit and dog tibias are the most frequently used bone sites for performance of such investigations [12,13].

The aim of the present study was to examine the surface characteristics and values of removal torque of an implant surface subjected to sandblasting with 125 μm Al_2O_3 particles with a following immersion in biomimetic fluid and to compare that surface with a machined implant surface.

Material and Methods

Forty-eight conical implants were initially made of second-grade titanium alloy with the following chemical composition: Fe max – 0.15; C max – 0.05; Si max – 0.08; N max – 0.04; Ti min – 99.6; O max – 0.1; H max – 0.008. The diameter of implants was 4mm at the head and 2.6 at the apex, all implants were of 8 mm length and of large variable thread design. Half of them were subjected to sand blasting and immersion in biomimetic fluid at 37 °C for four weeks with daily replenishment of solution until the moment of placement; another 24 implants were left with untreated machined surface.

Simulated body fluid solution was prepared by dissolving reagent-grade NaCl, NaHCO3, KCl, Na2HPO4·7H2O, MgCl2·6H2O, CaCl2 and NaSo4 in distilled water containing buffering agent, HCl and (CH2OH)3CNH2 at pH 7.25.

Roughness values were evaluated in accordance with recommendations established by Albrektsson & Wennerberg. Commercially pure titanium plates were used as initial material to obtain the abovementioned parameters. The samples were made of the same titanium grade material and were rectangular in shape (5×8).

Mean roughness (Sa) and maximum peak-trough distance (St) were measured with the help of an Aristoplan confocal laser-scanning microscope (Leica, Germany). Measurements were made using a 20x eyepiece under vertical resolution less than 20 nm. For separation of waviness, the profile roughness calculations were made with a Gaussian filter. Applied cut-off values (λc) were 0.8 mm and 0.25 mm.

Animals

Twelve dogs were selected for the study. Experiments were performed in the experimental center of Tashkent Medical Academy. The procedures in this study were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Maximal effort was made to minimize animal trauma and the number of animals necessary for the acquisition of reliable data. All experiments were approved by our local ethics committee.

All surgical stages associated with implant insertion and assessments of removal torque were carried out under general anesthesia with rometar 2 mg/kg and 0.5 ml atropine; maintenance with novocaine 2% local infiltration. Postoperative care protocol included penicillin G and nonsteroidal anti-inflammatory drugs for 5 days. The selected site of implantation was the internal part of the animal's tibia. In each limb we inserted two implants (four implants in every animal): one machined surface implant was placed in the proximal epiphysis of the dog's tibia, and one sand blasted implant immersed in biomimetic fluid was placed in the distal epiphysis of the same tibia. Incisions were made in appropriate locations with a split thickness flap technique. Drillings were made under cool physiological saline irrigation. After insertion of an implant into the prepared bed, the periosteum was sutured with chromic gut 3/0, and nylon 3/0 was applied for the skin.

The second surgical steps took place 6 and 16 weeks later. After administration of general anesthesia to the animals, incisions were made again in the implant zones and the heads of 24 implants (12 machined and 12 incubated sand blasted) were exposed. After 16 weeks the same procedure was carried out with the remaining 24 implants.

In both study intervals, after removing the locking screws, the torque gauge (BTG150CN-S TOHNICHI) with a 20 cN·m - 150 cN·m scale of force registration was applied for the measurements of the removal torque.

Results were statistically processed using the software package Statistica 6.1. A probability value of $P<0.05$ was considered statistically significant.

Results and Discussion

The mean 3-dimensional roughness value of biomimetically treated implant surfaces was 1.34±0.24 μm and the mean roughness value measured for the machined surfaces was 0.33±0.04 μm ($P<0.05$). As to the average parameters of maximum peak-trough distance, these were equal to 2.85 for machined and 24.25 for incubated sandblasted implants.

Machined implants demonstrated 49.5±10.3 removal torque values after the 6-week healing period. But for the immersed sandblasted implants the same parameter was equal to 72.7±15.98 cN·m. During a 16-week recovery period, these values increased up to 77.5±15.16 cN·m and 89.7±11.83 cN·m for machined and biomimetically treated sandblasted implants, respectively, $P<0.05$.

