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NATIONAL TRANSPLANT REGISTRY: FROM WHO GUIDING PRINCIPLES ON NATIONAL REGISTRY FOR DONOR ORGANS, DONORS AND RECIPIENTS

Закон Российской Федерации от 22 декабря 1992 года № 4180-1 "О трансплантации органов и(или) тканей человека" определил условия и порядок трансплантации органов и(или) тканей, опираясь на современные достижения науки и медицинской практики, а также учитывая руководящие принципы Всемирной организации здравоохранения по трансплантации человеческих клеток, тканей и органов.

Сложившаяся к настоящему времени государственная система учета донорских органов и тканей человека, доноров и реципиентов осуществляется согласно Федеральному закону от 13.07.2015 года № 271-ФЗ "О внесении изменений в Федеральный закон "Об основах охраны здоровья граждан в Российской Федерации". Порядок учета сформулирован в приказе Минздрава России от 8 июня 2016 г. № 355н "Об утверждении порядка учета донорских органов и тканей человека, доноров органов и тканей, пациентов (реципиентов), форм медицинской документации и формы статистической отчетности…".

В нашем журнале ежегодно публикуются сообщения трансплантационного Регистра Российского трансплантологического общества (РТО). Трансплантационный регистр РТО существует более 10 лет. По-

Law No. 4180 1 of the Russian Federation "On Transplantation of Organs and (or) Human Tissues" (passed on December 22, 1992) defined the conditions and procedure for organ and (or) tissue transplantation, based on modern breakthroughs in science and medical practice, and also taking into account the WHO Guiding Principles on Human Cell, Tissue and Organ Transplantation.

The current Russian national registry for donor organs and human tissues, donors and recipients, is implemented in accordance with Federal Law No. 271-FZ of July 13, 2015 "On Amending the Federal Law On the Basics of Protecting Citizens' Health in the Russian Federation". The registry procedure is formulated in Order No. 355n of Russia's Ministry of Health (June 8, 2016) "On approval of the registry procedure for donor organs and human tissues, organ and tissue donors, patients (recipients), medical documentation forms and statistical reporting forms…".

The Russian Journal of Transplantology and Artificial Organs annually publishes reports from the Transplant Registry of the Russian Transplant Society (RTS). This Transplant Registry has existed for more than 10 years. The need for it came up a long time ago and it has increased year after

требность в нем возникла давно, и год от года она возрастала по мере развития донорства и трансплантации органов в стране, с расширением географии трансплантационных программ и увеличением числа центров трансплантации. Созданию регистра способствовали консолидация медицинского сообщества трансплантологов в Российское трансплантологическое общество (октябрь 2008 г.), а также объединение ведущих трансплантологов страны под эгидой профильной комиссии по трансплантологии Минздрава России (май 2009 г.).

Данные Регистра РТО предоставляются в международные регистры: International Registry of Organ Donation and Transplantation (IRODaT); Registry of the European Renal Association – European Dialysis and Transplant Association, ERA – EDTA Registry; Registries of the International Society for Heart and Lung Transplantation – ISHLT Registries.

С 2016 г. Регистр РТО используется в качестве инструмента контроля качества и полноты сбора данных в информационной системе учета донорских органов и тканей человека, доноров и реципиентов.

Таким образом, формирование существующего современного Национального трансплантационного регистра явилось результатом поступательного движения от рекомендаций ВОЗ к государственной системе учета донорских органов, доноров и реципиентов.

year with the development of organ donation and organ transplantation in the country, expansion of the geographical coverage of transplantation programs and increase in the number of transplant centers. The registry was created thanks to consolidation of the medical community of transplantologists into the Russian Transplant Society (October 2008), as well as collaboration between the country's leading transplantologists under the auspices of the relevant transplant commission of the Russian Ministry of Health (May 2009).

Data from the RTS Registry is submitted to international registries, such as the International Registry on Organ Donation and Transplantation (IRODaT), Registry of the European Renal Association – European Dialysis and Transplant Association (ERA-EDTA Registry), and Registries of the International Society for Heart and Lung Transplantation (ISHLT Registries.

Since 2016, the RTS Registry has served as a tool for quality control and data collection integrity in the information system for registering donor organs and human tissues, donors and recipients.

Thus, creation of the existing modern National Transplant Registry resulted from a progressive movement from WHO Guiding Principles on the national system of registering donor organs, donors and recipients.

С уважением
академик РАН С.В. Готье

Sincerely,
Sergey Gauthier, Academician,
Russian Academy of Sciences
Editor-in-Chief, The Russian Journal of Transplantology and Artificial Organs

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BASELINE PULMONARY HYPERTENSION IN HEART TRANSPLANT RECIPIENTS: 9 YEARS OF EXPERIENCE AT ALMAZOV NATIONAL MEDICAL RESEARCH CENTRE

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Objective: to assess the impact of baseline pulmonary hypertension (PH) on early and long-term outcomes following heart transplantation (HT). **Materials and methods.** From January 2010 to December 2018, 112 HTs were carried out. Based on right heart catheterization results, all recipients were divided into 2 groups: Group 1 – with PH (n = 76; mean pulmonary arterial pressure (mPAP) ≥25 mm Hg), Group 2 – without PH (n = 36; MPAP <25 mm Hg). The average age of Group 1 patients was 46.4 ± 14.9 years, baseline pulmonary vascular resistance (PVR) was 3.5 ± 1.5 Wood units, PVR after reversion test (nitric oxide – 80 ppm, iloprost 20 μg) – 2.8 ± 1.0 Wood units, systolic PAP (sPAP) – 50.1 ± 13.4 mm Hg. The average age in Group 2 was 47.3 ± 12.2 years, baseline PVR – 2.1 ± 0.8 Wood units, sPAP – 27.4 ± 5.3 mm Hg. The dynamics in indicators of early postoperative period (duration of mechanical ventilatory support, use of vasodilators and inotropic support and the length of stay in intensive care unit (ICU), 30-day mortality) and long-term post-HT echocardiography results were assessed. **Results.** Due to acute right-ventricular failure (RVF) developing after heart transplantation, veno-arterial extracorporeal membrane oxygenation (VA-ECMO) was done in 8 patients (11%) from Group 1 and one patient (3%) from Group 2. Presence of PH did not affect duration of mechanical ventilatory support, inotropic support, and length of stay in ICU. Levosimendan therapy in the early postoperative period was more often performed in Group 1 (n = 29) than in Group 2 (n = 6) (p = 0.048). Nitric oxide inhalation was also more often administered in Group 1 (n = 54); Group 2 (n = 7), (p = 0.003). Sildenafil therapy after HT was comparable in both groups Group 1 (n = 25); Group 2 (n = 6), (p = 0.048). In early post-HT stages, 14 patients died, 30-day mortality was comparable in both groups (p = 0.12). Six months after HT, no differences were found in the sPAP (p = 0.21) and PVR (p = 0.07) levels. **Conclusion.** Patients with baseline PH after HT have a more severe early postoperative period, including a higher RVF incidence, with the need for ECMO implantation. A PVR level >3.5 Wood units is not a threshold for HT. Patients with baseline PVR >3.5 Wood units following HT show comparable results with patients without baseline PH. This allows such patients (baseline PVR >3.5 Wood units) to be considered for inclusion in the heart transplant waiting list. In addition, 30-day mortality and duration of mechanical ventilatory support after HT in patients with and without baseline PH did not differ. Regardless of the baseline level of sPAP and PVR, all patients showed improvement in these parameters after HT. Six months after HT, no differences were found in sPAP and PVR levels in the patients, regardless of whether there was baseline PH or not.

Keywords: heart transplantation, heart failure, pulmonary hypertension, PH, pulmonary artery pressure, PAP, pulmonary vascular resistance, PVR, vasodilators.

INTRODUCTION

Selecting a therapeutic tactics for managing patients with severe cardiac pathology and associated pulmonary hypertension (PHT) remains one of the unresolved issues involved in choosing candidates for the heart transplant waitlist. According to ESC (European Society of Cardiology) guidelines, PHT is defined by elevated mean pulmonary artery pressure (mPAP) ≥25 mmHg [1]. C. Roldaan divides PHT into 3 groups: mild (sPAP 35–45 mmHg), moderate (sPAP 46–60 mmHg), and severe (sPAP >60 mmHg) [2]. There is no common opinion among researchers that could help to identify contraindications for heart transplantation (HT) in PHT. G.F. Delgado et al. and M.C. Deng et al. consider 2.5 WU as the limit value for pulmonary vascular resistance (PVR) when wait listing HT candidates [3, 4]. H. Ross et al. suggest that sPAP >50 mmHg and PVR >3–4 WU, measured after pharmacological test, should be considered a contraindication for HT [5]. According to J. Kettner et al., severe PHT (sPAP >50 mmHg) is no longer a contraindication for HT [6]. According to S. Klotz et al., HT candidates with pre-HT reversible PHT can be comparable with patients without PHT, since, despite a significantly higher risk of complications, long-term

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survival after orthotopic cardiac transplantation was not affected [7].

PHT increases the risk of early post-transplant acute right ventricular failure (RVF), which often requires the use of auxiliary circulatory devices (extracorporeal membrane oxygenation (ECMO) devices) and levosimendan. It is associated with severe complications [8, 9]. For instance, in a study by M.V. Mogollón Jiménez et al., which analyzed 39 heart transplantations in recipients with PHT (mPAP exceeded 35 mmHg in 30.8% of patients), early postoperative mortality increased to 50% [10]. In a study by S. Klotz et al., within 30 days after HT, RVF manifestations developed in 64% of recipients with PHT, and only 27% of patients without PHT [7]. Z. Zeng et al. found no significant difference in mortality rates in the PHT and non-PHT groups [11]. S. Klotz et al. found that PHT was reversible in most patients after HT [12]. In this case, there was no statistically significant difference in survival rates among the PHT and non-PHT groups [12, 13]. E. Gude et al. showed that a year after HT, recipients with mPAP >20 mmHg had a lower survival rate than those with mPAP <20 mmHg [14]. Currently, there is no clear consensus on PHT severity threshold values for wait-listing cardiac transplant candidates.

In PHT patients, B. Lindelow et al. observed rapid positive dynamics starting from the early post-transplant period – decreased PAP – without any other concomitant therapy [15]. After HT, all parameters normalize in patients (decreased PAP and PVR) [16]. In addition, HT outcome in PHT patients depended on different approaches and had no single relationship with the borderline values of these indicators for wait-listing cardiac transplant candidates. Various studies have demonstrated the inexactness of the effect of baseline PHT on the early post-transplant period and on mortality. Thus, issues related to selection and management of patients with associated PHT after HT require further investigation. The work was aimed at evaluating the effect of baseline PHT on early and long-term survival after heart transplantation.

MATERIALS AND METHODS

From January 2010 to December 2018, 112 orthotopic cardiac transplantations were performed via a bicaval technique. The recipients included 82 men and 30 women. Their average age was 46.7 ± 14.0 years. Before HT, the left ventricular ejection fraction (LVEF) was 22.3 ± 10.1%. Heart failure was caused by: coronary heart disease – 49% (n = 55), dilated cardiomyopathy – 28% (n = 31), non-compact myocardium – 8% (n = 9), arrhythmogenic right ventricular dysplasia – 3.6% (n = 4), congenital heart defect (CHD) – 3.6% (n = 4), chronic rheumatic heart disease – 2.7% (n = 3), hypertrophic cardiomyopathy (HCM) – 2.7% (n = 3), cardiac sarcoidosis – 0.9% (n = 1), cardiac amyloidosis – 0.9% (n = 1) and restrictive cardiomyopathy (RCM) – 0.9% (n = 1). Prior to HT, 13 recipients were implanted with mechanical circulatory support (MCS) devices: extracorporeal membrane oxygenation system (n = 6), biventricular MCS Berlin Heart EXCOR (n = 8). This group of patients had signs of NYHA FC II–III chronic heart failure (Simpson LVEF – 8 to 29%, TAPSE <10 mm), multiple organ failure (MOF).

Prior to HT, all patients underwent right heart catheterization in order to assess PVR level and central hemodynamics indicators. The patients were divided into 2 groups: group 1 – with pulmonary hypertension (mPAP ≥25 mmHg), group 2 – without pulmonary hypertension (mPAP <25 mmHg). In group 1, 47% (n = 36) of patients underwent a test for PHT reversibility with inhalation of nitric oxide (80 ppm) or iloprost 20 μg [17].

The average age of patients in group 1 (n = 76) was 46.4 ± 14.9 years, PVR value was 3.5 ± 1.5 (1.25 to 8.30) WU, PVR value after reversibility test was 2.8 ± 1.0 (0.7 to 5.0) WU, sPAP – 50 ± 13 (27 to 97) mmHg. In 6 patients from group 1 who were implanted with MPC Berlin Heart EXCOR as a bridge before HT, the average age was 19 to 39 years, PVR value was 2.9 to 4.5 WU, PVR value after reversibility test was 2.5 to 4.6 WU, sPAP – 42 to 58 mmHg. In group 2 (n = 36), the average age was 47.3 ± 12.2 years, PVR – 2.1 ± 0.8 (0.8 to 3.7) WU, sPAP – 27.4 ± 5.3 (14 to 36) mmHg. The age of patients between the two groups did not differ (p = 0.096). Pulmonary wedge pressure (PWP) in group 1 was 20.7 ± 6.8 (4 to 32) mmHg (including for recipients with Berlin Heart EXCOR – 19 to 28 mmHg), in group 2 – 11.1 ± 4.4 (3 to 20) mmHg (p = 0.023).

Also, 23 patients from group 1 were treated with levosimendan [18] and 4 with sildenafil before HT; 20 of them had PHT during surgery. Among associated diseases, it is necessary to note that 15 patients had chronic obstructive pulmonary disease while 33 had a history of pulmonary embolism.

Immediate outcomes were evaluated based on duration of inotropic support, mechanical pulmonary ventilation (MPV), being in the intensive care unit (ICU), frequency of deaths, use of levosimendan, sildenafil or inhaled nitric oxide. The use of dopamine, dobutamine and epinephrine was considered an inotropic support after HT. Vasopressor support was performed with norepinephrine.

To assess long-term results, transthoracic echocardiography (echocardiography) was performed 6 months after HT. The formula $10 \times (Vmax \times Th/VTI_{RVOT}) + 0.16$ was used to calculate PVR, while S.P. Nagueh 1.24 × E/Em + 1.9 was used to calculate PWP [19]. Echocardiography also assessed the presence of tricuspid valve regurgitation and mitral valve regurgitation.

Statistical data processing was performed using the SPSS 21.ORU program. In cases of normal distribution of indicators, the Student's t-test was used to evaluate the statistical significance of differences between groups. Data were presented in the form of "mean value ± stan-

dard deviation (M ± SD)". For a distribution other than normal, the nonparametric The Mann–Whitney U test was used to estimate differences; data were presented as median (Me) [25th; 75th percentile]. When describing groups of less than 20 patients, data were presented as median, minimum and maximum values of the symptom. Fisher's exact test was used to evaluate the differences in qualitative parameters. The criterion of statistical significance of findings was considered $p < 0.05$. This study was conducted in accordance with the principles of the Helsinki Declaration.

RESULTS

Early postoperative period in all patients had manifestations of biventricular heart failure. There was no significant difference in duration of patients' inotropic support in the early postoperative period (5 [4; 10] and 5 [3; 7] days, Mann–Whitney U test $p = 0.21$). Presence of PHT did not affect length of MPV after heart transplantation (1 [1; 2] and 1 [1; 2] days, Mann–Whitney U test $p = 0.8$). Due to post-HT acute RVF, 8 patients (11%) from group 1 and 1 patient (3%) from group 2 were implanted with ECMO systems using veno-arterial technique; 3 patients (4%) from group 1 and 1 (3%) from group 2 underwent tricuspid valve replacement (TVr) (Batista procedure) due to development of post-HT degree 4 tricuspid regurgitation (TR). Two recipients from group 1 had early graft dysfunction; there was no such complication in group 2. Endomyocardial biopsy in patients who underwent a TVr revealed no signs of rejection in the first follow-up month.

The study showed that the number of patients who had a complicated post-HT period and were in ICU for more than 10 days were more in group 1 (39%, n = 30) than in group 2 (19%, n = 7), chi-square $p = 0.04$. There were no statistically significant differences in the length of stay in ICU between the two groups – 8 [6; 13] and 7 [6; 10] days, respectively, $p = 0.18$.

However, in the early postoperative period, levosimendan therapy was more often performed in group 1 (n = 29) than in group 2 (n = 6), chi-square $p = 0.048$. Also, a significantly larger number of patients received nitric oxide inhalations in group 1 (n = 54) than in group 2 (n = 7), chi-square $p = 0.003$. Six-month outcomes of the use of sildenafil in the early postoperative period after transplantation were comparable in both groups: in group 1 – 25 patients, in group 2 – 6 patients, chi-square = 0.048.

In early post-HT stages, 14 patients died in the study population; 30-day mortality was comparable in both groups: group 1 – 12 (15%) recipients, group 2 – 2 (6%) recipients, chi-square $p = 0.12$. The difference in group 1 was that within 1 month after HT, five recipients died against the background of ECMO implanted due to acute RVF.

Six months after HT, the groups showed no differences in sPAP (34.2 ± 7.1 and 33.8 ± 4.8 mmHg, $p = 0.21$) and PVR (1.8 ± 0.6 and 1.5 ± 0.4 WU, $p = 0.07$) levels. In group 1, there was decreased sPAP in all patients after HT (48.3 ± 12.5 and 34.0 ± 7.0 mmHg, respectively, $p < 0.001$). In this case, 32% (n = 24) from group 1 still had PHT (sPAP 40.4 ± 4.8 mmHg) 6 months after HT. Patients from group 1 experienced decreased PVR level (3.7 ± 1.4 and 1.8 ± 0.5 WU, respectively, $p < 0.001$) six months after HT. In particular, positive dynamics were detected in recipients who before HT were on MPC Berlin Heart EXCOR: sPAP with 50 (42 to 58) mmHg. to 36 (29 to 38) mmHg; PVR from 4.1 (2.9 to 4.50) to 2.1 (1.9 to 3.4) WU. Six months after HT in patients from group 2, there was no evidence suggesting PHT; sPAP and PVR were within normal limits.

After 6 months, post-HT PWP levels in both groups did not significantly differ (12.4 ± 6.1 and 11.1 ± 4.7 mmHg, respectively, $p = 0.27$). However, in group 1, post-HT PWP levels were significantly lower than pre-HT PWP levels (17.0 ± 7.9 and 11.5 ± 5.3 mmHg, respectively, $p < 0.001$).

Six months after HT, incidence and severity of mitral regurgitation (MN) and TR were comparable – MN was detected in a quarter of patients in both groups: 26% (16 of 61) in group 1, 21% (7 of 34) in group 2, chi-square $p = 0.64$, and TR was found among most patients: 64% (39 of 61) in group 1, 65% (19 of 34) in group 2, chi-square $p = 0.44$. Incidence of MN (grade 2–4) in group 1 was 31% (5 of 16), in group 2 – 29% (2 of 7) and TR (grade 2–4): in group 1 – 36% (14 of 39), in group 2 – 32% (6 of 19), chi-square $p = 0.75$.

DISCUSSION

Irreversible PHT in patients undergoing pharmacological therapy is a contraindication for HT due to the high risk of postoperative RVF [6, 8]. HT surgeries at Almazov National Medical Research Centre has shown that the risk of early post-transplant complications is higher in recipients with baseline PHT. Some studies have demonstrated that despite the high risk of RVF, long-term survival after orthotopic cardiac transplantation is not affected by baseline PHT [7, 12]. This is consistent with our findings at Almazov National Medical Research Centre. We found out that PVR >3.5 WU is not a contraindication for heart transplantation. Besides, post-transplant outcomes in these patients are comparable to those with no baseline PHT.

Baseline PHT increases the risk of acute RVF in the early postoperative period after HT [15, 20]. The use of nitric oxide and other vasodilators perioperatively can be effective in reducing the risk of post-transplant RVF [12, 21]. TR is one of the most common post-HT complications [20]. It can be caused by various factors. At the same time, some studies attribute TR to progression of cell rejection [20, 22]. In our study, only 4 out of 89 pa-

tients needed TVr; they had no histological signs of graft rejection in the early postoperative period. Increased TR in a follow-up long-term period may be associated with the risk of tricuspid valve injury during endomyocardial biopsies, which requires further investigation.

Developing PHT long after HT is associated with decreased survival rate [14, 23]. According to B. Lindelow et al., all HT recipients have lower PVR levels [15]. In turn, Gude et al. claim that RVF and increased PVR levels at 6 months, 2 and 3 years after HT are prognostically unfavorable factors [14]. S. Klotz et al. claim that reversible PHT patients have similar outcomes with non-PHT patients [12]. Moreover, the presence of combined (pre-capillary and post-capillary) PHT before HT is attributed to persistence of increased PVR 1 year after surgery [24]. According to retrospective evaluation of our results, in 32% (n = 24) of patients with baseline PHT, sPAP levels were above 35 mmHg 6 months after heart transplantation, despite positive dynamics.

The use of sildenafil, levosimendan, and nitric oxide in patients with heart failure brings down PAP and PVR levels [25, 26]. Possible use of vasodilators after cardiac transplantation has not been sufficiently studied. A retrospective evaluation of our findings showed that the use of various tactics (levosimendan, sildenafil, nitric oxide) for treating PHT patients is effective in the early post-transplantation period.

CONCLUSION

1. Patients with baseline PHT after HT have a more severe early postoperative period, including higher incidence of RVF, which require ECMO implantation.
2. A PVR >3.5 WU is not a contraindication for heart transplantation. Patients with baseline PVR >3.5 WU after HT achieves comparable results compared with patients without baseline PHT. This makes such patients potential cardiac transplant candidates.
3. However, 30-day mortality and length of ventilator support after HT did not differ in patients with and without baseline PHT.
4. Regardless of baseline sPAP and PVR levels, these indicators improved significantly in all patients after HT.
5. 6 months after HT, no differences were found in the sPAP and PVR levels, regardless of whether the patient had baseline PHT or not.

The authors declare no conflict of interest.

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LIFE EXPECTANCY OF HEART RECIPIENTS WITH DONOR-TRANSMITTED CORONARY ATHEROSCLEROSIS

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Introduction. Heart transplantation (HT) is an extreme treatment for chronic congestive heart failure. One of the ways of reducing deficit of donor organs was to expand the criteria for selection of donors in favor of heart retrieval from older donors. This became one of the causes increasing the risk of donor-transmitted coronary atherosclerosis (DTCA). The impact of endovascular DTCA correction on postoperative survival of heart recipients remains poorly studied. **Objective:** to estimate the life expectancy of heart recipients with donor-transmitted coronary atherosclerosis. **Materials and methods.** The life expectancy of 518 heart recipients who underwent coronarography during the first week after HT was evaluated. When hemodynamically significant stenosis of the coronary arteries was detected, percutaneous coronary intervention (PCI) was performed as planned. The average age of recipients was 46.92 ± 1 year (10 to 72 years). 90% of them were men. Recipients' initial UNOS status was distributed as follows: UNOS 1a – 217 people, UNOS 1b – 89 and UNOS 2 – 212. Two groups were formed based on coronary angiography results. Group 1 included patients with DTCA signs, Group 2 was the control group (without DTCA). The first group was divided into 2 subgroups – a subgroup with DTCA signs, but without hemodynamically significant lesions (without PCI), and a subgroup with DTCA, where PCI was performed (PCI). **Results.** The age of recipients in both groups (DTCA and without DTCA) did not differ – 47.54 ± 1.01 and 46.64 ± 0.64 years, respectively. Donors were older in the DTCA group (50.2 ± 0.7 years) than in the control group (41 ± 0.5 years) (p = 0.0005). Survival in the control group averaged 58.25 ± 1.17 months, and in the DTCA group – 53.16 ± 0.36 months (p = 0.033). The difference in life expectancy of patients who underwent PCI (51.18 ± 2.9 months) and patients of the control group (58.25 ± 1.17 months) was not statistically significant (p = 0.88). In the group where graft showed signs of atherosclerotic changes in the coronary arteries, the cause of donor brain death from cerebrovascular accident was more common than in the control group. **Conclusion.** The risk of DTCA is associated with the donor's age and the death of the donor brain from vascular causes. Endovascular correction of atherosclerotic lesions of coronary arteries makes it possible to neutralize the impact of transplant coronary artery stenosis on long-term outcome of HT surgery.

Keywords: heart transplantation, donor-transmitted coronary atherosclerosis, percutaneous coronary intervention.

INTRODUCTION

HT is the only extreme therapy for chronic congestive heart failure [1, 2]. However, donor organ shortage limits its capabilities [3]. Therefore, optimization of HT treatment approaches is required. Recently, expanding the donor selection criteria has been used to address the problem of donor organ shortage [4, 5]. These changes led to retrieval of hearts from older donors, which in turn resulted in increased risk of DTCA [6]. Life-time pre-transplant diagnosis of atherosclerosis in potential donors is not always possible. Some transplant centers perform HT already with the presence of atherosclerotic lesions in coronary arteries, which were earlier detected in a potential cardiac donor via preliminary coronary angiography and intravascular ultrasound (IVUS). Under such conditions, HT requires simultaneous myocardial revascularization by coronary artery bypass grafting (CABG) or delayed percutaneous coronary intervention (PCI) [7–9]. However, in most cases, DTCA requires correction already in the post-transplant period. PCI has become the preferred method of revascularization. Assessing the prognosis for survival in such patients after DTCA correction remains a pressing issue.

Objective: to estimate the life expectancy of heart recipients with DTCA.

MATERIALS AND METHODS

A total of 518 angiograms of heart recipients who underwent treatment at our Center from 2013 to 2018 were analyzed retrospectively. Follow-up results of heart recipients who underwent coronarography within a week

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after HT were presented. Endovascular revascularization was performed during the first month whenever hemodynamically significant lesions were detected. The age of the subjects ranged from 10 to 72 years (average 46.92 ± 1), 90% were men. Recipients' initial UNOS status was distributed as follows: UNOS 1a – 217 people, UNOS 1b – 89 and UNOS 2 – 212. All the patients were divided into two groups: group 1 included patients who, according to coronary angiography, had signs of DTCA, while group 2 was the control group (without signs of DTCA). The first group was divided into 2 subgroups – a subgroup with DTCA signs, but without hemodynamically significant lesions, and a subgroup with DTCA, where atherosclerotic lesions in the coronary arteries were hemodynamically significant and required PCI. All patients underwent standard examinations, which included electrocardiography (ECG) and echocardiography (echo). ECG included 12-lead recordings on a Siemens Megacart device (Germany). An echo was performed on a GE Vivid E9 device (USA). The examination included 2D echo to determine the left ventricular volumetric characteristics using the area-length method – left ventricular end-diastolic volume (LVEDV) and left ventricular end-systolic volume (LVESV) – with calculation of the left ventricular ejection fraction (LVEF). Pulmonary blood pressure was estimated via doppler echocardiography. Degree of mitral regurgitation was estimated from 0 to 3. Coronary angiography was performed using the Judkins technique on a Siemens Axiom Artis angiography system (Germany) and Philips Allura Xper imaging system (Netherlands). PCI was performed via femoral arterial access using standard doses of heparin (5000 units) and stenting of the affected segments of arteries. In all cases, we performed complete revascularization, usually in one procedure. Stenosis of over 70% in the main branches (arterial diameter of at least 2.5 mm) were eliminated in all cases. In addition to evaluating recipients, the gender, age, and causes of death of heart donors were analyzed. These studies were processed by parametric statistics methods using Microsoft Excel and IBM SPSS Statistics 22.0. The study presented the arithmetic mean values of indicators and standard errors of mean. Significance of differences was evaluated via nonparametric tests: the Wilcoxon test for paired comparisons of dependent variables and the Mann–Whitney U test for comparing independent variables. Survival analysis was performed using the Kaplan–Meier estimator.

RESULTS

The average age of recipients in the DTCA and non-DTCA groups did not differ – 47.54 ± 1.011 and 46.64 ± 0.640 years, respectively. The initial UNOS status for different groups is presented in table 1.

Men made up 75% of heart donors in both groups. The average age of donors was significantly higher in the DTCA group than in the control group – 50.2 ± 0.7 and 41 ± 0.5 years, respectively (p = 0.0005). It should be noted that in the group with atherosclerosis, donors' mortality rate from cerebrovascular accident was over 15%, which may indicate systemic atherosclerotic processes. The characteristics of donors are presented in table 2.

Survival rates did not significantly differ among the groups – 26.54 ± 0.945 months in the DTCA group and 29.47 ± 0.95 months in the control group. Kaplan–Meier survival analysis for the groups is shown in Fig. 1.

The impact of initial UNOS status on survival in the post-transplant period was considered. The survival rates (depending on the initial UNOS status) are shown in Fig. 2.

Analysis of recipient subgroups in the DTCA group showed a significant difference in the average age of the donor, which was greater in the subgroup of patients who underwent endovascular revascularization. All other donor characteristics investigated did not differ (Table 3).

Table 1

Initial UNOS status of patients

Parameter	PCI subgroup, n = 65	DTCA subgroup, without PCI, n = 101	Control group, n = 352	DTCA group, n = 166
UNOS 1a	31 (47.5%)	47 (46.5%)	139 (39.5%)	78 (47%)
UNOS 1b	9 (14%)	19 (19%)	61 (17%)	28 (17%)
UNOS 2	25 (38.5%)	35 (34.5%)	152 (43.5%)	60 (36%)

Table 2

Main parameters of donors

Parameter		Control group, n = 352	DTCA, n = 166	p
Donor age (years)		41.40 ± 0.593	50.20 ± 0.714	0.0005
Donor gender	M.	270 (77%)	126 (76%)	0.525
	F.	82 (23%)	33 (20%)	0.525
	Unknown	0 (0%)	7 (4%)	0.525
Brain death caused by stroke		214 (61%)	126 (76%)	0.029

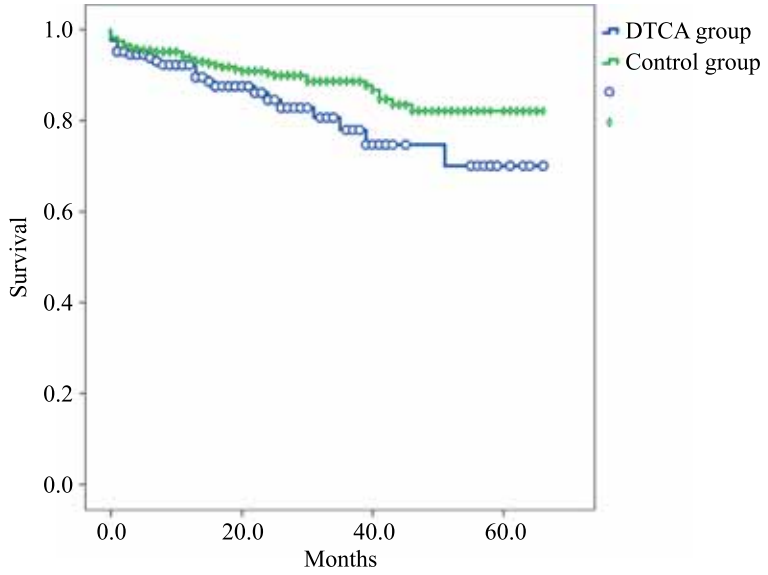

Fig. 1. Kaplan–Meier survival curves of patients after heart transplantation

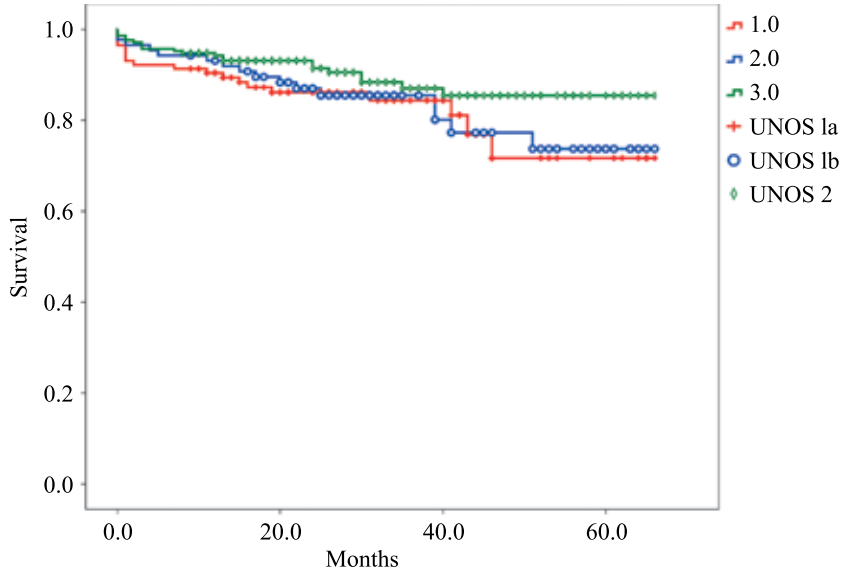

Fig. 2. Kaplan–Meier survival curves of patients after heart transplantation, depending on the initial UNOS status of the recipient

Table 3

Characteristics of donors in DTCA patient subgroups

Parameter		DTCA subgroups		p
		PCI, n = 65	Without PCI, n = 101	
Donor age (years)		52.65 ± 0.932	48.67 ± 0.976	0.011
Donor gender	M.	53 (81.5%)	73 (72.1%)	0.525
	F.	10 (15%)	23 (23%)	0.525
	Unknown	2 (3.5%)	5 (4.9%)	0.525
Brain death caused by stroke		50 (77%)	76 (75%)	0.029

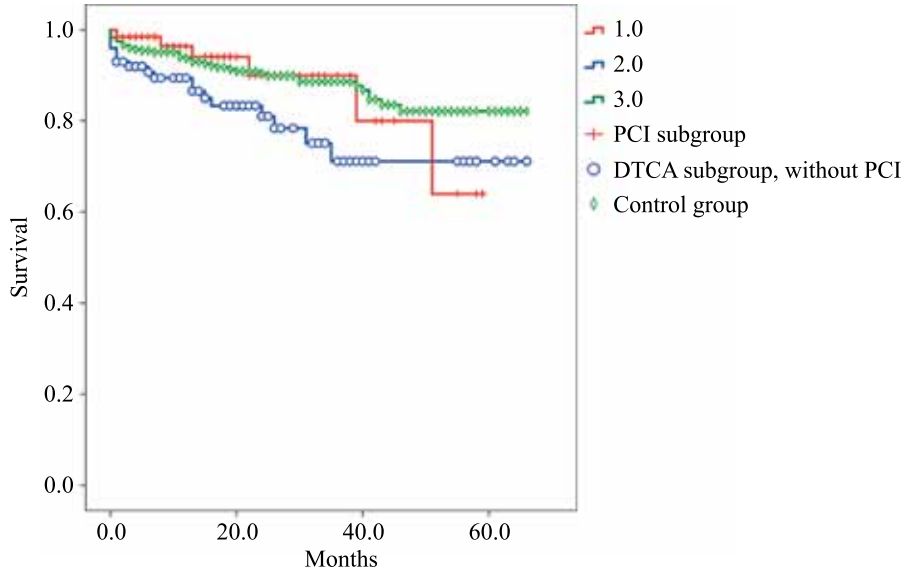

Fig. 3. Kaplan–Meier survival curves of patients after heart transplantation

Analysis of survival rates in the DTCA subgroup showed that the PCI group had better survival. Survival curves for subgroups are shown in Fig. 3.

CONCLUSION

Data presented suggests that the older the donor, the higher the risk of atherosclerosis and its transmission to the heart recipient. Donor brain death resulting from vascular causes may indirectly indicate generalized atherosclerotic processes in the body. Heart transplantation performed for UNOS status 2 recipients is prognostically more favorable for further survival than for UNOS status 1 recipients.

The authors declare no conflict of interest.

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EXTRAHEPATIC MALIGNANT NEOPLASMS AFTER LIVER TRANSPLANTATION: THE EXPERIENCE OF A SINGLE TRANSPLANT CENTER

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Objective: to evaluate the incidence of *de novo* malignant neoplasms (MN) after liver transplantation (LT) and compare with indicators among the general Russian population. **Materials and methods.** The study included 182 patients who had at least a 6-month follow-up period after LT and had no extrahepatic malignancies before LT. All data were analyzed retrospectively. Statistical processing of the results was carried out using the Statistica program for Windows v.10. **Results.** MN incidence was 5.5% (10 of 182 patients). The average period from transplantation to diagnosis of *de novo* neoplasm was 47.8 months (8 to 144 months). The patients were 3 men and 7 women. Types of *de novo* tumors included digestive system tumor (2 out of 10), hematologic malignant tumor (3 out of 10), skin cancer – melanoma (1 out of 10), urologic cancer (1 out of 10), gynecological (2 out of 10) and base of tongue cancer (1 out of 10). Five patients (50.0%) died, mortality was higher than in other LT patients ($Z = –2.6$; $p = 0.009$). The average follow-up period after detection of neoplasms was 18.8 months. Incidence of malignant neoplasms following LT was 10 times higher than among the general Russian population. No significant differences were found in the incidence of late acute rejection between 10 patients with MN and other 172 patients ($Z = 0.18$, $p = 0.8$). Among surviving patients, 2 patients with lymphomas received tacrolimus immunosuppression monotherapy, while 3 had everolimus-based immunosuppression. **Conclusion.** Incidence of *de novo* extrahepatic malignancies after LT is significantly higher than in the general population. To reduce the incidence of neoplasms in the future, patients should undergo regular screening, proliferative signal blockers should be prescribed, although their effectiveness requires further research.

Keywords: liver transplantation, de novo malignant neoplasms, incidence, tacrolimus, everolimus.

INTRODUCTION

Over the past decade, liver transplantation (LT) has become the treatment of choice for patients with liver failure in end-stage liver disease [1]. Breakthroughs in surgical technology, advances in immunosuppressive therapy, and optimized patient monitoring approaches have all led to improved survival outcomes. In most transplant centers, 1-year post-liver transplant survival is 91% and higher [1, 2]. Longer life expectancy in liver transplant recipients has led to higher incidence of cardiovascular diseases and extrahepatic MN [3, 4]. Obviously, organ recipients are 3–7 times more likely to develop extrahepatic MN than the general population due to the oncogenic effects of prolonged immunosuppression [5–7]. The cumulative frequency is 3–5% by three years and 11–20% by ten years after orthotopic liver transplantation (OLT) [1]. D. Collett et al. report that *de novo* malignancy rate reaches 10% by 10 years after LT [8].

In this retrospective study, the authors analyzed the incidence of post-LT *de novo* extrahepatic malignancies, as well as the types and risk factors.

MATERIALS AND METHODS

Analysis included data from 182 patients who underwent liver transplantation at the Granov Russian Research Center of Radiology and Surgical Technologies from 1998 to 2017. All were observed on an outpatient basis for at least 6 months and had no pre-transplant extrahepatic malignancies. Liver transplants were obtained from dead donors. After LT, basiliximab (Simulect®) induction therapy and a standard immunosuppressive therapy (IST) regimen were administered: calcineurin inhibitors (CNIs) (cyclosporin/tacrolimus), corticosteroids and mycophenolic acid. Proliferation signal inhibitors (everolimus) were prescribed to patients with hepatocellular cancer, as well as with CNI nephrotoxicity. After discharge, patients were observed monthly during the first year and then at intervals of 2–3 months. In each

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outpatient visit, clinical and laboratory examinations were performed to determine the main indicators of the functional state of the liver. Also carried out were comprehensive abdominal ultrasound, and multispiral computed tomography (MSCT) of the thorax and abdomen, fibrogastroduodenoscopy (FGDS), MRI – once per year during follow-up and according to indications. CNI concentration in the long term for tacrolimus was 3–5 ng/mL, for cyclosporine at point C0 – 100–150 ng/mL.

The results were statistically processed in statistics program Statistica v.10 for Windows. Descriptive and nonparametric statistics methods were used. The Mann–Whitney U test was used for intergroup comparisons. Data with $p < 0.05$ were considered significant.

RESULTS

Post-LT extrahepatic malignancies were detected in 10 out of the 182 patients. Of the 10 patients, there were 7 women and 3 men, the average age was 46.1 ± 9.4 years and 51 ± 10 years in LT and at the stage of MN diagnosis, respectively. The MN was detected within 8 to 144 months (average 47.8 months, median 36 months) after LT. Average follow-up period from the time MN was diagnosed was 18.8 months. MN incidence was 5.5% of all patients who were discharged for outpatient treatment and survived 6 months after LT. Post-transplant lymphoproliferative disorder (PTLD) was the most common disease – 3 patients (30%). Other MN localizations were: duodenal carcinoid – 1 (10%), gastric adenocarcinoma – 1 (10%), cervical cancer – 1 (10%), uterine adenocarcinoma – 1 (10%), skin melanoma – 1 (10%), base of tongue cancer – 1 (10%), renal cell cancer – 1 (10%).

Of the 10 recipients, 7 (70%) underwent surgery, 3 of which were radical, the rest were cytoreductive or diagnostic in nature. Persistent remission was achieved in 3 patients operated upon for uterine adenocarcinoma, renal cell carcinoma, skin melanoma, and in 2 cases of combined treatment for lymphoma. Treatment of non-Hodgkin's lymphoma after surgery was done according to the R-CHOP polychemotherapy regimen. During polychemotherapy, the IST scheme was modified – only tacrolimus was retained at a minimum concentration of not more than 3 ng/mL. Currently, in 1 patient, the duration of remission is 5 years, 1 patient is in remission for 12 months, having a satisfactory function of liver transplant amidst prolonged-release tacrolimus monotherapy (see table). One patient refused treatment and died from lymphoma progression. Epstein–Barr viral load carriage, which is considered a predictor for development of lymphomas, was detected in 2 out of 3 patients.

In the early postoperative period, 2 patients died of infectious complications: after gastric resection that was performed due to low-grade adenogenic cancer of body of the stomach, and after palliative duodenal resection for peritoneal carcinomatosis. One patient died from progression of cervical cancer one year after verification of diagnosis (T3M1N0) and combined radiation therapy and chemotherapy; one patient died of stroke (hemorrhagic stroke) after successful radiation treatment for base of tongue cancer (table). Thus, out of 10 patients with extrahepatic MN, 5 died (50%). Compared to the group of patients without MN, mortality in the studied small group was significantly higher ($Z = –2.6$, $p = 0.009$).

After cancer detection, the immunosuppression regimen was modified by prescribing a proliferation signal inhibitor (everolimus), without compromising liver transplant function. An exception was 2 patients with lymphomas, since there are no guidelines for the use of everolimus in such patients, there are data from clinical studies [9].

There were no significant differences in incidence of detected late rejection episodes and effect of bolus administration of corticosteroids in MN patients and other patients ($Z = 0.18$, $p = 0.8$).

The average follow-up period in the group of 10 people from the moment MN was detected was 18.8 months. However, the group was small, and calculations included data from those who died in the early period after non-radical surgical interventions performed in the late stage of the disease.

DISCUSSION

Records show that extrahepatic cancer in patients undergoing liver transplantation is more common than in the general population [10–13]. According to various centers, incidence of cancerous tumors varies from 2.6 to 26% [14–16]. Incidence of *de novo* cancer is 3 to 5% 1–3 years after liver transplantation and 11 to 20% 10 years after transplantation [1, 2, 16]. The most common malignancies after liver transplantation are skin cancer, lung cancer, PTLD, and Kaposi sarcoma [6, 8].