Implant surfaces are the subject of prolonged studies in order to reach the fastest and safest clinical consolidation of artificial root abutments. At present, machined or first-generation implant surfaces clearly have been surpassed by newer second-generation ones. Sandblasting procedures with or without etching (Tioblast and SLA surfaces), anodic oxidation (TiUnite surface by Nobel Biocare),, laser modified micro- and nano-structured surface (Brånemark BioHelix Implant), calcium phosphate coated implants, plasma spraying, sputter-deposition, and biomimetic precipitation are techniques for which several authors should be given a special mention [2-5,14].

Numerous in vitro studies confirmed that the topographical surface characteristics of titanium implants influence blood clot retention, protein adsorption, platelet adhesion, degree of tissue inflammation, osteogenic cell response, and finally the rate of healing [15-18]. It has been already established that the gingival tissue and bone marrow cell response could be considerably influenced by the chemical composition of the implant surfaces [19-21].

Therefore, biomimetically produced titanium implant surfaces may be useful in facilitating early bone ingrowth into porous surfaces without the possibility of fibrous tissue encapsulation and eventual coating failure, which may occur with other types of titanium implant surface manufacturing processes because of the presence of chemical impurities.

Conclusion

The present study demonstrated the rapid recovery time for biomimetically incubated sandblasted dental implants in comparison to machined surface implants based on findings of early (6 weeks healing period) removal tests. Although there was established only a 13.4% difference in values of removal torque after a 16-week healing period (instead of 32% after 6 weeks of recovery) between two groups of implants which could be associated with delayed bone integration.

Competing interests

The authors declare that they have no competing interests.

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CASE REPORT

Autologous Bone Marrow Mesenchymal Stem Cell Transplantation in Liver of a Patient with Liver Cirrhosis: Case Report

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Abstract

This report presents a clinical case describing successful endovascular transplantation of autologous bone marrow mesenchymal stem cells (MSCs) into liver of a patient with liver cirrhosis.

Keywords: *mesenchymal stem cells; transplantation; liver cirrhosis.*

Introduction

Liver cirrhosis (LC) remains a major challenge of modern medicine. According to the Global Burden of Disease 2010 study, liver cirrhosis caused 31 million Disability Adjusted Life Years (DALYs), or 1.2% of global DALYs, in 2010, and one million deaths, or 2% of all deaths worldwide in that year [1,2]. Currently, the arsenal of tools aimed at improving the functional state of the liver and other organs and systems affected by LC has been significantly increased, but the results of treatment remain unsatisfactory. A relatively radical method for treatment of LC patients is liver transplantation. However, in the near future it will not be possible to provide patients with donors in the demanded volumes, as well as organize this service in many hospitals, for a number of scientific and economic reasons [3]. The above data have caused a search for more affordable and effective methods for treatment of LC patients. In this respect, the data on the successful results of transplantation of different kinds of stem cells used to stimulate reparative processes in damaged tissues of various organs have a great scientific and practical interest [4-8]. Below we present a clinical case describing successful endovascular transplantation of autologous bone marrow mesenchymal stem cells (MSCs) into liver of a patient with liver cirrhosis.

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Case presentation

The 56-year-old man who is the subject of this report was admitted to the surgical department of the Institute of Emergency and Reconstructive Surgery, named after V.K. Gusak of NAMS of Ukraine, with acute gastrointestinal bleeding. The patient reported general weakness, malaise, fatigue, bloating, and bloody vomiting. He had the experience of drinking alcohol, although he did not suffer from chronic alcoholism. He denied any history of viral hepatitis.

A physical examination revealed a severe clinical condition; the skin and sclera were pale, slightly icteric. The abdominal palpation revealed a slight pain in the right upper quadrant and epigastric zone. The liver was enlarged and splenomegaly was marked. Hemodynamic instability was noted (BP - 90/50 mmHg, pulse - 100 bpm). The heart sounds were muffled. Auscultation of the lungs revealed vesicular breathing, which was weakened in the lower lung fields.