The Ministry of Health of the Russian Federation estimated the incidence of cancer in Russia in 2018 at 425.4 new cases per 100,000 population [17]. Based on data from our center (Granov Russian Research Center of Radiology and Surgical Technologies), incidence of *de novo* extrahepatic cancer in patients after LT was about 10 times higher than in the general population of the Russian Federation – 5.5%.

Various researchers have identified the main risk factors for development of post-LT cancer. These include old age, alcohol consumption, smoking, oncogenic viruses, excessive insolation and prolonged immunosuppressive therapy [2, 5, 18, 19]. In addition, immunosuppressive therapy contributes to suppression of immune control and lower resistance to certain oncogenic viruses [18, 20, 21]. In the studied group of patients, as well as in a similar Turkish study [22], the average age of recipients at the cancer detection stage was above 50 years – a potential risk factor for cancer development. Unlike other researchers, we did not identify lung cancer among the entire group of recipients. This is likely since there were

Table

Demographic characteristics of patients with post-transplant *de novo* malignancies

S/N	Sex	Age (years)	Diagnosis	IST	MN type	Stage	Treatment	Remission period (months)	Observation period (years)	IST in MN	Status
1	f	59.8	Polycystic kidney disease	CyA	Renal squamous cell carcinoma	T1aN0M0	Surgery	78	21.1	EVL	Alive
2	f	52.2	Primary biliary cholangitis	TAC + MPA	Duodenal carcinoid	T4NxM1	Surgery	0	0.9	TAC	Dead
3	m	34.6	Unspecified cirrhosis	CyA	Splenic lymphoma	–	Refused treatment	0	3.2	CyA	Dead
4	m	57.5	CHC	TAC + MPA	Gastric adenocarcinoma	T2NxM0	Surgery	0	3.1	EVL	Dead
5	f	48.5	Budd–Chiari syndrome	TAC	Endometrial adenocarcinoma	T1aNxM0	Surgery	64	13.4	EVL + TAC	Alive
6	f	45.5	Unspecified cirrhosis	TAC + MPA	Cervical cancer	T3bNxM0	Radiation, chemotherapy	0	9.9	EVL + TAC	Dead
7	m	65.2	Unspecified cirrhosis	TAC + MPA	Skin melanoma	T1N0M0	Surgery	24	8.5	EVL	Alive
8	m	53.8	CHC	EVL + TAC	Base of tongue cancer	T4N1M1	Radiation therapy	6	1.6	EVL	Dead
9	f	56.5	CHC	TAC	Non-Hodgkin's lymphoma	II	Surgery, chemotherapy	56	8	TAC	Alive
10	f	34.9	Retransplantation	TAC	Non-Hodgkin's lymphoma	IVA	Surgery, chemotherapy	12	6.4	TAC	Alive

Note. IST – immunosuppressive therapy; CHC – chronic hepatitis C.

more women in the observed patient population; smokers were no more than 20% of all outpatients.

Skin cancer, which is one of the most common types of post-transplant malignant neoplasms, was observed in only 1 patient (10%) in our population. It was detected after melanocytic nevus trauma and was radically operated on.

Chronic alcohol consumption and long-term tobacco smoking were present in only 1 patient (10%) who developed base of tongue cancer and was subjected to conformal irradiation with good clinical effect. However, the patient died of hemorrhagic stroke 6 months after therapy.

PTLD (30%) was dominant in the group of patients participating in our study. Outcomes of treatment for this disorder can be considered satisfactory, since in 2 cases, long-term remission was achieved. Unfortunately, one patient refused treatment – lived in a region far from the transplantation center – and died from progression of the disease.

Tacrolimus (8 patients) and cyclosporine (2 patients) were used as the immunosuppressive agents. Pulse methylprednisolone therapy was performed in 3 patients in the early postoperative period. In our series, we did not use antithymocyte immunoglobulin preparations, which are associated with more frequent PTLD [23]. None of the patients had hyperimmunosuppression during outpatient follow-up period. CNI concentration was monitored regularly and did not exceed 3–5 ng/mL for tacrolimus and 100–150 ng/mL for cyclosporine over a 12-month period after LT. Given the possible trigger effects of the Epstein–Barr virus for PTLD, preoperative screening and subsequent molecular genetic monitoring of this infection may be advisable; there is still insufficient data for mandatory preventive measures [24].

Thanks to improvements in transplantation technologies, liver transplant recipients are living longer, the population of recipients naturally ages, and the risk of developing MN thus increases [25]. Patients should be informed of such risks. Since cancer of the skin, head, neck, lungs and lymphoma is often develop after transplantation [5, 6, 26, 27], patients at risk may need more frequent outpatient visits, possibly preventative administration of proliferation signal inhibitors after discharge from the transplant center.

The use of proliferation signal inhibitors for prevention of MN recurrence or metastasis offered some hope [5] due to their ability to suppress neoangiogenesis. But literature data are contradictory and relate mainly to kidney transplant recipients [28–31]. Nevertheless, all

patients with newly diagnosed MN received everolimus in an average daily dose of 3.5 mg. Blood concentration was maintained at a level of at least 5–8 ng/mL. Increased concentration led to severe side effects. Despite everolimus use, the disease progressed in some cases. This was obviously associated with late diagnosis of MN, possibly with insufficient dose of the drug. The question of reducing incidence of post-LT malignant neoplasm with everolimus in combination with low-dose tacrolimus requires further study [32].

CONCLUSION

Incidence of *de novo* extrahepatic malignancies after liver transplantation is significantly higher than in the general population. To reduce the incidence of malignant neoplasms in the future, risk factors should be considered, cancer screening should be done, there should be regular outpatient visits and full instrumental examination of such patients. If a tumor is detected, proliferation signal inhibitors should be prescribed.

The authors declare no conflict of interest.

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DELISTING OF LIVER TRANSPLANT CANDIDATES FOLLOWING RECOMPENSATION OF CHRONIC LIVER DISEASES – PATIENT CHARACTERISTICS AND PREDICTORS OF DELISTING: A PROSPECTIVE STUDY

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Objective: to identify predicting factors at the listing stage that could be associated with recompensation followed by patient's delisting. **Materials and methods.** A prospective case-control study was conducted. The "case" cohort included 19 adult patients who initially were wait-listed as a result of decompensated liver diseases of various origin, but later were delisted due to recompensation. The "control" cohort consisted of 61 patients who were listed during the same period for decompensation and died in the waiting list. **Results.** A logistic regression model was used to determine independent predictors of delisting following recompensation. Plasma albumin concentration and white blood cell count at listing became significant predictors of recompensation ($p = 0.024$ and $p = 0.019$, respectively). ROC (Receiver Operating Characteristic) curve analysis was used to compare the predictability of identified predictors. The area under the ROC curve (AUC) for plasma albumin concentration was 0.938 [95% confidence interval (CI) 0.882–0.995; $p < 0.001$]. The AUC for the white blood cell count was 0.924 [95% CI 0.865–0.982; $p < 0.001$]. The odds ratio for recompensation outcome, if the plasma albumin concentration at listing was $\geq 3.1 \times 10^9$/L, was 14.639 (95% CI 2.16–99.12). The odds ratio for recompensation outcome, if the plasma albumin concentration at listing was ≥39.1 g/L, was 3.06 (95% CI 1.58–5.95). **Conclusion.** Liver injury could be reversed after the factors leading to decompensation have ceased to exist. Independent predictors of recompensation and subsequent delisting of patients were: white blood cell count $\geq 3.1 \times 10^9$/L and plasma albumin concentration ≥39.1 g/L at listing for liver transplantation.

Keywords: liver transplant waiting list, delisting following recompensation of liver function, predictors of delisting.

INTRODUCTION

Liver transplant (LT) is the only option leading to higher survival of patients with end-stage liver disease when all other treatment methods are unsuccessful [1, 2]. The LT has a successive outcome due to the lack of alternative therapy and good survival rates of patients in the post-transplant period (90% and 80% in the first year and for the following five years, respectively) [3]. An important component of the LT procedure is the patients' selection and their inclusion in the waiting list (WL). After the LT candidates were wait listed, specialists monitored the somatic status and provided for the dynamic clinical and laboratory control, carried out pathogenetic and / or symptomatic therapy, and in the case of life-threatening complications, surgical treatment [3, 4]. The LP waiting list may include three main types of patients [1, 2]. The first group comprises patients with acute liver failure, who in most European centers are included in the so-called LT emergency waiting list. These patients have priority over all other liver recipients and receive a transplant within a few hours or days [5]. The second group in the LT waiting list are patients with decompensated liver cirrhosis (LC). The LT timing is determined by the MELD (Model for End Stage Liver Diseases) index. Priority is given to patients with a very high MELD, in which the LT terms are from several days to several weeks. The LT timing for patients with moderate to low MELD levels varies from several months to several years (1). The third group in the liver waiting list consists of patients with hepatocellular carcinoma (HCC) on the background of compensated LC. Lack of donor organs is a limiting factor in the LT development both in various countries and globally [6], which, in turn, contributes to the mortality of the wait

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listed patients or a critical deterioration in liver function leading to negative outcomes in the perioperative period and in the long term [7]. Nevertheless, in everyday clinical practice the liver function occasionally improves and the recompensation develops, even among the wait listed patients [8]. In particular, a change in the paradigm of liver decompensation and the development of recompensation became possible after modern antiviral agents were introduced into clinical practice. The use of drugs with direct antiviral effect (DAE) in patients with HCV cirrhosis awaiting LT showed a significant clinical improvement leading to their delisting [9–11]. It was possible to identify recombination predictors through analysis of the wait listed patients with alcoholic liver disease (ALD). In this, at listing, MELD <20 and serum albumin ≥32 g/l are predictors of the recompensation development in the ALD patients and their subsequent delisting [12]. In this regard, the present work was aimed at identifying, at the listing stage, the predictive factors (predictors) which could be associated with recompensation development followed by patient's delisting.

MATERIALS AND METHODS

From 2015 to 2019, 198 LT candidate patients have been included in the WL. Of these, 39 patients underwent orthotopic LT (OLT). The data obtained during a prospective case-control study of 80 LT candidate patients observed at the Center for Surgery and Donor Coordination of the Rostov Regional Clinical Hospital were analyzed. The case cohort included 19 adults with decompensated liver diseases of various etiologies included in the WL and subsequently delisted due to recompensation. The control cohort consisted of patients (n = 61) with decompensated liver diseases who were wait listed at the same time interval and died during the decompensation period.

Exclusion criteria were severe cardiopulmonary pathology; continued alcohol intake at the time of the study; hepatocellular carcinoma; patients included in the WL due to decompensation and delisted due to reasons other than recompensation; patients included in the WL for reasons other than decompensation (recurrent cholangitis with primary sclerosing cholangitis); patients included in the WL for advanced thrombosis of the portal vein and its stems, the Budd Chiari syndrome, sinusoidal obstruction syndrome, polycystic liver disease, amyloidosis; patients included in the WL for transplant or with other organs' transplants in history; patients with acute liver failure.

The demographic and clinical data were obtained from the continuously updated electronic database of the Center for Surgery and Donor Coordination of the Rostov Regional Clinical Hospital.

At listing and with the development of recompensation, all patients were measured for the original and updated indices: MELD [13, 14], MELD-Na [15] and Charlson comorbidity index [16].

The study was approved by the Ethics Committee at the Rostov Regional Clinical Hospital. The primary endpoint of the study was the identification of the at-listing factors associated with subsequent delisting of the patients due to recompensation, i.e., possible recombination predictors identification.

The reasons for listing the patients with liver function decompensation were failure of all previous therapeutic measures, development of ascites or hepatic hydrothorax, indications for antibiotic relief of spontaneous bacterial peritonitis, jaundice, presence of hepatic encephalopathy (HE) and / or varicose gastrointestinal bleeding, MELD ≥16. In all ALD patients included in the WL, withdrawal symptoms persisted for at least 3 months as evidenced by the narcologists and psychiatrists.

The clinical diagnosis of recompensation of patients included in WL due to decompensation of liver function was based on the absence of ascites, "hepatic" hydrothorax, and peripheral edema despite the cessation of diuretics, the absence of hepatic encephalopathy and the need for its preventive therapy, MELD <15. All patients with recombination have been followed-up for six months to confirm a stable "recompensation status" confirmed by examinations by specialists and the subsequent decision on delisting.

All patients in the WL passed clinical blood and urine tests, biochemical analyses, studies of hemostasis parameters, HBV and HCV screening and diagnostics, liver elastography and biopsy. Some patients underwent ascitic fluid analysis.

The patients in both cohorts with HCV and HBV infection received antiviral therapy, including direct antiviral drugs (HCV) and nucleoside reverse transcriptase inhibitors (HBV). In the patients with autoimmune diseases, the therapy included immunosuppressants, glucocorticosteroids. All patients underwent pathogenetic therapy with non-selective β-blockers and diuretics. The HE patients got L-ornithine-L-aspartate intravenously in combination with lactulose and rifaximin per os. In some patients, extracorporeal hemocorrection (plasma sorption and CVVHDF) was applied.

Some patients in both cohorts underwent surgery for recurrent varicose bleeding: transjugular intrahepatic portosystemic shunt (TIPS) and azygoportal disconnection (APD, RF patent No. 2412657) by the original procedure [17].

The statistical analysis of data was made with the IBM SPSS Staticrics version 21. To check the normality of distribution of the obtained data, Kolmogorov–Smirnov test was used. Sample data with a normal distribution are represented by arithmetic means (M) and standard deviation (SD) with 95% confidence interval (CI). The statistical significance of the differences between the compared parameters in the normal distribution was determined by Student t-test. Without normal data distribution, non-parametric tests were used, Wilkoxon for

paired comparisons of dependent variables, Mann–Whitney U test, Pearson's chi-squared test for comparison of independent variables. Quantitative indicators in samples with a distribution beyond normality were presented as the median and interquartile range (between the 25th and 75th percentiles). For qualitative data, frequencies and fractions (%) were calculated. Differences between the compared parameters were considered statistically significant provided the error less than 0.05 ($p < 0.05$). Regression analysis (logistic regression) was used to determine the recombination predictors. The odds ratio for significant outcome predictors was calculated by recompensation with 95% CI. To assess the quality of the constructed regression models (predictive ability of the model), ROC (Receiver Operating Characteristic) curves were built and the area under the AUC (Area Under Curve) was calculated. The AUC ROC not differing from 0.5 [18] was taken as a zero hypothesis. Survival was assessed by Kaplan–Meier technique.

RESULTS

Characteristics of the case group patients (recompensation)

The group of the patients with recompensation included 10 men (52.63%) and 9 women (47.37%) with a mean age at the time of inclusion in the WL 48.4 ± 10.3 years. BMI 25.5 ± 3.3 kg/m^2. The average stay in WL was 31.7 ± 12.1 days. At the time of listing, MELD-Na was <20 in 21.1% of cases, =20 in 63.2% of cases, and 21–30 in 15.7% of cases. The hepatorenal syndrome (HRS) was diagnosed in 21.05% of patients. Expressed HE was diagnosed in 84.21% of cases, latent HE in 15.69% of cases. By etiology, patients with end-stage liver disease were distributed as follows: LC in the outcome of chronic hepatitis C – 9 patients (47.37%), LC in the outcome of ALD – 5 patients (26.33%), primary biliary cirrhosis (PBC) – 1 patient (5.26%), PBC and autoimmune hepatitis (AIH) – 1 patient (5.26%), cryptogenic LC – 3 patients (15.79%). Eleven LC patients (57.89%) had class C and 8 patients (42.11) – class B by the Child–Pugh score. Charlson comorbidity index was 9.05 ± 2.48.

57.9% of patients in this group were treated casually, 89.5% of patients received non-selective β-blockers, 100% of patients received diuretics and HE therapy (intravenous administration of L-ornithine-L-aspartate in combination with lactulose and rifaximin per os). Besides the drug therapy, patients got azygoportal disconnection by the original technique (31.6% of cases), a single endoscopic esophagus veins ligation (10.5% of cases) and extracorporeal hemocorrection (plasma absorption combined with CVVHDF) (5.3% of cases).

Characteristics of the control group patients (fatal cases with liver function decompensation development)

The group of the patients with fatal cases included 36 men (59.02%) and 25 women (41.98%) with a mean age at the time of inclusion in the WL 48.2 ± 11.3 years, BMI 25.3 ± 6.6 kg/m^2. The average stay in WL was 9.8 ± 8.4 days. At the time of listing, MELD-Na was <18 in 3.3% of cases, 19–25 in 42.6% of cases, 26–35 in 34.4% of cases, >35 in 19.7% of cases. Hepatorenal syndrome (HRS) was diagnosed in 65.6% of patients. Expressed HE was diagnosed in 95.1% of cases, latent HE in 4.9% of cases. By etiology, patients with end-stage liver disease were distributed as follows: LC in the outcome of chronic hepatitis B – 2 patients (3.3%), LC in the outcome of chronic hepatitis B+D – 2 patients (3.3%), LC in the outcome of chronic hepatitis C – 17 patients (27.8%), LC in the outcome of ALD – 16 patients (26.2%), primary biliary cirrhosis (PBC) – 5 patients (8.2%), AIH – 2 patients (3.3%), primary sclerosing cholangitis (PSC) – 5 patients (8.2%), cryptogenic LC – 9 patients (14.7%). Sixty LC patients (98.4%) had class C, one patient (1.6%) – class B by the Child–Pugh score. Charlson comorbidity index was 9.11 ± 2.66.

31.2% of patients in this group were treated casually, 91.9% of patients received non-selective β-blockers, 100% of patients received diuretics and HE therapy (intravenous administration of L-ornithine-L-aspartate in combination with lactulose and rifaximin per os). Besides the drug therapy, patients got azygoportal disconnection by the original technique (4.92% of cases), TIPS (4.92% of cases), a single endoscopic esophagus veins ligation (13.11% of cases). Thirty patients got laparocentesis (49.18% of cases).

Comparison of parameters in the case and control groups

When checking the distribution of the obtained data with the Kolmogorov–Smirnov test, the age parameters of the patients, the number of leukocytes and platelets at the time of inclusion in WL, the albumin concentration at the time of inclusion in WL, MELD, MELD-Na, and Charlson corresponded to the normal distribution. Those were analyzed by parametric statistics. All other parameters (HE degree, alkaline phosphatase activity, Na concentrations, creatinine and bilirubin at the time of inclusion in WL, INR and BMI at the time of inclusion in WL) did not correspond to the normal distribution and nonparametric statistical methods (Mann–Whitney test, U-test, Chi-square) were used for their analysis.

Tables 1 and 2 show the demographic, clinical, laboratory parameters, BMI, comorbidity, MELD, MELD-Na in the groups of patients with recompensation (n = 19) and fatal cases in the period of stay in the WL (n = 61).

Table 1

Comparative characteristics of parameters of patients with recompensation (delisting) and deaths in the period of stay in the waiting list – listing (normal distribution)

Parameter	Recompensation (n = 19) M ± SD	Deaths (n = 61) M ± SD	p value
Age	48.42 ± 10.32	48.23 ± 11.26	0.57
WBC at the time of inclusion in the waiting list, $\times 10^9$/l	3.66 ± 0.38	2.55 ± 0.68	0.026
PLT at the time of inclusion in the waiting list, $\times 10^9$/l	84.37 ± 31.31	53.02 ± 33.37	0.912
Plasma albumin at the time of inclusion in the waiting list, g/l	39.21 ± 3.36	27.74 ± 6.33	0.015
MELD at the time of inclusion in the waiting list	15.73 ± 3.56	25.12 ± 8.43	<0.001
MELD-Na at the time of inclusion in the waiting list	15.77 ± 3.55	25.45 ± 8.44	<0.001
Charlson index at the time of inclusion in the waiting list	9.05 ± 2.48	9.11 ± 2.67	0.864

Table 2

Comparative characteristics of parameters of patients with recompensation (delisting) and deaths in the period of stay in the waiting list – listing (lack of normal distribution)

Parameter	Recompensation (n = 19) Median (25^{th}–75^{th} percentile) or quantity (%)	Deaths (n = 61) Median (25^{th}–75^{th} percentile) or quantity (%)	p value
Male gender	10 (52.6%)	36 (59%)	0.623
HE degree	2 (2-2)	3 (2-3)	<0.001
ALP at the time of inclusion in the waiting list, U/l	265.0 (180.0–300.0)	389.0 (296.5–500.5)	0.001
Na at the time of inclusion in the waiting list, mmol/l	139.0 (138.0–141.0)	136.0 (135.5–138.5)	0.001
INR at the time of inclusion in the waiting list	1.4 (1.3–1.5)	1.8 (1.6–2.35)	<0.001
Creatinine at the time of inclusion in the waiting list, μmol/l	114.0 (86.0–120.0)	148.0 (111.5–202.5)	<0.001
Bilirubin at the time of inclusion in the waiting list, μmol/l	49.0 (38.0–72.0)	82.0 (55.0–142.5)	0.001
BMI at the time of inclusion in the waiting list, kg/m^2	24.8 (23.6–28.3)	24.5 (20.6–27.9)	0.459

The patients with respective outcomes (recompensation/death) were subjected to regression analysis (logistic regression). Significant recompensation predictors were the parameters of albumin in blood plasma and leukocyte levels at the time of inclusion in the waiting list ($p = 0.024$ and $p = 0.019$, respectively).

Odd ratio (OR) for the recompensation outcome (delisting) provided the WBC count at the time of inclusion in the waiting list was $\geq 3.1 \times 10^9$/l, was 14.639; 95% CI 2.16–99.12. OR for the recompensation outcome (delisting) provided the albumin content in blood plasma at the time of inclusion in the waiting list was ≥39.1 g/l, was 3.06 (95% CI 1.58–5.95).

The AUC were calculated for albumin concentration and leukocyte level at the time of inclusion in the waiting list; the ROC curves were built for these parameters (Fig. 1). AUC ROC for albumin concentration was 0.938 [95% CI 0.882–0.995; $p < 0.001$]. AUC ROC for leukocyte levels was 0.924 [95% CI 0.865–0.982; $p < 0.001$].

The development of patient recompensation was analyzed by Kaplan–Meier technique. The survival function in the model was identified with the recombination development at certain times for specific patients. Figure 2 shows the waiting time for the recompensation development for patients (the period from inclusion in WL to the recompensation development and delisting).

DISCUSSION

It was shown that in the group of patients with developed recompensation of liver function at the time of inclusion in WL the leukocytes level, albumin concentration in plasma, Na in blood appeared significantly higher than in the group of deceased patients with decompensation. In the group of patients with recompensation at the time of inclusion in WL INR, HE degree, alkaline phosphatase, creatinine, bilirubin, MELD and MELD-Na were also significantly lower than in the group of the patients deceased at the decompensation stage.

The recompensation development in patients with LC of various etiologies is associated with a number of plausible factors. Fibrosis and portal hypertension have been shown to decrease after successful antiviral therapy of HCV-associated LC [18–21]. In particular, a significant decrease in the hepatic venous pressure gradient (HVPG) was established after achieving a stable virologic response resulted from HCV antiviral therapy in patients with decompensated LC and portal hypertension [20, 21]. A multicenter European study showed that recompensati-

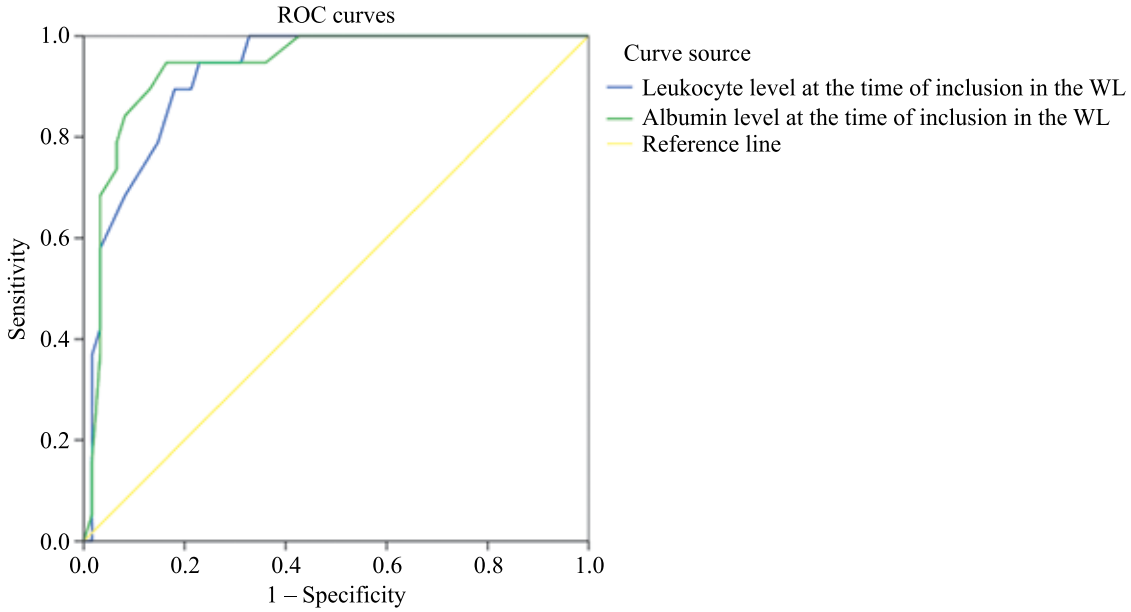

Fig. 1. ROC curve for leukocyte and albumin levels in the blood of patients at the time of inclusion in the waiting list as predictors of the development of recompensation

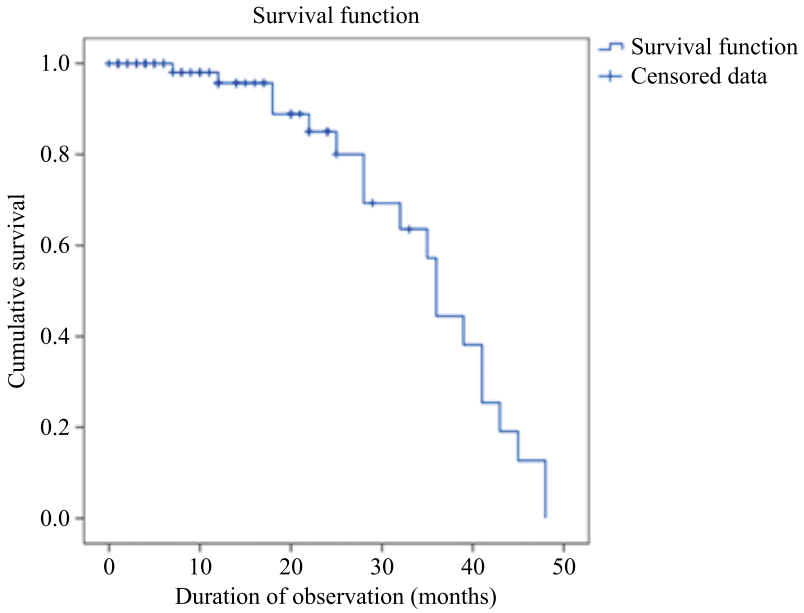

Fig. 2. The waiting time for the recompensation development (survival function) in the analysis of the survival rate by Kaplan–Meier

on due to antiviral therapy caused delisting of patients (19.2% of cases) who were previously included in WL due to decompensated LC in the outcome of chronic hepatitis C [22]. The authors came to an important conclusion that treatment of patients included in WL with direct antiviral agents before transplant, causing recompensation and delisting, can significantly reduce the LT number, which is important against the background of organ deficiency and high prevalence of HCV-associated liver diseases.

Another study showed that in 30.9% of cases, patients with successful antiviral therapy for HCV-associated decompensated LC develop recompensation followed by delisting [23]. However, in two years after delisting, four patients were re-included in WL (relisted). In one case, the patient developed HCC, in three cases ascites developed.

As a result of antiviral therapy in patients with HBV infection, the potential for the reverse development of not only compensated, but also decompensated LC was demonstrated [24–26]. Jang et al. [27] found antiviral therapy of LT candidates with HBV-associated decompensated LC to cause recombination followed by delisting in about $^{1}/_{3}$ of patients.

Candidate patients for LT with LC due to the development of obesity and progression of non-alcoholic steatohepatitis (NASH) got bariatric surgery [28]. In 33.3% of patients, a regression of the disease developed followed by delisting. However, subsequently, as the follow-up period increased to 7 years, the authors noted the development of sarcopenia and malnutrition in 71.4% of cases in patients with delisting.

Aravinthan et al. [12] found that of 77 LT candidate patients who developed recompensation with subsequent delisting, 61% had ALD, 16% – HCV-associated LC, 5% – ALD/HCV-associated LC. In the remaining patients, recombination followed by delisting in 4% of cases was associated with HBV-induced LC, in 5% of cases with AIH, in 4% of cases with NASH, in 1% of cases with PSC, in 1% of cases with cryptogenic LC, in 3% of cases with sarcoidosis. The authors suggest the application of TIPS, antiviral therapy of HCV and HBV infection, and treatment of AIH with azathioprine as probable factors that caused the development of recompensation in the patients [12].

In our study, the factors for the development of recompensation followed by delisting of patients are likely successful antiviral therapy of HCV-associated LC, immunosuppressive therapy of autoimmune liver diseases, HE therapy, administration of diuretics and non-selective β-blockers. Probable factors also include the application of TIPS, azygoportal disconnection by the original technique, and endoscopic ligation of the esophagus veins.

Using logistic regression, it was found that leukocytes level and albumin concentration at the time of inclusion in WL are independent predictors of the disease recompensation and patient delisting. The model has high predictive ability, sensitivity and specificity, as evidenced by AUC for both independent variables (0.924 and 0.938, respectively) and ROC curves.

Decrease in the leukocyte level in LC patients can presumably be associated with portal hypertension. For instance, spleen enlargement in LC patients is often accompanied by the development of hypersplenia that serves as the main cause of cytopenia and thrombocytopenia [29]. The exact effector mechanisms associated with splenomegaly and hypersplenia remain unclear. Nevertheless, the most probable causes of these phenomena are hemodynamic disturbances due to portal hypertension, damage to spleen tissue, and the inflammation-induced release of signaling molecules [30, 31]. The recompensation development, as our data show, is associated with a significant difference between the leukocyte levels in the compared groups at the time of inclusion in WL, which probably reflects a lesser degree of hypersplenia, and, accordingly, portal hypertension. This assumption is confirmed by the OR calculation, which showed that in patients with leukocyte levels $\geq 3.1 \times 10^9$/l at the time of inclusion in WL, the probability of developing recompensation (delisting) increases by 14.639 times.

Our data show that the second independent predictor of the recompensation development with subsequent delisting was the concentration of albumin in the blood plasma at the time of inclusion in WL. Belli et al. [22] in a multicenter European study found that the recompensation predictors for patients with HCV-associated LC due to successful antiviral therapy were MELD and serum albumin concentration at the time at the time of inclusion in WL. Aravinthan et al. [12] showed that in patients with ALD (decompensated LC), both of these indicators at the time of inclusion in WL turned out to be independent predictors of the recompensation development and subsequent delisting.

Hypoalbuminemia is an independent risk factor for patient mortality as a marker of malnutrition [32–34], and an increase in plasma albumin concentration is a predictor of patient recompensation [12, 22]. By calculating OR, we showed that in patients with albumin concentration ≥39.1 g/l at the time of inclusion in WL, the probability of recompensation developing (delisting) increases by 3.06 times.

We found that at the time of inclusion in WL, MELD and MELD-Na were significantly lower in the group of patients with recompensation than in the group of patients who died due to decompensation. At the time of inclusion in WL, low MELDs increased the likelihood of recompensation development, and high MELDs, on the contrary, were negative predictors for patients with decompensated HCV cirrhosis who received antiviral therapy and decompensated LC of the alcoholic etiology [12, 22].

Recompensation of patients with decompensated diseases is a clinical conclusion not corresponding to the concept of "recovery". There are various points of view specialists have on the definitions of this condition: "recompensation", "access to transplant", "avoidance of additional complications", etc. [36]. Regression of fibrosis after elimination of the HCV virus is a lengthy but probably proven process [37, 38]. However, despite the eradication of the HCV virus, fibrosis can not only avoid regress, but progress. Perhaps this is due to the fact that it the line between the "return and no return points" are very difficult to find, especially if we take into account that the elimination of liver damage factors (eradication of the HCV virus, withdrawal symptoms in the case of ALD) do not lead to normalization of vasculature alterations in LC patients [36].

CONCLUSION

The present study showed that the reversibility of liver damage (recompensation) after the cessation of factors causing its decompensation is a likely process. It should be emphasized that the concept of "recompensation" is a clinical conclusion that is not synonymous with the concept of "recovery" and requires physicians to continuously monitor patients and make proper decisions

(reinclusion in WL, relisting) if the condition worsens. It seems possible that there exists a "critical point of no return" after which the decompensation of liver function becomes irreversible. When candidates for LT are included in the WL, independent predictors of the liver recompensation development and subsequent delisting of patients are the number of blood leukocytes $\geq 3.1 \times 10^9$/l and the concentration of plasma albumin ≥39.1 g/l.

The authors declare no conflict of interest.

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PECULIARITIES OF THE MORPHOLOGY OF LIVER BIOPSY SAMPLES OF DONORS ABOVE 60 YEARS OF AGE

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Objective: to study the differences in the frequency of pathological processes in liver biopsy samples of donors older than 60 years (group 1) and donors currently recognized as "standard" by age – 60 years and younger (group 2). **Material and methods.** Of the total pool of 300 consecutive donors with brain death, there were 28 (9.3%) donors over 60 years old (61 to 73 years old; 19 men and 9 women). **Results.** The frequency of pathology is independent of gender in both groups ($p > 0.05$). In elderly donors, compared with "standard" donors, mild ($p < 0.05$) and significantly more often severe ($p < 0.05$) albuminous degeneration are significantly less frequent, and there is only a tendency ($p > 0.05$) to more frequent mild hepatic steatosis. Dystrophic processes are the result of more severe ischemic injury to the liver of elderly donors. Ischemic liver injury determines the risk of more frequent biliary complications, which require careful monitoring and maintenance at an optimal level of hemodynamics for donors in the intensive care unit. Based on other morphological parameters, the liver of donors above 60 years of age does not significantly differ ($p > 0.05$) from the liver of donors 60 years and younger. **Conclusion.** To expand the donor pool, age restrictions should be removed when selecting a liver for transplantation, thereby maximizing the use of donor potential.

Keywords: elderly liver donors, "standard" liver donors, albuminous degeneration of hepatocytes, fatty hepatosis, liver fibrosis.

Liver transplantation (LT) is the only effective treatment for end-stage acute or chronic liver disease. However, there is worldwide shortage of organs to perform these surgical procedures. Many waitlisted patients do not live up to LT. That is why medical researchers are now searching for additional ways of increasing the pool of donors. Transplantation of a part of the liver (transplantation of a part of the liver of a living donor and transplantation of a split liver) is helping to overcome donor organ shortage to a certain extent [1]. Besides, using organs from expanded criteria donors is one of the ways of increasing the number of LT. An expanded criteria donor is any deceased donor over the age of 60, a donor with hepatitis C virus, a donor whose cold ischemia has lasted for over 12 hours, a non-heart-beating donor, or a donor with hypernatremia, macrovesicular steatosis >30%. In recent years, the most discussed issues include the wisdom of using elderly donors for liver transplantation [2].

Pre-transplant histopathological evaluation is known to be an effective, accurate and reliable tool for assessing the quality of liver retrieved from deceased donors. Pre-transplant biopsies are critical in selecting donor livers for transplantation, especially in cases of expanded criteria donor. The biopsies should be performed more often to avoid unnecessary loss of organs suitable for transplantation and prevent transplantation of inappropriate organs [3].

The objective of this study is to examine differences in the incidence of pathological processes in liver biopsy specimens from donors over the age of 60 (group 1) and among donors currently recognized as "standard" in age – 60 years and below (group 2).

MATERIALS AND METHODS

Donor maps were analyzed. Histological evaluation of incision liver biopsy samples of 300 consecutive donors with brain death was performed. Biopsies were carried out prior to cold storage of liver.

Biopsy specimens of donor liver were fixed in 10% neutral-buffered formalin at pH 6.8–7. They were dehydrated in alcohols of an ascending strength and poured into paraffin blocks. Sections (4–5 μm thick) were prepared on a Leica RM 2145 microtome. After dewaxing, the histological sections were stained with hematoxylin and eosin, as well as with Masson's trichrome. Histological preparations were studied using a Leica DM6000 B microscope. Level of albuminous degeneration of hepatocytes, fatty hepatosis, liver fibrosis, and liver inflammation were evaluated.

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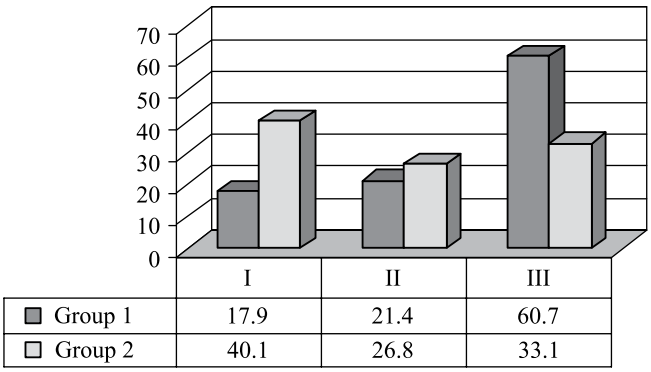
Results were statistically processed using software package Statistica 7.0 and Excel spreadsheets. The results were processed using student's t-test and chi-square test. Significance of differences was taken at $p \leq 0.05$.

RESULTS

In our study, out of the total pool of consecutive 300 brain dead donors, there were 28 (9.3%) donors over 60 years old (19 men and 9 women), aged 61 to 73 years (group 1). The control group (group 2) included 272 donors (196 men and 76 women) aged 18 to 60 years. In both groups, no relationship between incidence of pathological changes and gender was found ($p > 0.05$).

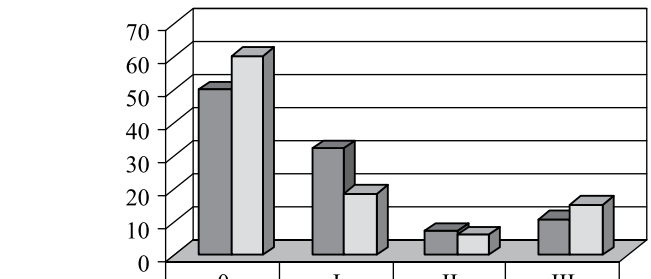
Severity of ischemic liver injury was evaluated by the level of albuminous degeneration of hepatocytes in the elderly (group 1, n = 28) and in the "standard" (group 2, n = 272) donors (Fig. 1).

In mild degree (1), predominantly granular dystrophy was observed in hepatocytes. Only small areas of hydropic degeneration were found in the liver lobules. This degree of potentially reversible albuminous degeneration was found in five (17.9%) biopsy specimens of elderly donors and 109 (40.1%) biopsy specimens of "standard" donors. That is, slight degree of liver injury was significantly more common ($p < 0.05$) in group 2 donors. At a moderate degree (2), hepatocytes had predominantly hydropic degeneration, but there were also small ballooning degeneration foci (group 1: n = 6; 21.4% and group 2: n = 73; 26.8%). The frequency of moderate degree (group 2) of albuminous degeneration in both groups did not significantly differ ($p > 0.05$). In severe degree (3), hydropic and ballooning degeneration were common in equal measure in all hepatocytes (group 1: n = 17; 60.7% and group 2: n = 90; 33.1%). Such severe degree of albuminous degeneration was significantly more likely ($p < 0.05$) observed in group 1 donors. Thus, in comparison with "standard" donors, age-related donors are reliably less likely to have mild degree (1), but more likely to have severe degree (3) of albuminous degeneration in the liver.



	I	II	III
Group 1	17.9	21.4	60.7
Group 2	40.1	26.8	33.1

Fig. 1. Comparative frequency of various degrees of albuminous degeneration in liver biopsy specimens of elderly (group 1) and "standard" (group 2) donors



	0	I	II	III
Group 1	50	32.1	7.1	10.7
Group 2	60.3	18.5	5.9	15.1

Fig. 2. Comparative frequency of various degrees of fatty hepatosis in liver biopsy specimens of elderly (group 1) and "standard" (group 2) donors

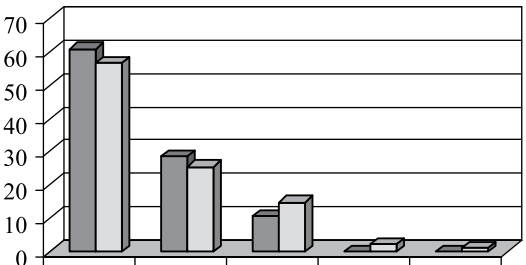
Based on the generally accepted classification, we divided fatty hepatosis into four degrees. Zero (0) degree – fatty hepatosis is absent in the absence or presence of fatty degeneration of up to 5% of hepatocytes; mild (1) degree of fatty hepatosis – fatty degeneration of more than 5% but not more than 33% of hepatocytes with fatty degeneration; moderate (2) degree of fatty hepatosis – fatty degeneration of more than 33% but not more than 66% of hepatocytes with fatty degeneration. Severe (3) degree of fatty hepatosis – more than 66% of hepatocytes with fatty degeneration.

Figure 2 shows the comparative incidence of various degrees of fatty hepatosis in liver biopsy specimens of age-related (group 1) and "standard" (group 2) donors.

Fatty hepatosis was less common in group 1 donors (n = 14; 50.0%) than in group 2 (n = 164; 60.3%). In contrast, mild and moderate fatty hepatosis were more common in age-related donors (degree 1: n = 9; 32.1%; degree 2: n = 2; 7.1%) and not in "standard" donors (degree 1: n = 51; 18.5%. degree 2: n = 16; 5.9%). Severe fatty hepatosis was more common in the young (n = 41; 15.1%) and not in the elderly (n = 3; 10.7%) donor group. However, all these differences were not significant ($p > 0.05$).

Extent of fibrosis was staged according to the METAVIR scoring system: F0 (no fibrosis), F1 (portal fibrosis without septa), F2 (portal fibrosis with few septa), F3 (numerous septa without cirrhosis), and F4 (cirrhosis).

Figure 3 shows the comparative frequency of various degrees of fibrosis in liver biopsies in age-related (group 1) and "standard" (group 2) donors. Liver fibrosis was absent in more than half of the observations, both in the age-related (n = 17; 60.7%) and in the "standard" (n = 154; 56.6%) donors. F1 fibrosis was found in 8 (28.6%) and 69 (25.4%) biopsies in group 1 and group 2, respectively. F2 fibrosis was detected in 3 (10.7%) and 40 (14.7%) biopsies, respectively. Severe fibrosis (F3 and F4) were absent in elderly donors and were rare in "standard" donors (F3: n = 6; 2.2%. F4: n = 3; 1.1%).



	F0	F1	F2	F3	F4
Group 1	60.7	28.6	10.7	0	0
Group 2	56.6	25.4	14.7	2.2	1.1

Fig. 3. Comparative frequency of varying degrees of fibrosis in liver biopsy specimens of elderly (group 1) and "standard" donors (group 2)

Differences in the absence or presence of fibrosis in the groups are not significant ($p > 0.05$).