Blood test: HB - 76 g/L, Er - $3.7^{x}10^{12}$/L, platelets - 180,000/mL, and leukocytes – $5,6^{x}10^{9}$/L; ESR - 56 mm/h; total protein - 76 g/L; albumin - 28 g/l; total bilirubin - 36 mmol/l; ALT – 136 IU/L and AST – 185 IU/L; blood glucose was 3.5 mmol/L.

Hepatitis viruses were not found in the patient. Abdominal sonography revealed hepatomegaly, splenomegaly, and a moderate amount of free fluid; the structure of the liver was non-uniform and the diffuse changes of parenchyma were noted. Portal vein diameter was 1.2 cm; the portal blood flow

was 420 ml/min. During EGD, we identified varicose veins of the esophagus and stomach of III degree, which were the cause of the bleeding. Bleeding was stopped by clipping these veins. A liver biopsy of the avascular zone was performed under local anesthesia and ultrasound control. Monoject ABC needles (Sherwood Medical) were used. The severity of cirrhosis was evaluated by Child-Pugh criteria. The Child-Pugh class B was defined.

The patient underwent intensive treatment and preventive measures aimed at preventing re-bleeding, improving the functional capacity of the liver and kidneys. A week later, the patient was discharged to home in good condition with the recommendation to observe a strict diet that excluded alcohol. A month later, after re-examination, we harvested bone marrow for transplantation of autologous MSCs into the liver. The patient was aware of the planned method of transplantation, possible side effects, and complications. Written informed consent was obtained.

The culture of autologous MSCs was isolated from the bone marrow obtained by puncture of the iliac crest (2 months before MSCs transplantation). Bone marrow aspirate was diluted by Hank's balanced salt solution (HBSS) in a ratio of 1:2.5. Density gradient separation medium Histopaque®-1077 was added to a 50-ml centrifuge tube (1 ml per 1 ml of the bone marrow). The bone marrow cell suspension was carefully layered on top of the gradient. After that, the tube was centrifuged at room temperature at 1800–2000 rpm for 30–40 minutes. The interphase cells containing MSCs were collected in a 15-ml centrifuge tube with a small amount of HBSS and re-suspended. The tube was centrifuged at 800–1000 rpm for 8–10 minutes. Supernatant was removed; the precipitate was re-suspended in HSSS and centrifugation was repeated twice. After that, the precipitate was mixed with growth medium containing DMEM/F12 (Sigma, USA), 20% FBS ("Biolot", Russia), and mitogens, and then suspension was seeded onto 75 cm^2 plastic flasks at a density of 1-2x10^5 cells/cm^2 and placed in a carbon dioxide incubator for 3 days (37^0C, 5% CO2). Next, the medium was replaced and non-adherent cells were removed. The medium replacement took place every 2 days. The term of autologous MSCs cultivation was 42 days.

For identification and characterization of MSCs, the basic criteria recommended by the International Society of Cell Technologies in 2006 were applied:

(a) MSCs must be adherent to plastic under standard tissue culture conditions;

(b) MSCs must express certain cell surface markers such as CD73, CD90, and CD105, and lack expression of other markers including CD45, CD34, CD14, or CD11b, CD79alpha or CD19 and HLA-DR surface molecules;

(c) MSCs must have the capacity to differentiate into osteoblasts, adipocytes, and chondroblasts under in vitro conditions.

On day 42 after MSC cultivation, the patient was re-admitted to the hospital for SMSCs transplantation into the liver. Manipulation was carried out in a specially equipped operating room to perform endovascular interventions. After pre-sedation, under local anesthesia and X-ray control, we catheterized *a. hepatica propria* through the femoral approach; then the cultured autologous MSCs were intro-arterial injected. The manipulation underwent well. Complications directly related to the manipulation were not. As seen in Figure 1, the arterial tree of liver surface was impaired. After the procedure, the patient was transferred to the surgical department and discharged home 3 days later in satisfactory condition.