DISCUSSION

A retrospective study of biopsies of 300 consecutive donor livers showed that mild albuminous degeneration of hepatocytes (conditional "norm") in the liver of age-related donors is less common ($p < 0.05$), while severe albuminous degeneration of hepatocytes is significantly more common ($p < 0.05$) than in the liver of "standard" donors. The same trend was observed with respect to mild fatty hepatosis, although these differences were not significant ($p > 0.05$).

We believe that symptoms of both albuminous degeneration and fatty hepatosis are a manifestation of ischemic liver injury. They are associated with unstable hemodynamics in age-related donors, in whom cardiovascular pathology is typically more common and more severe than in young donors [4, 5]. A more severe ischemic injury to the liver of elderly donors is one of the main causes of more common biliary complications [6–8], although graft and patient survival rates do not differ significantly [6–10]. Therefore, the main task involved in improving outcomes of liver transplantation from elderly donors is to reduce ischemic injury to the donor liver. One may agree with the opinion expressed by Ghinolfi et al. [11] that *ex situ* normothermic machine perfusion (NMP) might perhaps be one of the future ways, which might minimize ischemia-reperfusion injury to liver grafts. The authors examined 10 primary liver transplantation recipients of older grafts (≥70 years) after NMP. Patients with similar characteristics served as control, but liver graft was used after cold storage. Electron microscopy showed decreased mitochondrial volume density and steatosis and increased volume density of autophagic vacuoles in the liver at the end of transplantation in NMP versus cold storage patients ($p < 0.001$). Thus, histological evidence showed that the use of *ex situ* NMP with older liver grafts (≥70 years) reduced ischemia-reperfusion injury. However, the clinical benefit of this method remains to be demonstrated.

In our study, there were no statistically significant differences in the incidence of various degrees of fatty hepatosis and fibrosis depending on the age of donors ($p > 0.05$). There are reports showing evidence that with liver graft fibrosis after transplantation, fibrosis does not progress, it even regresses. So, after LT (101 patients) with mild to moderate fibrosis (F1 and F2), the degree of the condition did not progress in 40% of patients; decreased fibrosis was observed in 30%. When observed for an average of 71 months, 63 patients (63%) maintained transplant function, six (6%) had liver transplantation, while 35 patients died. Graft survival was 82% and 69% after one year and five years, respectively. It was found that differences in graft survival are not statistically significant depending on the extent of liver fibrosis: five-year graft survival (73% for F1 and 62% for F2, $p = 0.24$). In addition, a group of recipients of liver graft with fibrosis was compared with control group of 208 consecutive patients who received liver graft without fibrosis. The 5-year graft survival rate did not significantly differ between the groups (69% in the liver fibrosis group versus 75% in the non-fibrosis group, $p = 0.19$). The 5-year survival rate of patients also did not statistically differ between the groups (73% survival in the liver fibrosis group versus 79% in the non-fibrosis group, $p = 0.2$). In patients with HCV, differences in 5-year graft survival rate were also not statistically significant: 60% in the fibrosis group versus 70% in the non-fibrosis group ($p = 0.22$). This study demonstrated that allografts with mild to moderate degrees of fibrosis achieve acceptable long-term survival after liver transplantation [12].

So, our study showed that, with exception of more severe ischemic liver injury in elderly donors, there are no significant differences in the histology of the liver of elderly and "standard" donors. In the absence of liver diseases, it retains its biological youthfulness, unlike the heart and kidney, regardless of the chronological age of the donor. In this regard, geriatrics data on heterogeneity of the aging process are also of great interest – the higher the individual's chronological age, the less he corresponds to his biological age. Geriatric-like measures can be used to select livers of chronologically old, but biologically young donors [13]. The use of chronologically old, but biologically young liver donors will expand the donor pool and reduce waitlist mortality [14].

CONCLUSION

When compared with "standard" (< 60 years) donors, older donors (≥60 years of age) are significantly less likely to have mild ($p < 0.05$) and significantly more likely to have severe ($p < 0.05$) albuminous degeneration. There is only a tendency ($p > 0.05$) towards more common mild fatty degeneration. Degenerative processes result from more severe ischemic injury to the liver of elderly donors

that are known to have more common and more severe cardiovascular pathology. Ischemic liver injury leads to more frequent biliary complications. In order to reduce the severity of ischemic liver injury in elderly donors and the risk of biliary complications, careful monitoring and maintenance of optimal hemodynamics in donors in the intensive care unit is required. Based on other histological parameters, the liver of donors >60 years of age, in the absence of liver congenital or acquired disorders, does not significantly differ ($p > 0.05$) from the liver of donors ≤60 years. To expand the donor pool, age restrictions should be removed when selecting a liver for transplantation. This would maximize the use of donor potential.

The authors declare no conflict of interest.

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CONVECTION FLOW OPTIMIZATION IN ONLINE HEMODIAFILTRATION

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Objective: to evaluate the dependence of the magnitude of convection flow in online hemodiafiltration (OLHDF) on ultrafiltration control method and patients' individual characteristics. **Materials and methods.** The study included 36 stable dialysis patients (20 male and 16 female). The substitution rate was conducted manually based on transmembrane pressure (TMP). In some cases, devices with automatic filtration rate control unit AutoSub *plus* were used. The filtration rate (FR), TMP, blood flow rate (Qb), specific filtration rate (SFR, ml/min/mm Hg^{-1}) were recorded. **Results.** The maximum SFR in various patients ranged from 0.51 to 0.80 ml/min/mm Hg^{-1}; average value was 0.62 ± 0.07 ml/min/mm Hg^{-1}. There was significant correlation of SFR with hemoglobin level (r = –0.55). SFR reduced during hemodiafiltration (on average – by 23 ± 4%). SFR was significantly affected by Qb (r = 0.70). Maximum SFR was achieved with a TMP of 140–220 mm Hg; with TMP over 250 mm Hg, a decrease in SFR was noted, an increase in Qb was required for further increase in FR. Individual stability of SFR was noted during serial observations; fluctuations in a particular patient did not exceed 10%. Substitution volume for the HDF session was 18.0 ± 3.3 L, the FR/Qb ratio was 24.7 ± 5.2%. Substitution volume of 21 L was not achieved in 17 of 36 patients. The use of automatic FR adjustment system made it possible to increase the substitution volume (SV) by 12–18%. **Conclusion.** Achieving maximum convection volume in OLHDF requires individualizing treatment parameters. The use of FR automatic control allows maximum possible convection flow.

Keywords: online hemodiafiltration, specific filtration rate, substitution volume.

Online hemodiafiltration (OL-HDF) is a technique incorporating all modern technological achievements. It is mostly considered as the gold standard in renal replacement therapy requiring hemodialysis [1]. The main advantage of OL-HDF from the point of view of a more complete correction of uremia is the possibility of active transmembrane transfer of substances with significant molecular weight when creating high-speed filtration flow of water molecules from the blood circuit to the dialysate circuit. With a known membrane and a known high sieving coefficient with respect to the substance, the filtration volume achieved during treatment session can be considered a surrogate indicator of treatment efficacy with respect to elimination of this substance [2]. Thus, modern dialysis membranes used in OL-HDF have a sieving coefficient for β2-microglobulin (11,800 Da) of at least 0.6. The assumption that more significant elimination of medium- and high-molecular-weight uremic substances should have a positive effect on the outcomes of long-term hemodialysis has been confirmed in recent studies. It was demonstrated that higher survival rates are achieved with high filtration volume – over 20 liters per treatment session [3]. In real clinical practice, ensuring such high ultrafiltration rate is often fraught with several difficulties caused by both the parameters of the procedure and the patient's features [4].

The objective of this study is to evaluate the dependence of convection flow on ultrafiltration adjustment method and on patients' individual characteristics.

PATIENTS AND RESEARCH METHODS

The study included 36 patients (20 men and 16 women) aged 21 to 82 (59.6 years average). The subjects were treated with programmed hemodialysis for at least 6 months. Vascular access was achieved through arteriovenous fistula or vascular prosthesis in 29 patients, and through tunneled dual-lumen catheters in 7 patients. Four-hour OL-HDF sessions were performed on Fresenius 4008 and 5008 devices and FX60 and FX80 hemodiafilters (Fresenius Medical Care, Germany) at a fixed dialysis fluid flow rate of 500 mL/min and there was post-dilution introduction of substitution fluid. The substitution rate was controlled in manual mode based on TMP indicators. Here, FR, TMP and actual Qb were recorded. During treatment sessions, we also investigated the SFR corresponding to the filtration flow value (FR, mL/min) and the actual transmembrane pressure (TMP, mmHg). In some cases, automatic dialysate flow control systems with 1.5 coefficient with respect to Qb and automatic FR adjustment unit AutoSub plus were used. AutoSub plus was based on constant measurement of pressure pulsation in the air trap of the venous line, as

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part of Fresenius 5008 dialysis machines. The data was statistically processed using a Microsoft Office Excel spreadsheet.

RESULTS AND DISCUSSION

With fixed duration (4 hours) of therapy session and average Qb = 322.5 ± 27.1 mL/min, maximum SFR for different patients ranged from 0.51–0.80 mL/min/mmHg^{-1}, the average value was 0.62 ± 0.07 mL/min/mmHg^{-1}. There was significant correlation of SFR with hemoglobin level (r = –0.55). There was no dependence of SFR on proteinemia, albuminemia, glycemia and total cholesterol levels. During treatment sessions, decreased SFR (on average by 23 ± 4%) was observed. This can be explained both by systemic blood concentration amidst decreased volume of circulating blood and by compaction of secondary protein membrane on the surface of the dialysis membrane. The secondary protein membrane is actively formed precisely at high filtration rate [2]. Among the parameters of the procedure, the value of SFR was significantly influenced by Qb (r = 0.70).

Maximum SFR values were achieved in TMP 140–220 mm Hg. Further increase in filtration rate, and accordingly, TMP value, led to exponential decrease in SFR value (see figure).

With TMP >250 mm Hg, a fall in SFR levels became especially noticeable and subsequent increase in FR often led to alarming levels of TMP. In such situation, increasing Qb was required to restore FR.

During serial observations, individual SFR stability was noted, session-to-session fluctuations in a particular patient did not exceed 10%. Average substitution volume per HDF session was 18.0 ± 3.3 L, while filtration fraction (FR/Qb ratio) was 24.7 ± 5.2%. In 17 of the 36 patients, substitution volume 21 L was not achieved during HDF. This observation was primarily due to the fact that in this group of patients, there were no reserves for increasing the blood flow rate. Use of automatic FR adjustment system based on blood viscosity measurement allowed increasing the total convection volume by 12–18%, while SFR was not observed below 0.4 and no TMP alarms were noted. At the same time, in 4 out of the 12 patients who used this system, the threshold substitution volume for treatment session was not achieved because Qb could not be increased.

According to current research, the substitution volume achieved during HDF session is a key factor in improving the final outcomes of program dialysis [2]. At present, the threshold value is 21 L without taking into account the ultrafiltration volume aimed at eliminating interdialytic hyperhydration [3]. In routine clinical practice, achieving such high FR requires intense treatment regimens, including creating a high transmembrane pressure gradient [4]. It is known that HDF with high TMP values increases the number of alarms requiring the intervention of medical staff [5]. In addition, in this situation, the sieving of high molecular weight substances, including albumin, significantly increases [6]. Although several authors consider albumin elimination as a positive factor contributing to removal of protein-bound uremic toxins [7], significant albumin loss can reduce plasma albumin concentration. SFR and TMP levels help in evaluating convection flow intensity. With these two indicators, excessive hemoconcentration in the extracorporeal circuit and significant albumin sieving are avoided. Increasing blood flow rate is the main reserve to ensuring adequate (or maximum for a given patient and given duration of HDF session) substitution volume without resorting to extreme filtration regimes and, accordingly, significant drop in SFR. Clear, meaningful management of FR is becoming increasingly important, given the permanent tendency towards increase in hydraulic permeability and sieving coefficients of high molecular weight membranes used in wide clinical practice. In this regard, improvement and widespread introduction of automatic substitution rate control systems that increase the volume achieved during a therapy session, and also provide stable procedure requiring no human intervention, seems a promising approach.

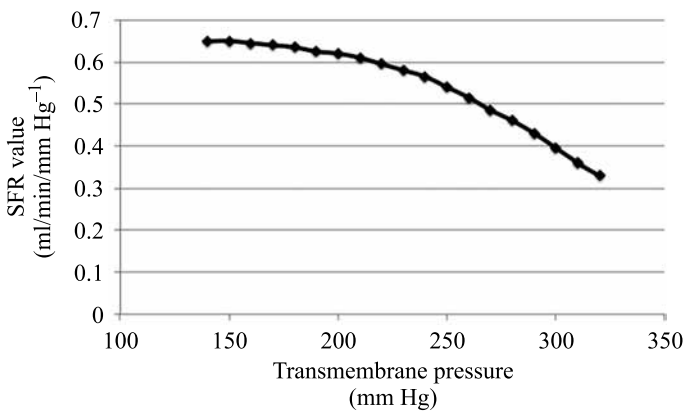

Fig. SFR dependence on TMP during OL-HDF session with progressive increase in FR

CONCLUSION

Achieving maximum convection volume in HDF requires individualizing treatment prescription. At the same time, it is optimal that filtration is done in the most efficient rank of transmembrane pressure of up to 250 mmHg. The main limitation in total substitution volume in HDF is the inability to ensure adequate blood flow rate.

With modern automatic filtration rate control systems, maximum possible convection flow is provided in specific conditions in real time without episodes of excessively high TMP and extracorporeal blood flow stops.

Identifying the substitution volumes required for patients of different sexes and ages with various anthropometric and clinical data, as well as the effect of intense filtration regimes on treatment outcomes, require further investigation.

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COMPARATIVE ANALYSIS OF THE SECRETORY CAPACITY OF ISLETS OF LANGERHANS CULTURED WITH BIOPOLYMER-BASED COLLAGEN-CONTAINING HYDROGEL AND TISSUE-SPECIFIC MATRIX

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Introduction. Creation of a biomedical cell product – a bioengineered pancreatic construct – is hampered by problems associated with maintaining the viability of functionally active isolated islets of Langerhans (ILs). Both biopolymer and tissue-specific scaffolds can contribute to maintaining the structure and function of isolated ILs *in vitro* and *in vivo*. The most preferred tissue-specific scaffolds for cells can be obtained via decellularized pancreas matrix scaffold (DP matrix scaffold). **Objective:** to conduct a comparative analysis of the secretory function of isolated ILs of rats cultured in biopolymer-based collagen-containing hydrogel (BCH) and tissue-specific DP matrix scaffold, respectively. **Materials and methods.** ILs from rat pancreas was isolated using classical collagenase technique with some modifications. ILs were cultured in BCH and tissue-specific scaffold under standard conditions. Tissue-specific DP matrix scaffold was obtained through decellularization of rat pancreas. The DP matrix scaffold was examined for cytotoxicity and DNA presence; it was subjected to morphological study. The secretory function of ILs was studied through enzyme-linked immunosorbent assay (ELISA). **Results.** The secretory function of islets cultured in BCH and DP scaffolds is significantly higher than in the monoculture of islets. The advantage of using tissue-specific DP matrix scaffolds when creating bioengineered constructs of the pancreas over BCH matrix scaffolds was identified. **Conclusion.** BCH and tissue-specific DP scaffolds contribute not only to preserving the viability of isolated ILs, but also to prolonging their secretory capacity for 10 days, compared with ILs monoculture.

Keywords: islets of Langerhans, biopolymer-based hydrogel, decellularized pancreas, matrix scaffold, cultivation, insulin secretion.

INTRODUCTION

The creation of a biomedical cell product – a bioengineered construct of the pancreas – is hampered by problems associated with maintaining the viability of isolated Langerhans islets (LI) [1, 2].

In the process of isolation, LIs are known to lose vascularization, innervation, and their connection with the extracellular matrix (ECM) which plays a significant role in the regulation of many aspects of the islets' physiology, including survival, proliferation and insulin secretion [3, 4]. The structure conservation and isolated LIs functioning *in vitro* and *in vivo* can be facilitated by ECM biomimetic matrices with the properties characteristic of the native pancreatic microenvironment [5–7]. Among biomimetics simulating the ECM composition, there is the biopolymer microheterogenous collagen hydrogel (MHCH matrix), a multicomponent product made of natural compounds which includes partially hydrolyzed collagen peptides, glycoproteins, uronic acids, and biologically active ECM substances, including growth factors necessary for the vital functioning of cells [8].

At incubation with collagen-containing matrices, isolated LIs are known to retain integrity, viability, and secretory function for a long time in comparison with LI monocultures [9, 10]. It was shown earlier that the cultivation of isolated rat LI with the MHCH matrix contributes to the preservation of the vitality and characteristic structure of LIs *in vitro* for 14 days [11].

With all their advantages, resorbable matrices of biopolymer materials do not possess tissue specificity. Recently, the development of bioengineered constructs based on tissue-specific matrices of decellularized tissues with the preservation of the structural, biochemical and biomechanical properties of the native ECM with subsequent recellularization by cellular components has been intensively forming [12, 13]. When developing protocols for P decellularization, it is important to take into account the conservation of its architectonics and

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microvasculature with the most complete removal of cellular material, including DNA, to minimize the immune response during implantation of the bioengineered P structure with minimal damage to ECM components [14, 15]. The presence of such native ECM components in the decellularized P matrix (DCP matrix) as structural proteins (various types of collagen, elastin, fibronectin and laminin), glycoproteins, and cell adhesion factors, allows creating conditions for prolonged vital activity of islet cells and simulate ECM almost completely [16]. The 3D ECM structure determines the topographic location of endocrine P cells, which also affects the survival and secretory activity of LIs [17]. The islets cultured in the presence of the DCP matrix have been shown to increase insulin secretion compared with isolated LIs in monoculture [18].

The **purpose** of the present study was to make the comparative analysis of secretory activity of isolated rat LIs cultured with biopolymer microheterogenous collagen-containing hydrogel and tissue-specific matrix.

MATERIALS AND METHODS

Research animals

The studies were performed on mature male rats of the Wistar breed (180–220 g) from the laboratory bank of the FGUP OPKH Manikhino. The laboratory animals were acclimatized and kept in accordance with the interstate standard GOST ISO 10993-2-2009, "Medical devices. Assessment of the biological effects of medical devices" Part 2. "Animal Handling Requirements".

All manipulations with animals were carried out in accordance with the "Rules for Working with Research Animals" of 1973 and the Rules adopted by the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (ETS 123, Strasbourg, 1986).

Hydrogel mimetic ECM

As an ECM hydrogel mimetic, the injection form of the MHCH matrix was selected from the *Sphero*®GEL composition line (BIOMIR Service JSC, Russia), intended for use in cell technologies with the following characteristics: average microparticle size 145.79 ± 0.09 microns; modulus of elasticity 1170 ± 12 Pa; viscosity modulus 62.9 ± 7.9 Pa; swelling – not lower than 86.6 ± 3.0 mass. %; resorption time up to 9 months. Previous studies have shown that this MHCH matrix is optimal for creating cell and tissue engineered constructs [19].

Tissue-specific ECM

As a tissue-specific ECM, a matrix derived from decellularized rat P tissue (DCP matrix) was selected.

For decellularization, a subtotally removed rat P crushed manually using eye scissors to a fragment size of not over 1×1×2 mm was used. P fragments were processed at room temperature under continuous stirring (MultiBio RS-24 rotational system, 5 rpm) and sequentially in 0.1% solution of sodium dodecyl sulfate (SDS) in distilled water for 3 hours, in a 0.1% SDS solution on 1N NaCl for 3 hours and in a 0.1% SDS solution in phosphate-buffered saline (PBS, pH = 7.35) for 18 hours. At the final stage of the preparation of the DCP matrix, the decellularized fragments of pancreatic tissue were washed from surfactant residues for 72 hours in three PBS shifts with an antibiotic/antimycotic. Once a day the solution was changed. Samples of the DCP matrix (DCP fragments in PBS) were put in cryovials, frozen and subjected to γ-sterilization (1.5 Mrad). The sterile DCP matrix was stored at 4–6 °C and immediately before the experiment was additionally crushed to an average microfragment size of 500 ± 45 μm to reduce the degree of microheterogeneity.

Morphological study of DCP matrix

For morphological studies, DCP matrix samples were fixed in 10% buffered formalin, dehydrated in ascending concentration spirits, kept in chloroform/ethanol mixture, in chloroform, and embedded in paraffin. 4–5 μm sections from the RM2245 microtome (Leica, Germany) were dewaxed, rehydrated and stained with hematoxylin and eosin for total collagen (Masson), elastic fibers (Unna–Taenzer), and DAPI stained for the qualitative determination of nuclear material in a DCP matrix.

DNA quantification in the DCP matrix

To determine the degree of immunogenicity of the decellularized material by the residual amount of nuclear material in a DCP matrix, DNA was isolated and fluorescent stained [20].

DNA was isolated from DCP matrix samples with the DNeasyBlood & TissueKit (QIAGEN, Germany) according to the manufacturer manual. For DNA quantification according to the protocol, PicogreenQuant-iT fluorescent dye™ was used (Invitrogen, USA), with its action activated by 480 nm wavelength radiation. The obtained thermoionic emission was analyzed on a Spark 10M microplate reader (TecanTrading AG, Switzerland) at 520 nm wavelength. To determine the absolute amount of DNA, a bacteriophage λ DNA calibration curve (Invitrogen, USA) in the range of 0.0–1000 ng/ml was used.

Study of DCP matrix for cytotoxicity

The cytotoxicity of *in vitro* DCP matrix samples was evaluated by direct contact in accordance with the interstate standard GOST ISO 10993-5-2011 "Medical devices. Assessment of the biological effects of medical

devices. Part 5. Study of cytotoxicity: *in vitro* methods" on the culture of mouse fibroblasts, line L929. A sample culture medium with 10% fetal calf serum (HyClone, USA) was taken for negative control. A single element aqueous standard of 10,000 μg/ml (Sigma-Aldrich, USA) was used as a positive control sample. All procedures were performed in aseptic conditions. The culture was visually evaluated with the Eclipse TS100 (Nikon, Japan) inverted microscope.

The metabolic activity of fibroblasts after contacting matrix samples was evaluated after 24 h with prestoBlue™ CellViabilityReagent vital dye (Invitrogen™, USA) according to the manufacturer protocol. The change in optical density was recorded with Spark 10M microplate reader (TecanTrading AG, Switzerland) with SparkControl™ Magellan V1.2.20 software at 570 and 600 nm wavelengths.

The obtained data were statistically processed with Microsoft Excel 2007. All results are presented in the form of "mean value ± standard deviation". Differences were considered significant at $p < 0.05$.

Langerhans islets isolation and identification

The procedure for isolating LIs from P of mature rats (n = 6) was carried out on the basis of classical protocols using collagenase [21, 22] with some modifications [11].

Islets were identified by dithizone staining. The dye selectively stained LIs, while the acinar cells remained unstained [22]. Resuspended isolated LIs were subsequently used in the experiment.

LIs cultivation in the presence of MHCH and DCP matrices

Freshly isolated LIs were resuspended in DMEM/F12 (1/1) medium with 10% ETS, 2 mM L-glutamine, 1 M Hepes and 50 mg/ml gentamicin and approximately equal number of islets (n = 300 ± 25) were introduced into three 25 cm^2 culture flasks. The islets cultured without matrix addition (culture flask 1) served as control. 0.2–0.3 ml of carefully resuspended MHCH matrix (experimental group I) and 0.2–0.3 ml of a suspension of DCP matrix with an average microfragment size of 500 ± 45 μm (experimental group II) were added to culture flasks 2 and 3, respectively.

All culture systems were incubated under standard conditions at 37 °C in a CO_2 incubator in humidified atmosphere with 5% CO_2. The cultivated islets were monitored and photographed with Nikon Eclipse TS100 digital camera inverted microscope (Nikon, Japan). The culture medium was changed on the 1^{st}, 2^{nd}, 3^{rd}, 6^{th}, 8^{th} and 10^{th} days to take samples for subsequent tests on insulin content.

Enzyme-linked immunosorbent assay for the determination of insulin content in the culture medium

Before determining insulin content in the samples from the culture flasks at the indicated incubation times, the growth medium was removed and replaced with fresh nutrient medium. After 1 hour of incubation under previous conditions (37 °C, 5% CO_2), growth medium samples were taken out and frozen (–23 °C) for the subsequent study [23].

To determine the basal concentration of insulin in the culture medium, the solid-phase sandwich method Rat Insulin ELISA Kit (Thermo scientific, USA) was used according to the manufacturer's manual. In this option, ELISA uses a pair of antibodies specific for spatially remote epitopes of the studied antigen, thus allowing high sensitivity and specificity in the antigen (insulin) determination.

The optical density was measured with Spark 10M microplate reader (TecanTrading AG, Switzerland) with Spark Control™ Magellan V1.2.20 software at 450 and 550 nm wavelengths to account for the microplate optical artifacts.

The obtained data were statistically processed with Microsoft Excel 2007. ELISA quantitative results were calculated using a linear calibration curve. All results are presented in the form of "mean value ± standard deviation". Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Morphological study of DCP matrix

A preliminary morphological study confirmed the classical pattern of the rat P structure without signs of ischemic damage (Fig. 1). Decellularized P samples used in the experiment showed the integrity of the stroma architectonics as a whole and were represented by a laced fiber mesh net – like structure. The surviving cells and individual cell nuclei in the samples were not detected. Specific DAPI staining confirmed the absence of cell nuclei and fragments of nuclear material in the matrix, thereby indicating the effectiveness of the procedure for the decellularization of pancreatic tissue (Fig. 2, a). Samples Masson staining made it possible to visualize collagen fibers in the obtained matrix composition (Fig. 2, b), and orsein staining also revealed the presence of elastic fibers, which indicated the preservation of the main fibrillar proteins of the matrix (Fig. 2, c).

DNA quantification in the DCP matrix

Quantitative analysis of the native and decellularized rat P tissue showed that the matrix of decellularized P compared to the original tissue was significantly ($p < 0.05$) purified from DNA (Table 1).

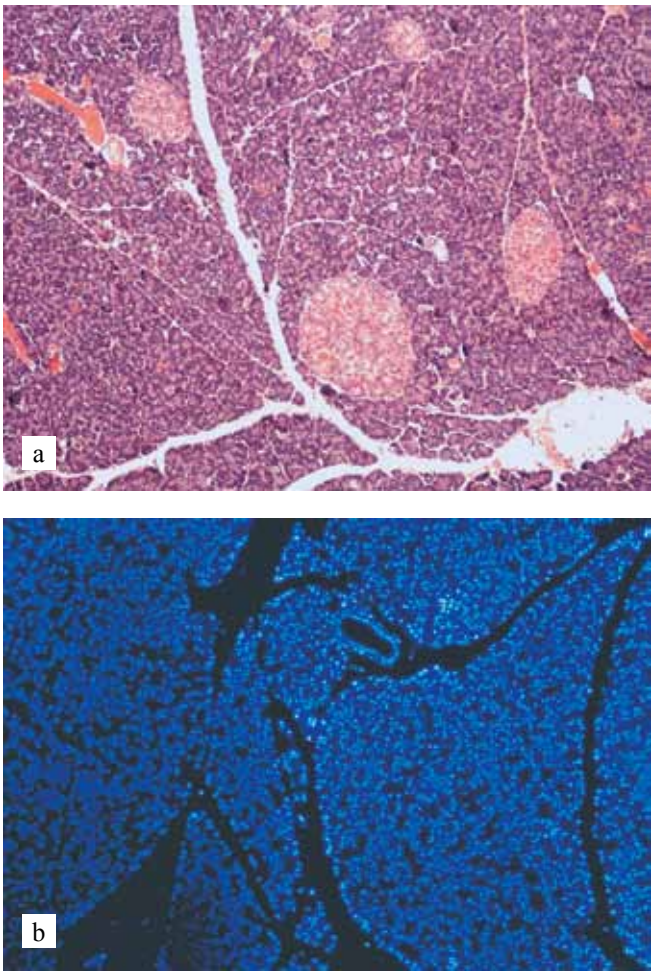

Fig. 1. Histological structure of rat pancreas: a – H&E staining; b – nuclear DAPI staining. ×100

Table 1

Quantitative content of DNA in native and decellularized rat pancreatic tissue

Rat P tissue sample	DNA, ng Mean ± SD
Native rat P	1,354.8 ± 168.7
Decellularized rat P	1.3 ± 0.3

Thus, decellularization resulted in not over 0.1% of DNA retained in the tissue, which indicated a high decellularization efficiency, and, accordingly, low immunogenicity of the obtained matrix (Fig. 3).

Cytotoxicity of DCP matrix

The results were analyzed by the evaluation scale of the degree of cell response after incubation with DCP matrix samples (n = 3).

Table 2 shows the values featuring the proliferative activity of L929 fibroblasts relative to the negative control (response degree 0). After contacting DCP-matrix samples, the proliferative activity of fibroblasts relative to the negative control remained above 90% (response degree 0), which showed the absence of cytotoxic effect of the samples of this matrix. The positive control in this experiment showed sharp cytotoxicity (response degree 4).

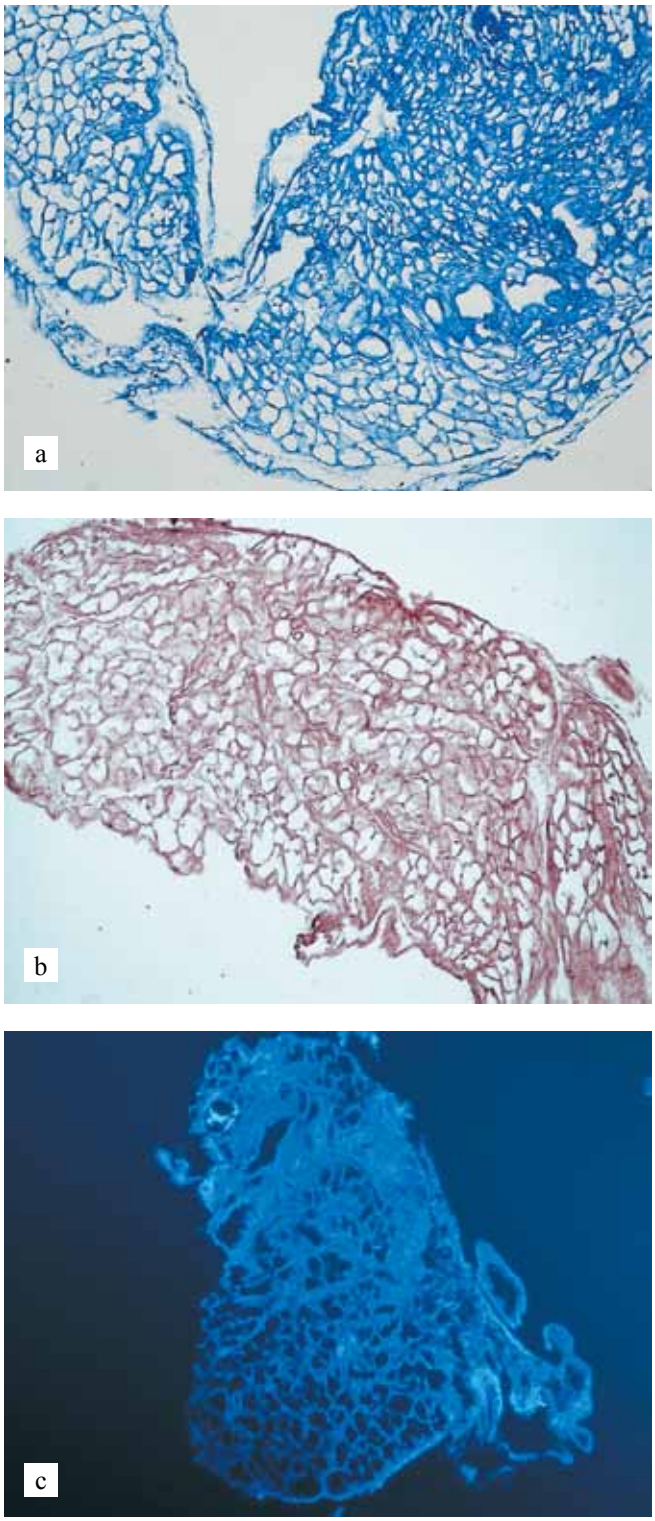

Fig. 2. Histological structure of decellular rat pancreas (DP matrix): a – Masson's trichrome staining demonstrated complete absence of cells and preservation of collagen fibres; b – Unna–Tentser's staining revealed preservation of elastic fibres; c – nucliar DAPI staining confirmed absence of nuclear material in DP matrix. ×200

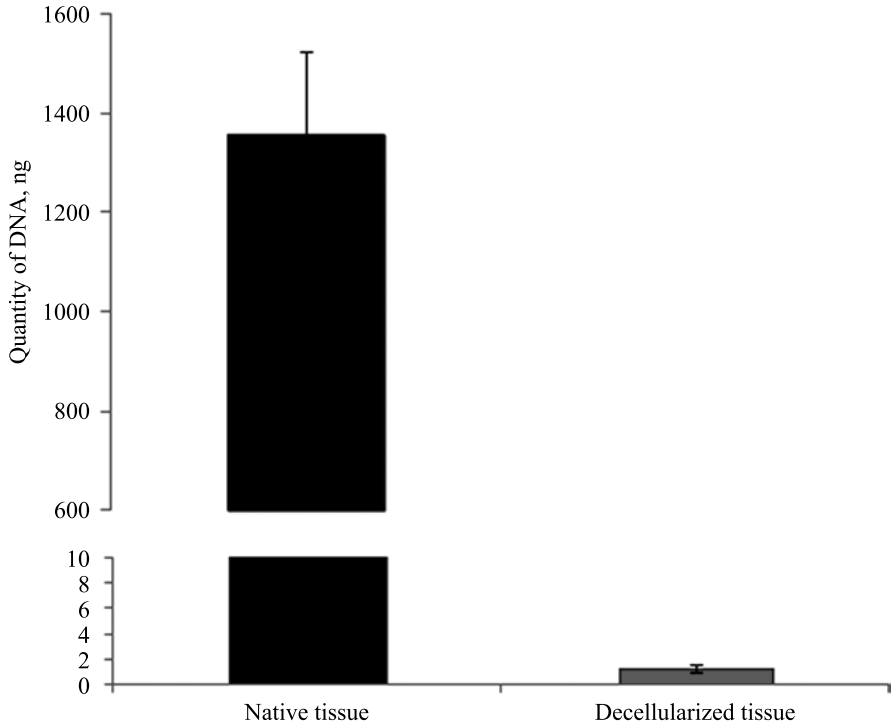

Fig. 3. Quantification DNA in native and decellularized rat pancreatic tissue

Table 2

Results of DP matrix cytotoxicity study

Sample No.	Sample name	Proliferating cells relative to the negative control (%)	Cell response degree
1	DCP matrix	96.25 ± 1.69	0
2	Positive control	7.84 ± 2.34	4

Morphofunctional properties of LIs upon incubation with MHCH and DCP matrices

The resulting LIs were round or oval in shape and maintained integrity, which indicated that their macrostructure was not affected during the isolation (Fig. 4).

After 24h of cultivation, significant morphological changes in the LI monoculture (control group) were not detected. However, after three days, the first signs of destruction were revealed: cavities appeared in some islets, and their surface became tuberous. At the turn of six days of cultivation, most of the control islets underwent fragmentation.

In the experimental group I, after one day of cultivation, a significant part of the islets showed adhesive properties and attached to the surface of the MHCH matrix, while the remaining islets continued to float (Fig. 5). This pattern practically did not change during the entire observation period (10 days). At this, LIs were visually preserved.

In contrast to the experimental group I, the islets cultured with the DCP matrix (experimental group II) did not exhibit adhesive properties and, being in close proximity to the matrix, remained intact up to 5 days of incubation. Subsequently, at least half of the cultured LIs were deposited on the surface of the DCP matrix (Fig. 6). During the entire observation period, as in the experimental group I, no pronounced signs of destruction of the islets were detected.

LI secretory function at incubation with MHCH and DCP matrices

After 24 hours of cultivation, the insulin concentration was higher by 26.2% (258.4 ± 9.7 μIU/mL) and 48.7% (304.9 ± 12.2 μIU/mL) in the experimental groups I and II compared to the control group (205.1 ± 11.5 μIU/mL), on the third day of incubation it was higher by 62.1% (149.0 ± 12.3 μIU/mL) and 102.9% (186, 5 ± 10.9 μIU/mL), respectively, compared to the control group (91.9 ± 7.8 μIU/mL) (Table 3).

The revealed differences in hormone concentration in the control and experimental groups at these time points can be explained by the positive effect of matrices on the functional ability of LIs. On the sixth day of cultivation, an even more significant difference was observed between the insulin concentrations in the experimental groups I and II (102.1 ± 10.6 and 138.3 ± 9.6 μIU/mL, respectively) and the control (29.2 ± 4.1 μIU/mL). This correlates with the morphological data on destructive

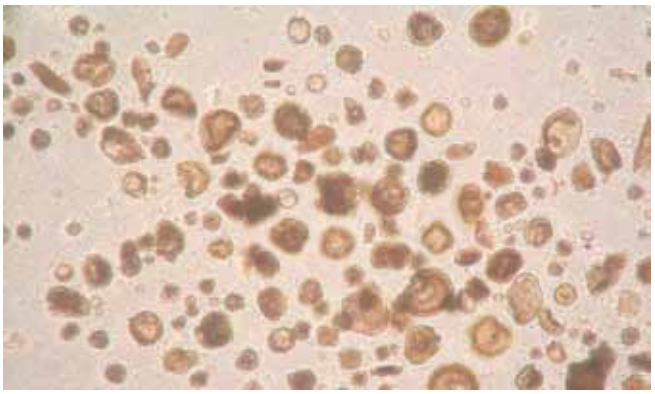

Fig. 4. Isolated Langerhans islets, inverted microscope. ×200

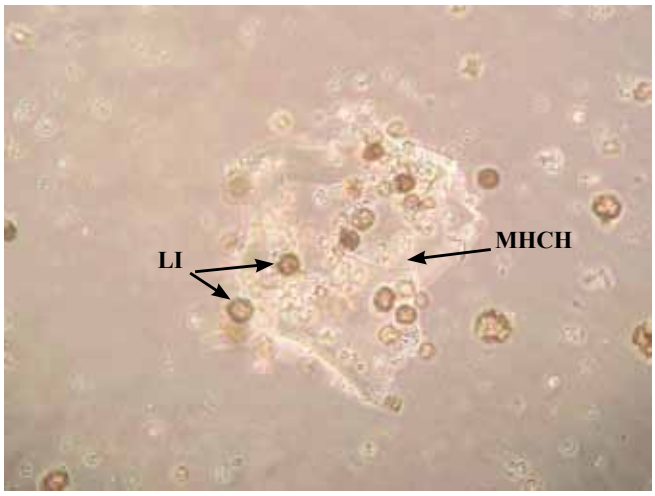

Fig. 5. Langerhans islets cultivated with MHCH, 7 days, inverted microscope. ×100

Table 3

Comparative analysis of insulin in experimental groups relative to the control group (LI monoculture), %

Days	LI + MHCH (experimental group I)	LI + DCP (experimental group II)
1	26.2 ± 3.8	48.7 ± 4.0
2	31.6 ± 6.2	71.6 ± 5.7
3	62.1 ± 8.3	102.9 ± 5.8
6	249.6 ± 10.4	373.6 ± 6.9

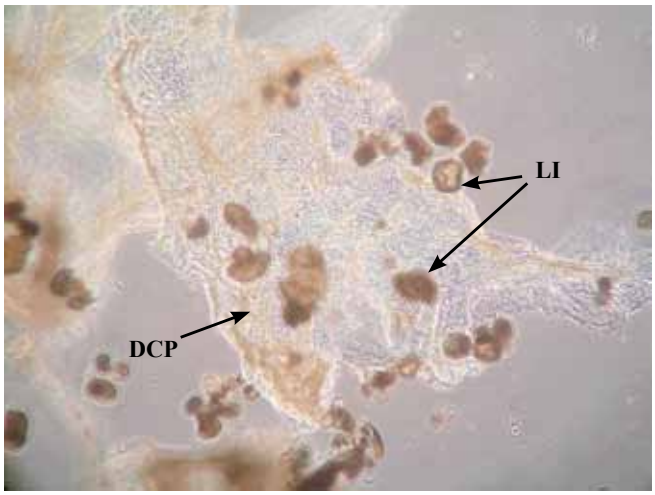

Fig. 6. Langerhans islets cultivated with decellular rat pancreas (DP matrix), 7 days, inverted microscope. ×100

changes occurring in the islets after three days of suspension cultivation. On the 8th to 10th day of incubation, no surviving islets were found in the control group; for this reason, it seemed irrelevant to study the culture medium. At the same time, at the same time points in the experimental groups, the insulin concentration remained practically unchanged: group I – 93.7 ± 6.2 μIU/mL, group II – 126.9 ± 8.9 μIU/mL, while the level of insulin secretion in group II (LI in the presence of a tissue-specific DCP matrix) was 35.5% higher than in group I (LI in the presence of MHCH matrix) (Fig. 7). Despite the fact that the insulin concentration, expressed in absolute terms, decreased with an increase in the cultivation period, the positive tendency of the effect of the matrix (MHCH and DCP) on the secretory function of the islets in percentage persisted throughout the entire observation period.

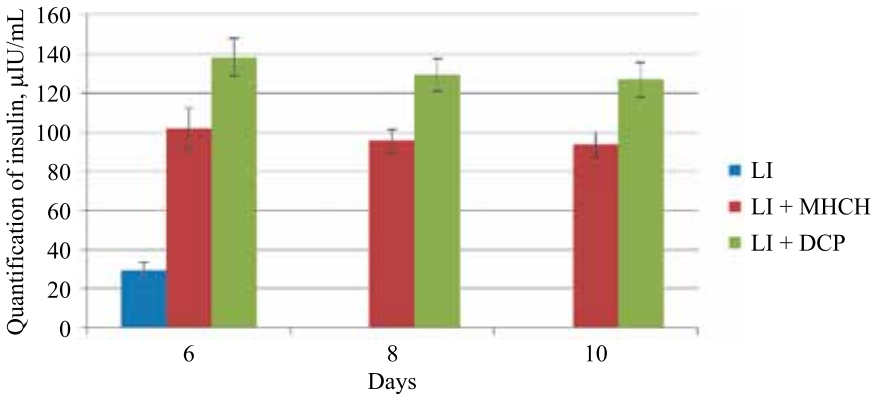

Fig. 7. Quantification of insulin in the control (monoculture LI) and experimental (LI, cultured with BMCH and DP matrix, respectively) groups

CONCLUSION

MHCH and tissue-specific DCP matrices contribute not only to the preservation of the viability of isolated LIs, but also to the prolongation of their secretory ability for 10 days compared to LI monoculture. In the experimental condition, the advantage of implying the tissue-specific DCP matrix to create a bioengineered P structure compared with the MHCH matrix is shown.

The authors declare no conflict of interest.