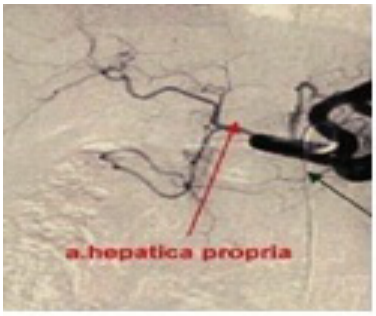

Figure 1. *SMSC transplantation into a. hepatica propria*

During the follow-up 2 months after transplantation, clinical improvement was revealed, as well as positive dynamics in the laboratorial parameters. We also noted a decrease in the severity of astheno-vegetative syndrome, disappearance of ascites, anemic and thrombocytopenic syndromes, improvement of protein-synthetic liver function (increased albumin levels), and a reduction phenomena cytolysis (decrease in the levels of thymol test and ALT). A follow-up examination at the end of the year after intra-arterial MSCs transplantation into liver revealed positive trends both in the subjective symptoms and laboratory-instrumental investigations. Objectively: a icterus ("jaundice") was not observed, the general condition of the patient was improved, and malaise has not occurred within the specified period of observation. Ascites was also not detected by ultrasound. Portal blood flow was 460 ml/min. Laboratory parameters also showed a positive trend: Hb 108 g/L; Er - 3.5x10^{12} /l; platelets - 210,000/ml; leukocytes - 3.8x10^9 /L, ESR - 22 mm/hour; total bilirubin - 22 μmol/L, total protein 98 g/L; albumin - 40 g/l; ALT - 86 IU/L; ACT - 92 IU/L; thymol test - 1.9; blood glucose was 4.2 mmol/L.

Thus, the above data showed high clinical efficacy of the intra-arterial administration of MSCs via the hepatic artery in a patient with liver cirrhosis.

Competing interests

The authors declare that they have no competing interests.

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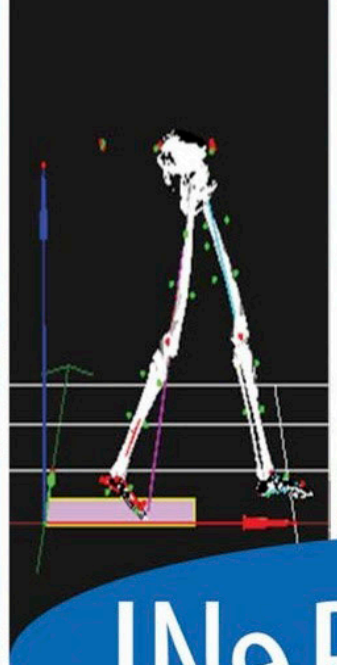

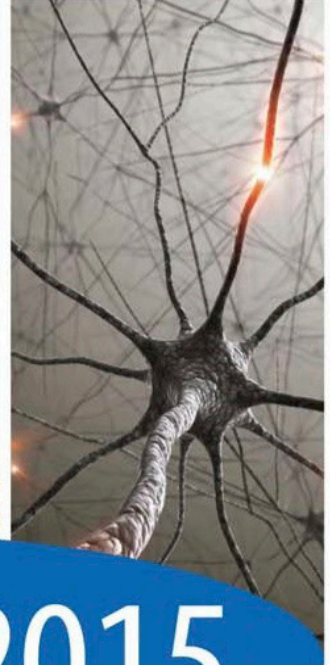
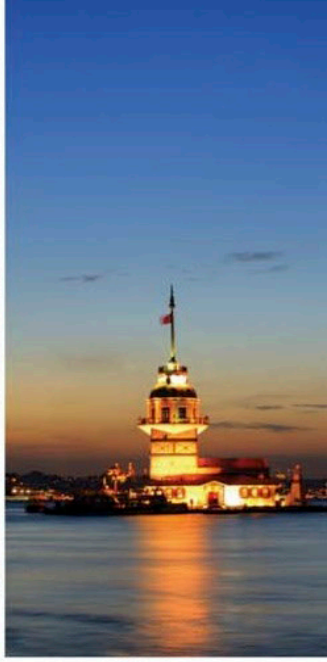

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