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EVALUATION OF THE EFFECTIVENESS OF COMBINED TREATMENT OF CORONARY HEART DISEASE – CORONARY ARTERY BYPASS GRAFTING, TRANSPLANTATION OF AUTOLOGOUS BONE MARROW MONONUCLEAR CELLS: A RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED STUDY

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Introduction. Despite resounding success in treatment of patients with coronary heart disease (CHD), researchers are yet unable to significantly reduce mortality in this disease. With this in mind, there are ongoing studies everywhere, which are aimed at investigating new techniques in order to boost the efficiency of existing standards. One of such promising techniques is cell/regenerative therapy with autologous bone marrow mononuclear cells (ABMMCs). However, even though ABMMCs have been studied for more than 10 years, there are no unambiguous data yet on several issues. **Objective:** to evaluate the outcome of ABMMC transplantation during coronary artery bypass grafting (CABG) surgery in combined treatment of CHD. **Materials and methods.** The data of 408 patients admitted to the clinic from 2013 to 2016 for planned surgical treatment of CHD were analyzed. The work included 117 people based on the design of the study. Patients were randomized in 3 groups: Group 0 (control group) – CABG surgery and intramyocardial injection of 0.9% NaCl solution, Group 1 – CABG surgery and intramyocardial injection of ABMMCs, Group 2 – CABG surgery, intramyocardial and intragraft injection of ABMMCs. The dynamics was assessed 12 months later – functional class of angina pectoris and heart failure, echocardiography, speckle tracking (assessment of the degree of myocardial deformation), treadmill test, 6-minute walk test, daily ECG monitoring, quality of life questionnaires, coronary angiography. Qualitative indicators were calculated using the Pearson's chi-squared test and Fisher criteria. Quantitative indicators were calculated using the Kruskal–Wallis and Wilcoxon tests. Factor analysis was used to identify certain severity factors and to study data homogeneity. Discriminant analysis was performed to investigate the leading characteristics that determine differentiation between the groups. For analysis of variance, taking into account various factors, the model of variance analysis for dependent samples – Repeated Measures ANOVA – was used. **Results.** In the observation groups, an improvement in both systolic and diastolic myocardial function was universally noted. A six-minute walk test showed statistically significant increase in Groups 1 and 2 compared with the control Group 0 – 315.06 ± 17.6 (433.54 ± 20.6), Group 1 – 319.8 ± 24.5 (524.4 ± 28.7), Group 2 – 329.9 ± 25.3 (452.7 ± 29.7) meters. A significant decrease in the functional class of exertional angina pectoris in Groups 1 and 2 was noted unlike in the control group. The percentage of functioning coronary shunts after a 12-month follow-up period was 87.6% in Group 0. In Groups 1 and 2, this ratio was 96.2% and 97.3%, respectively. Predictors of overall effectiveness were identified: smoking, initial diastolic myocardial dysfunction, left ventricular ejection fraction. **Conclusion.** In addition to surgical treatment of coronary heart disease, ABMMC transplantation can improve myocardial contractility, boost exercise tolerance, and increase the duration of the functioning of coronary shunts at the follow-up period of 12 months. The study showed the need for stage-by-stage analytical calculations with the aim of possible correction of further work.

Keywords: clinical trial, coronary artery bypass grafting, revascularization, reperfusion injury, coronary heart disease, speckle tracking, diastolic dysfunction, diastolic heart failure, autologous bone marrow mononuclear cells, intramyocardial injection.

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INTRODUCTION

The National Research Center for Preventive Medicine estimates that about 10 million able-bodied people in Russia suffer from CHD, and more than a third of them have stable angina pectoris. Economic burden resulting from such morbidity in 2016 alone exceeded 2.7 trillion roubles – 3.2% of the country's GDP. Among all cardiovascular diseases, CHD provides the highest economic burden – over 1 trillion roubles [1].

The World Health Organization (WHO) predicts a further increase in cardiovascular morbidity and mortality in both developed and developing countries, due to the aging population and lifestyles.

Despite the undeniable successes achieved in the treatment of patients with ischemic heart disease as a result of modern breakthroughs in rational pharmacotherapy, as well as development and implementation of various techniques of restoring coronary blood flow – bypass surgery, angioplasty and coronary artery stents, it has not yet been possible to significantly reduce mortality in this disease.

Mortality from cardiovascular disease (CVD) is projected to rise to 23.3 million by 2030. This indicator was 16.7 million in 2002 [2].

According to the American Heart Association, over 2150 Americans die of CVD each day (approximately 1 death every 40 seconds). It is noteworthy that about 155,000 people who died from this heart disease in 2011 were less than 65 years old [3].

In the Russian Federation, CVDs are the leading cause of death among the population (57% of total mortality rate). This figure is one of the highest in the world. CVD is the most common cause of hospitalization and disability in the Russian Federation.

Given these data, in order to combat age-related pathologies at the end of the 20th century, innovative programs were launched in economically developed countries to develop priority research and practical innovations on regenerative medicine as one of the promising methods for improving the effectiveness of existing standards.

Such strong focus on regenerative therapy is because stem cells promote neoangiogenesis. Such procedures can improve myocardial perfusion, extent of local and global contractility, and prevent myocardial remodeling and cell apoptosis [4–10].

The same results were obtained for ABMMC fraction [11–14].

However, even though ABMMCs have been studied for more than 10 years, there are no unambiguous data yet on several issues: type of cellular material, transplantation methods, volume of introduced cellular material, general safety and effectiveness.

These questions inspire researchers towards new studies [15–16].

In randomized, double-blind, placebo-controlled trial TAMIS (Autologous Bone Marrow Mononuclear Cells in the Combined Treatment of Coronary Heart Disease), ClinicalTrial.gov Identifier: NCT02059512, comprehensive assessment of the safety, efficacy and predicted outcome in combined treatment of ischemic heart disease (IHD) in combination with coronary artery bypass grafting (CABG) and ABMMC transplantation in patients with coronary artery disease and heart failure (left ventricular ejection fraction in reference values) was carried out.

The trial was conducted after approval by the ethics committee of Pavlov First St. Petersburg State Medical University (minutes No. 147 of February 26, 2013). The work was done as part of state assignment to the university, under a grant on the topic: "*Improving the methods of surgical treatment of coronary heart disease using cell technology*". Platform "Cardiology and Angiology" (115091630053).

MATERIALS AND METHODS

For the period of the trial from 2013 to 2016, the data of 408 patients who were admitted to the clinic of the Research Institute of Surgery and Emergency Medicine, Pavlov First St. Petersburg State Medical University for elective CABG surgery with cardiopulmonary bypass (CPB). According to angiographic study, these patients had 3 or more branches of stenosed coronary arteries. The study included 117 people who met the inclusion criteria: men and women from 18 to 80 years old, class III–IV angina pectoris, and informed voluntary consent. Exclusion criteria: intolerance to heparin and hydroxyethyl starch (HES), thyroid pathology (hypothyroidism, hyperthyroidism), concomitant pathology with predicted life expectancy of up to 3 years, infectious diseases, simultaneous participation in another study, patient's refusal to participate in the trial. Randomization was performed, according to the table of random numbers, in the following groups: group 0 (46 people) – control group – CABG surgery and intramyocardial injection of 0.9% NaCl solution, group 1 (34 people) – CABG and intramyocardial injection of ABMMCs, group 2 (37 people) – CABG, intramyocardial and intragraft injection of ABMMCs (Fig. 1).

Final quantity of the material obtained was calculated on a sample of patients – 37, 25 and 23 in groups 0, 1 and 2, respectively. The remaining patients included in the study felt satisfactory at the time of control examination, but for various reasons, there were no objective data on their clinical condition. They were excluded from final statistical analysis.

To evaluate the safety, efficacy, and prediction of outcomes of ABMMC transplantation in combination with CABG with CPB, several parameters were analyzed (Table 1). Analysis of the dynamics of these variables

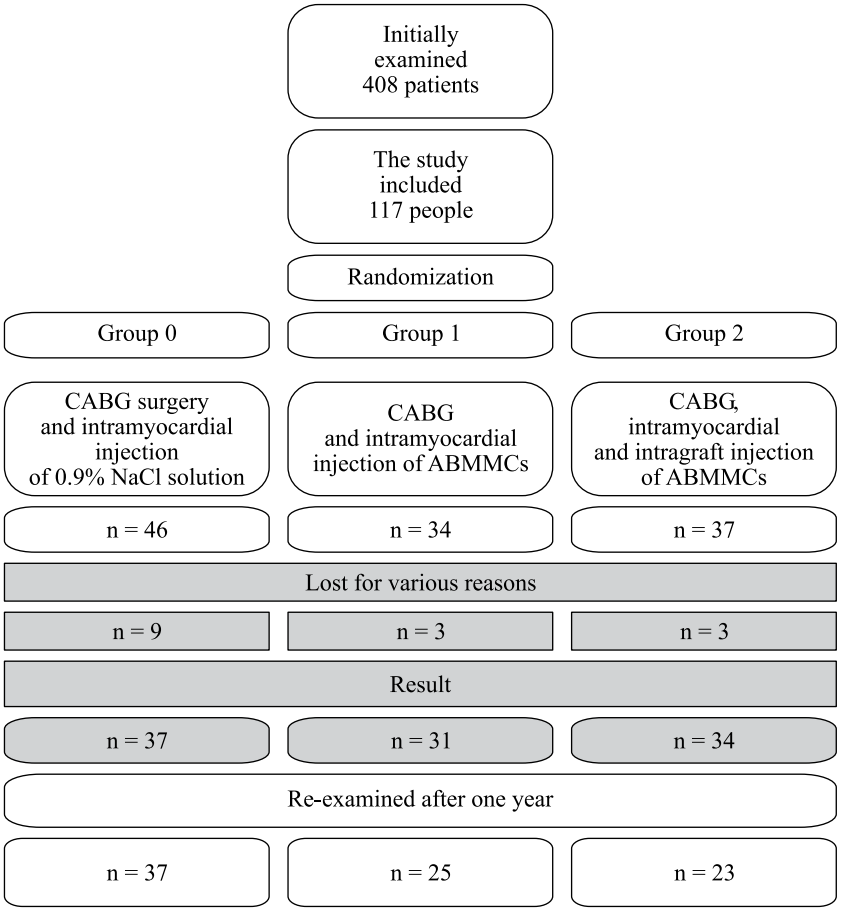


Fig. 1. Study design of TAMIS (Autologous Bone Marrow Mononuclear Cells in the Combined Treatment of Coronary Heart Disease)

was performed within the time frame established by the study design.

Initially, patients in the observation groups were comparable in terms of key parameters (Table 2).

Patient's baseline examination for inclusion in the study corresponded to the standard volume (laboratory, instrumental) before CABG surgery with CPB.

The risk of an adverse outcome for upcoming surgery was rated using the EuroScore II scale.

All patients received adequate medication according to international guidelines depending on the individual clinical situation. Correction was carried out as necessary during the hospitalization period.

Echocardiography was performed on a GE Vivid 7 ultrasound machine using standard technique.

Structural indicators obtained from the study were calculated according to recommendations by ASE and EAE 2015 [17].

Left ventricular linear and volumetric indicators were calculated taking into account the body mass index (BMI), which means that these parameters were taken into account.

The parameters for assessing the left ventricular diastolic function are: transmitral flow velocity (peak E/peak A) and their ratio, deceleration time of peak E (DT), and left ventricular isovolumetric relaxation time (IVRT).

Additional assessment of the left ventricular regional systolic function was done using an automatic functional imaging (AFI) application based on the 2D Strain function, which calculates myocardial tissue deformation by tracking the deformation by two-dimensional images. After software processing, the result was obtained in the form of 6 sectors (17-segment scheme), which shows maximum systolic longitudinal strain in arbitrary colors and numerical values, global strain data for each projection, and global strain averages for the entire left ventricle. The dynamics of these indicators were: initially, when the patient was included in the study, on the 7–14th day of postoperative period, and 12 months after the study.

Treadmill test was aimed at determining exercise tolerance, and at identifying clinical and electrocardiographic signs of myocardial ischemia. The patient was given a physical activity (treadmill), consisting of several stages of progressively greater workloads in accordance with the R. Bruce protocol.

At the same time, cardiac performance (pressure, pulse, electrocardiogram) and their changes depending on the load were recorded.

Table 1

Clinical study sections

I. Safety assessment for additional ABMMC transplantation	II. Efficacy evaluation	III. Prediction of treatment outcomes (influence of several parameters)
1. Assessment of the risk of adverse outcome of upcoming surgery according to the EuroScore II scale. 2. Assessment of intraoperative indicators of ACT; Hb, Ht, K+ at the end of CPB and at the end of surgery. 3. Heart rate recovery at the end of main stage of surgery (defibrillation/self-recovery). 4. Cardiopulmonary bypass time. 5. Duration of anoxia. 6. Volume of discharge through tube in day 1 and day 2 of postoperative period. 7. Level of CPK-MB, myoglobin, troponin I on the 1st and 3rd day of postoperative period. 8. Data obtained from echocardiography performed on the 7–14th day of postoperative period (assessment of myocardial contractility – presence of additional hypo- or akinetic zones compared with baseline). 9. Frequency of postoperative complications (hydrothorax, hydropericardium, rhythm disorders). 10. Length of ICU stay. 11. Length of hospital stay (bed-day).	1. Assessment of systolic and diastolic myocardial function (echocardiography). 2. Assessment of stress test data: treadmill test, daily ECG monitoring. 3. Assessment of data from 6-min walk test (6MWD). 4. Assessment of NYHA angina functional class and heart failure. 5. Quality of life assessment (Minnesota Questionnaire, Seattle Questionnaire, SF-36 Questionnaire). 6. Bypass patency 12 months after treatment (angiographic examination). 7. Assessment of myocardial condition (before and after treatment) – AFI 2D Strain, speckle tracking. 8. Dependence and duration of positive clinical effect on the size of introduced cellular material.	1. Age 2. Sex 3. Body mass index 4. Diabetes 5. Smoking 6. Family history of cardiovascular disease 7. Length of CHD history 8. Level of total cholesterol and its fractions 9. Leukocytosis and CRP levels (initial and dynamics of decline in postoperative period). 10. Creatinine level 11. Presence/absence of extracardiac arteriopathy 12. ABMMC injection intramyocardially or intracoronally 13. Bone marrow examination: number of nucleated cells, CD34+, CD133+.

Note. ACT – activated clotting time; Hb – hemoglobin; Ht – hematocrit; CPK-MB – MB fraction of creatine phosphokinase; CRP – C-reactive protein.

Table 2

Basic characteristics of patients in observation groups

	Group 0 (n = 37)		Group 1 (n = 25)		Group 2 (n = 23)		p
Age (years)	61 ± 8	45–79	61.7 ± 6.8	47–73	59.5 ± 5.4	51–70	0.44
Sex (m/f)	6/31		4/21		3/20		
BMI (kg/m^2)	28.6 ± 3.59	21.1–34.8	28.05 ± 4.46	22.9–44.8	28.09 ± 3.57	22.5–35.4	0.46
Diabetes	6	16.22%	5	20%	4	17.39%	0.93
High blood pressure (grade)							
2	1	2.70%	0	0	0	0	0.52
3	36	97.30%	25	100%	23	100%	
Arterial hypertension (grade)							
0	2	5.41%	3	12%	2	8.70%	
1	6	16.22%	5	20%	5	21.74%	0.82
2	14	37.84%	11	44%	7	30.43%	
3	15	40.54%	6	24%	9	39.13%	
Peripheral artery disease	11	29.73%	4	16%	6	26.09%	0.46
AMI before							
1	14	37.84%	17	68%	9	39.13%	0.13
2	4	10.81%	3	12%	6	26.09%	
3	2	5.41%	1	4%	1	4.35%	
Smoking	7	18.92%	2	8%	4	17.39%	0.78
Family history of CVD	19	51%	11	44%	10	43.48%	0.78
Debut angina pectoris (months)	71.9 ± 70.9	6–300	62.9 ± 79.2	5–240	76.9 ± 59.4	6–240	0.11
up to 5 years	23	62.2%	16	64%	12	52.2%	
6–10 years	8	21.6%	4	16%	8	34.8%	
>10 years	6	16.2%	5	20%	3	13.04%	

End of table 2

	Group 0 (n = 37)		Group 1 (n = 25)		Group 2 (n = 23)		p
Functional class of angina pectoris							0.85
3	32	86.49%	23	92%	21	91.30%	
4	3	8.11%	2	8%	1	4.35%	
painless	1	2.70%	0	0	1	4.35%	
NYHA heart failure functional class							0.24
2	29	78.38%	24	96%	21	91.30%	
3	3	8.11%	0	0	0	0	
TC (mmol/L)	4.49 ± 1.08	2.6–6.46	4.5 ± 1.24	3.05–7.69	4.6 ± 1.49	2.3–8.3	0.99
VLDL (mmol/L)	0.96 ± 0.35	0.41–1.65	0.94 ± 0.5	0.44–1.92	0.77 ± 0.3	0.26–1.34	0.30
HDL (mmol/L)	2.7 ± 0.89	1.34–4.44	2.58 ± 1.05	1.35–4.4	2.7 ± 1.26	0.76–5.63	0.90
LDL (mmol/L)	0.98 ± 0.31	0.62–1.73	1.16 ± 0.37	0.65–1.63	1.13 ± 0.29	0.56–1.58	0.20
TG (mmol/L)	2.04 ± 0.68	0.84 ± 3.59	1.69 ± 0.72	0.82–3.35	1.63 ± 0.62	0.57–2.92	0.07
AC	4.07 ± 1.32	2.1–6	3.27 ± 1.9	1.4–7.1	4.5 ± 5.2	1.3–24.4	0.3
Creatinine (mmol/L)	0.09 ± 0.02	0.06–0.1	0.09 ± 0.02	0.06–0.15	0.08 ± 0.02	0.06–0.1	0.05
CRP (mg/L)	5.9 ± 11.07	1–59.3	2.56 ± 1.96	0.7–7.7	5.3 ± 5.38	1–24.4	0.06
GFR (mL/min) CKD-EPI	79.89 ± 15.8	49–116	77.6 ± 16.03	42–111	84.2 ± 17.26	49–127	0.05
EuroScoreII	1.3 ± 0.68	0.7–3.6	1.07 ± 0.47	0.55–2.54	1.02 ± 0.4	0.5–1.85	0.24
Nitrates (took)	21	56.76%	11	44%	10	43.48%	0.49
Diuretics	5	13.51%	10	40%	3	13.04%	0.07
ACE inhibitors	18	48.65%	9	36%	16	69.57%	0.06
ARBs	5	13.51%	3	12%	4	17.39%	0.86
bETA-2 blockers	35	94.59%	20	80%	23	100.00%	0.05
CCB	5	13.51%	6	24%	4	17.39%	0.57
Antiplatelet drugs	18	48.65%	12	48%	9	39.13%	0.75
Statins	27	72.97%	16	64%	15	65.22%	0.7
Anticoagulants	6	16.22	0	0	2	8.7%	0.1

Note. BMI – body mass index; AMI – acute myocardial infarction; CVD – cardiovascular diseases;; TC – Total cholesterol; VLDL – very low-density lipoprotein; HDL – high-density lipoproteins; LDL – low density lipoproteins; TG – Triglycerides; AC – atherogenic coefficient; CRP – C-reactive protein; GFR – glomerular filtration rate; ACE inhibitors – angiotensin-converting-enzyme inhibitors; ARBs – angiotensin II receptor blockers; CCB – calcium channel blockers.

The 6-minute walk test (6MWT) was performed according to standard method.

An angiographic examination (coronarography) was performed under local (S. Lidicaini / S. Novocaini 0.5% – 30.0 mL) and intravenous anesthesia (S. Propofoli 1% – 200 mL) according to the Seldinger technique. Condition of coronary arteries was assessed in standard positions.

The SYNTAX Score was used to assess coronary artery disease severity. Risk stratification was evaluated based on summation of points obtained from this scale: low-risk group – 0–22 points, intermediate risk group – 23–32 points, high-risk group – >32 points.

Quality of life parameters were assessed using specialized questionnaires: SF-36, Minnesota Questionnaire, Seattle Questionnaire.

CABG surgery was performed based on the standard procedure in accordance with the Heart Team decision.

Bone marrow exfusion was performed in the operating room, in the position of the patient lying on the back, under general anesthesia to the skin incision from the sternum; bone marrow 140 mL in Teruflex 450/400 blood preservation bags (Terumo). Heparin 5000 U/100 mL of 0.9% NaCl solution was used as stabilizer. Thus, in one syringe, 7 mL of bone marrow had 3 mL of 0.9% NaCl solution and 150 units of heparin (15 units/mL).

Mononuclear fraction was isolated by hydroxyethyl starch precipitation in a density gradient.

Sequential removal of fatty inclusions, plasma, and red blood cells was performed using plasma extractor.

Before transplantation, additional mononuclear fraction filtration was carried out using a standard blood transfusion system and blood substitutes with a nylon liquid microfilter with minimum possible cell diameter of 200 μm.

Cell composition of mononuclear fraction was estimated using cytofluorimetry in a specialized laboratory.

Intragraft injection of ABMMCs was performed after application of distal anastomoses of 5 mL per bypass graft, 15-minute exposure.

Intramyocardial transplantation was carried out transepicardially at 0.2 mL per 1 cm^2 (1 mL divided into 5 points) – a total of 10 points in the blood supply pool of the left coronary artery from proximal to the distal sites.

A BD Miro-Fine Plus insulin syringe – 0.30 mm (30G) × 8 mm – was used for intramyocardial administration of both ABMMCs and 0.9% NaCl solution.

Data obtained were processed using the STATISTICA software for Windows program (version 7.0). Calculations were made taking into account exclusion of omissions, which were not considered when drawing up conclusions.

The normal/non-normal distribution was initially estimated to determine the method for statistical processing of obtained data. Nonparametric statistics methods were used to analyze data having a non-normal distribution.

Quality indicators were calculated using the Pearson (chi-squared) test and Fisher's exact test.

Quantitative indicators were analyzed using the Kruskal–Wallis and Wilcoxon tests.

Factor analysis was used to identify certain severity factors and study data homogeneity.

Discriminant analysis was performed to investigate the leading characteristics defining differentiation between the groups.

The model for analysis of variance of dependent samples – Repeated Measures ANOVA – was used for variance analysis, taking into account various factors.

A p-value less than 0.05 was used to assess the statistical significance of findings.

RESULTS

I. Safety assessment for additional ABMMC transplantation

ABMMC transplantation in combination with CABG surgery with CPB in patients with coronary and heart failure is a safe technique [18].

II. Efficacy evaluation

Systolic and diastolic myocardial function parameters, the state of heart valve based on echocardiography that was carried out 12 months after treatment were comparable and did not have statistically significant differences.

Similar data were obtained in analysis of left ventricular diastolic function indicators.

Dynamics of the treadmill test data tended to improve in the observation groups without statistically significant differences among the observation groups ($p = 0.7$). Ischemic changes were detected in 2 cases of group 0.

6MWD showed significant dynamics of increase in the number of meters covered in groups 1 and 2 compared with group 0 ($p = 0.03$) (Fig. 2).

During control examination, ECG daily monitoring data showed ischemic changes in 2 cases from group 0. No rhythm disorders were observed in the observation groups.

Functional tests after 12 months revealed class II-angina in 8 cases only in group 0 ($p = 0.001$).

Recurrent angina pectoris was noted in 8 group 0 patients. The average recurrence period was 6.5 ± 2.9 (1–11) months.

A decrease in heart failure functional class (diastolic) was observed in the observation groups without significant differences ($p = 0.13$).

There were improvements in quality of life parameters in observation groups, according to SF-36, Seattle and Minnesota questionnaires, without significant differences.

The percentage of functioning bypass grafts after a 12-month follow-up period was 87.6% (initially 113 superimposed bypass grafts, 99 were tracked over time) in group 0. In group 1, this ratio was 96.2% (79 and 76), and in group 2 – 97.3% (75 and 73) ($p = 0.048$).

In the presence of non-functioning bypass grafts, ischemic changes according to stress tests, as well as symptoms of at least class-II angina pectoris, native coronary arteries were stented. In group 0 – in 5 cases (13.5%) and in group 1 – in one case (4%) ($p = 0.38$).

Speckle tracking did not have significant differences in the observation groups at the control points of examination.

III. Prediction of treatment outcomes (influence of several parameters)

Analysis demonstrated that smoking has significant effect on 6MWD results – smaller distance was covered in the observation groups ($p = 0.048$).

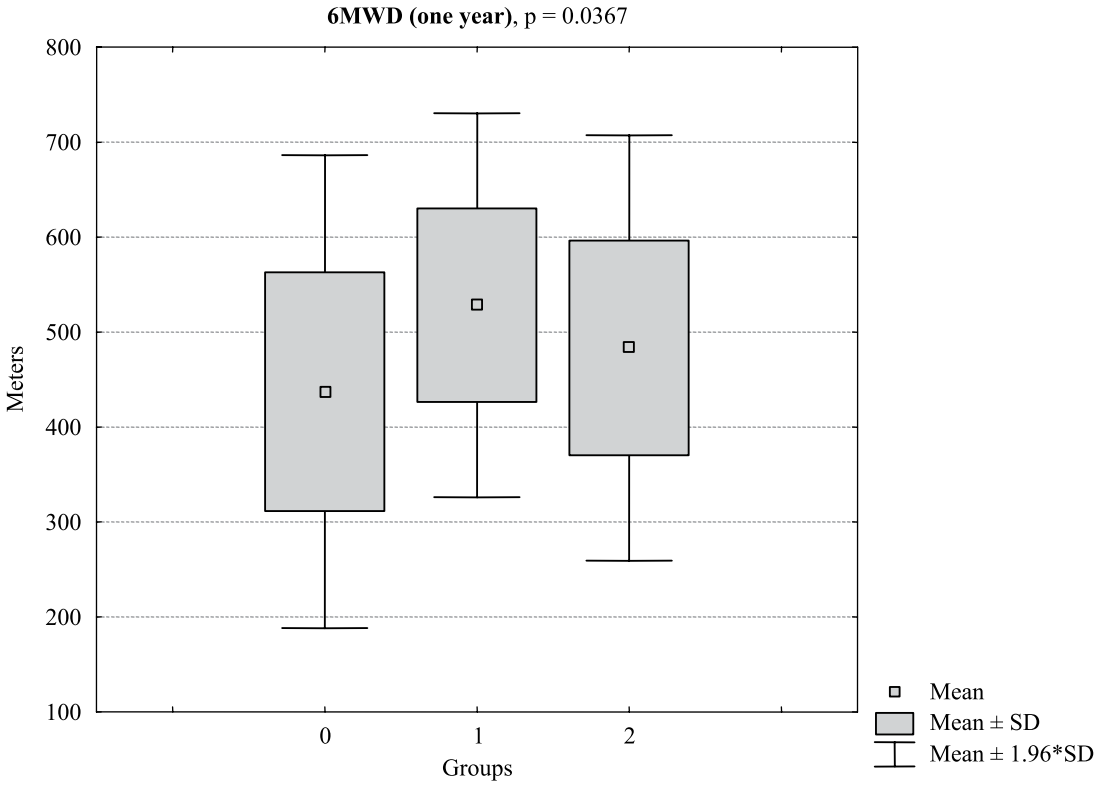
The effect of the initial left ventricular ejection fraction (LVEF) on dynamics of 6MWD parameters and the combined effect of initial LVEF and pulmonary artery pressure on the dynamics of 6MWD indicators were also noted.

The cell composition of obtained mononuclear fraction did not have statistically significant differences in the observation groups (Table 3).

When comparing ABMMC intramyocardial and/or intracoronal transplantation techniques, no statistically significant differences between the groups were found.

DISCUSSION

TAMIS (Autologous Bone Marrow Mononuclear Cells in the Combined Treatment of Coronary Heart Disease) is a single-center, randomized, double-, placebo-controlled trial. The aim of the study was to evaluate the safety, efficacy, and the influence of various factors on the overall effect of transplanted ABMMCs in the surgical treatment of coronary heart disease. This work was carried out after many years of research in the field of cell/regenerative medicine. Previously performed studies in groups of patients in the treatment of chronic IHD, both in isolation and in combination with surgical treatment (angioplasty and stenting, CABG), as well as in



	Initially		1 year later	
Group 0	315.06 ± 17.6	279.9–350.2	433.54 ± 20.6	392.2–474.9
Group 1	319.8 ± 24.5	270.8–368.8	524.4 ± 28.7	466.8–581.9
Group 2	329.9 ± 25.3	279.4–380.5	452.7 ± 29.7	393.3–512.1

Fig. 2. Dynamics of the number of meters covered during the 6-min walk test (6 MWT) in the observation groups

* – Avg. value ± St. Dev./interval

Table 3

Cellular composition of the mononuclear fraction obtained via flow cytometry

	Group 1 n = 25		Group 2 n = 23	
Total cytosis (10^8/L)*	20.3 ± 8.9	1–84.5	6.8 ± 3.2	2–12
(%)*	21.04 ± 2.9	16.09–28	20.0 ± 2.7	15–25
CD34+ (%)*	1.16 ± 0.22	0.9–1.57	1.18 ± 0.34	0.8–1.7
CD133+ (%)*	0.4 ± 0.08	0.3–0.5	0.38 ± 1.14	0.2–0.7

* – Avg. value ± St. Dev./interval.

the treatment of acute myocardial infarction (AMI) served as the basis for development of the design [19–23].

Positive outcomes were obtained in a separate observation group of patients with severe systolic heart failure (dilated cardiomyopathy) [24].

Preliminary calculations indicated the required capacity of the study.

Analysis of literature data pointed to some parameters that are integral to this kind of work. In particular, incidence of complications during such operations varies: 30 ± 10%. According to the formula:

$$n_1 = kn_2,\ n_2 = \frac{(z_\alpha + z_\beta)^2}{(\varepsilon - \delta)^2}\left[\frac{p_1(1-p_1)}{k} + p_2(1-p_2)\right]$$

Let's denote by n_1 the sample size, n_2 – the sample size in the control group. In the test group, there are k = 2/3 times more observations than in the control. Difference between the test and control frequencies $\varepsilon = p_1 - p_2$ [25].

So, with $p_1 = 0.2$, $p_2 = 0.4$, $\delta = 0.1$, we get $n_1 = 49.5$, $n_2 = 33$. In practice, the size of the control group was 36, the two test groups were 25 + 25 = 50.

The null hypothesis is that the frequency of complications in the test sample does not exceed the frequency in the control sample with equivalence limit δ, that is, Ho: $p_1 < p_2 + \delta$. The value p_1 can be $< p_2 + \delta$ by any value. An alternative hypothesis means that the frequency of complications in the test sample is significantly higher

than the control. Accordingly, these statistical indicators are significant.

In overall, this work has indicated an increase in the effectiveness of combined treatment of IHD by combining ABMMC transplantation and CABG surgery with CPB. In addition, common predictors of adverse outcomes, which were not previously considered as such, were identified.

Statistical analysis showed no significant differences in echocardiography data, both in relation to systolic and diastolic myocardial function. There was general tendency towards improvement of these indicators to a more significant degree in groups 1 and 2 without statistical significance in comparison with the control group.

Between observation group and control group, previous studies have found no significant differences in systolic and diastolic myocardial functions [26–29].

With this in mind, Doppler ultrasonography (speckle tracking) was included in the study design to improve the quality of the data obtained. The method showed significant changes in local myocardial contractility on the 7–14th day (average 10th day) of postoperative period. These changes were accompanied by reduced left ventricular ejection fraction (LVEF) and increased left ventricular linear and volumetric indicators (Fig. 3).

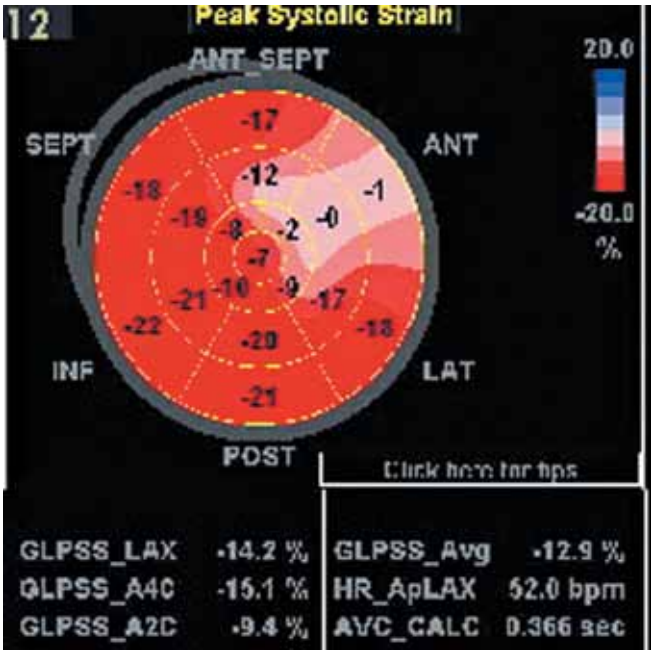
However, discriminant analysis showed that these changes were not significant.

At the same time, there was no significant increase in markers of myocardial injury (troponin I, CPK-MB, myoglobin), and ECG data were nonspecific. After a 12-month follow-up period, speckle tracking echocardiography was able to confirm improvement in revascularization areas. No statistically significant differences in echocardiography parameters were obtained either.

This pattern was directly associated with surgical intervention (with CPB) and reperfusion of previously ischemic myocardial segments (hibernation and stanning zones). Different volume of these segments, peculiarities of the individual anatomical structure of coronary arteries, and differences in surgical revascularization could lead to insignificant echocardiography results.

A step-by-step analysis of results obtained noted that left ventricular (LV) diastolic dysfunction can be a factor influencing treatment results, that is, the functional viability of bypass grafts (Table 4).

Initially



14th day of postoperative period

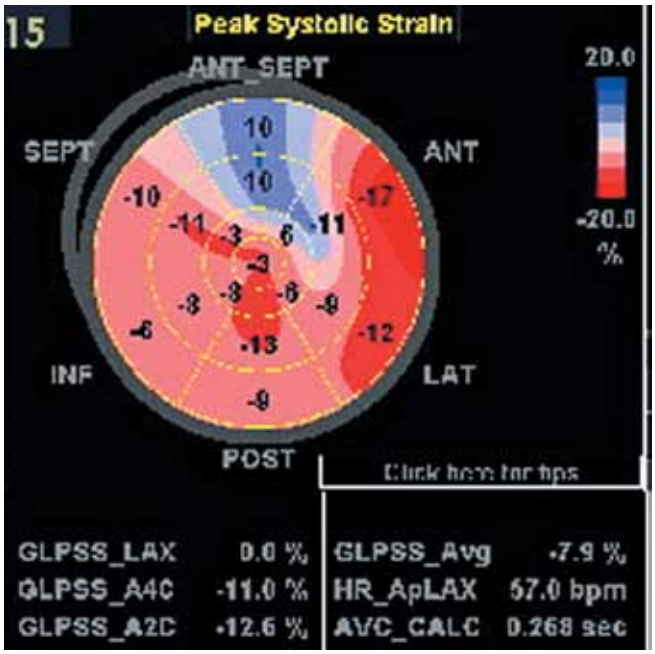


Fig. 3. Dynamics of speckle tracking echocardiography (initially and on the 14th day of postoperative period)

Table 4

Effect of diastolic myocardial dysfunction on the functional viability of bypass grafts after CABG surgery (echocardiography)

	Group 0	Group 1	Group 2
Initial number of patients with LV diastolic dysfunction (n)	19	16	15
Presence of LV diastolic dysfunction after 1 year of follow-up (n)	15 (78.9%)	7 (43.7%)	3 (20%)
Number of patients with non-functioning bypass grafts / with LV diastolic dysfunction after 1 year of observation (n)	10/8	1/0	2/1

During control examination in group 0, LV diastolic dysfunction remained in 78.9% of cases, 43.7 and 20% in groups 1 and 2, respectively. At the same time, LV diastolic dysfunction remained in 9 out of 13 patients with non-functioning bypass grafts, that is, 69.2% ($p = 0.04$).

Aggregate results confirmed the effect of LV diastolic dysfunction on duration of bypass graft functional viability, regardless of the type of surgery performed.

In this case, influence of LV diastolic dysfunction on the integral indicator – functional viability of bypass grafts – manifested through increased elasticity, decreased myocardial stiffness, decreased number of ischemic segments, and increased LV ejection fraction.

Previous studies on the use of ABMMCs have shown that myocardial diastolic function improved [30–31], but none of the studies reported a relationship with the functional viability of coronary bypass grafts.

Quality of life assessment was carried out comprehensively using the SF-36, Minnesota, and Seattle questionnaires. The main purpose of using all these questionnaires was to obtain reliable and comprehensive results. Statistical analysis did not show significant differences in the observation groups. The results are linked to possible error of subservience. This assumption was made, given that all patients were instructed to strictly abide by the prescribed recommendations (lifestyle, drug therapy, frequency of dynamic and follow-up examinations) at all stages of work. In case of deviation from these guidelines, patients were warned of exclusion from further research.

Probably, in order to obtain adequate data regarding quality of life parameters, it was necessary to identify patients who responded to therapy (responders) and did not respond (non-responders).

Cell substrate analysis and ABMMC administration method analysis also showed no statistically significant benefits. These results were associated with insufficient sampling required to calculate the indicated differences. Besides, the observation period of 12 months established by our study design was determined to be insufficient to obtain information on significant difference in the effectiveness of a particular cellular material delivery method.

Absence of statistically significant differences in the data, which previously confirmed the significant influence of ABMMCs on overall effectiveness of treatment, is most likely attributable to the small size of the sample, which makes it impossible to detect small but still significant differences. In addition, since this study was randomized, significant selective sampling was conducted in order to form equally-weighted groups as much as possible. The selection criteria may have been softer in earlier studies. Despite the probably small size of sample, we, nevertheless, obtained new results that were not previously reflected in any study. These findings have more significant implications for subsequent research in this section of scientific medicine.

CONCLUSIONS

ABMMC transplantation along with CABG surgery can boost the overall effectiveness of combination therapy for ischemic heart disease. It was noted that compared with the control group, the observation groups with additional transplantation of indicated type of cellular material achieved significant difference – higher exercise tolerance (6MWD), lower exertional angina functional class, and better bypass graft functional viability. The study showed the need for stage-by-stage analytical calculations with the aim of possible correction of further work.

The authors declare no conflict of interest.

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SIBS TRIBLOCK COPOLYMERS IN CARDIAC SURGERY: *IN VITRO* AND *IN VIVO* STUDIES IN COMPARISON WITH ePTFE

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Implantation of polymeric heart valves can solve the problems of existing valve substitutes – mechanical and biological. **Objective:** to comprehensively assess the hemocompatibility of styrene-isobutylene-styrene (SIBS) triblock copolymer, synthesized by controlled cationic polymerization in comparison with expanded polytetrafluoroethylene (ePTFE) used in clinical practice. **Materials and methods.** SIBS-based films were made by polymer solution casting method; *in vitro* biocompatibility assessment was performed using cell cultures, determining cell viability, cell adhesion and proliferation; tendency of materials to calcify was determined through *in vitro* accelerated calcification; *in vivo* biocompatibility assessment was performed by subcutaneous implantation of rat samples; hemocompatibility was determined *ex vivo* by assessing the degree of hemolysis, aggregation, and platelet adhesion. **Results.** The molecular weight of synthesized polymer was 33,000 g/mol with a polydispersity index of 1.3. When studying cell adhesion, no significant differences ($p = 0.20$) between the properties of the SIBS polymer (588 cells/mm^2) and the properties of culture plastics (732 cells/mm^2) were discovered. Cell adhesion for the ePTFE material was 212 cells/mm^2. Percentage of dead cells on SIBS and ePTFE samples was 4.40 and 4.72% ($p = 0.93$), respectively, for culture plastic – 1.16% ($p < 0.05$). Cell proliferation on the ePTFE surface (0.10%) was significantly lower ($p < 0.05$) than for the same parameters for SIBS and culture plastic (62.04 and 44.00%). Implantation results (60 days) showed the formation of fibrous capsules with average thicknesses of 42 μm (ePTFE) and 58 μm (SIBS). Calcium content in the explanted samples was 0.39 mg/g (SIBS), 1.25 mg/g (ePTFE) and 93.79 mg/g (GA-xenopericardium) ($p < 0.05$). Hemolysis level of red blood cells after contact with SIBS was 0.35%, ePTFE – 0.40%, which is below positive control ($p < 0.05$). Maximum platelet aggregation of intact platelet-rich blood plasma was 8.60%, in contact with SIBS polymer – 18.11%, with ePTFE – 22.74%. **Conclusion.** In terms of hemocompatibility properties, the investigated SIBS polymer is not inferior to ePTFE and can be used as a basis for development of polymeric prosthetic heart valves.

Keywords: polymeric heart valve prostheses, poly(styrene-b-isobutylene-b-styrene), polytetrafluoroethylene, hemocompatibility.

INTRODUCTION

Prosthetic heart valves are currently the preferred choice in correcting valvular defects. Functioning efficiency, being durable without the use of anticoagulant therapy and ability to be implanted in patients of various age groups are the key requirements for this type of valves [1, 2]. Prosthetic heart valves can be mechanical (with a rigid flap) or biological (based on xenomaterials), which have been used in clinical practice for several decades. Both types of prostheses have setbacks: the mechanical one has high thrombogenicity, while the biological one has limited durability as a result of xenotissue degradation [1, 2]. However, with higher number of younger patients and longer life expectancy of the population, there is need for a paradigm shift in the technology of valve structures and related materials today. Leaflet flap apparatus based on elastomeric polymers has prospects for successful use in clinical practice and can address the problem of bioprosthesis durability (reoperation). It can eliminate the need for permanent use of anticoagulants required for implantation of mechanical valve devices and provide hemodynamics that is comparable to physiological hemodynamics [3]. The ability to design a leaflet device with a given structure and properties is another advantage of synthetic products [4, 5]. In addition, biostable polymer matrices are attractive for use in heart valve tissue engineering [6].

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Despite the considerable number of researches geared towards finding suitable materials, polymer valves are still used in clinical practice due to high degree of instability of thermoplastic elastomers [7] and the thrombosis and calcification processes triggered by their use [8]. That is why the search for an effective combination of biostability and hemocompatibility properties is a pressing issue standing on the way of identifying polymer valve with clinical reality. Beginning in the 1950s, there were attempts to use a number of polymers as the basis for leaflet apparatus: polyurethanes [9], silicones [10], 3D cross-linked polyvinyl alcohol [11], polytetrafluoroethylene (PTFE and ePTFE) [12, 5], POSS–PCU nanocomposite [13], styrene-isobutylene-styrene (SIBS) triblock copolymer [14], etc. However, no ideal solution has yet been found.

SIBS block copolymers are of particular interest due to several factors: high hemocompatibility and biostability [15]; their properties can be controlled by varying the length and structure of polymer units; processing them by extrusion or injection molding is relatively easy; there is considerable experience in using them in medicine [16]. So, SIBS30 (30% styrene) manufactured by Innovia is the basis of modern cardiosurgical stents. It demonstrates high biocompatibility [17]. However, because pure SIBS30 has low physical and mechanical characteristics and given available experimental data on thrombosis [18], there was the need to look for new triblock copolymer modifications that could be used to develop a leaflet prosthetic heart valve, in particular, surface modification and creation of composites with reinforcing networks made of stronger polymers [19, 20].

This paper describes the synthesis of SIBS model polymer by controlled cationic polymerization. It also looks at the study of *in vitro* and *in vivo* hemocompatibility properties. The work uses, for the first time, an integrated approach to assessing hemocompatibility properties of the presented SIBS-based polymeric materials. This approach is especially important for adequately predicting the potential use of the polymers in valve structures.

MATERIALS AND METHODS

Synthesis of SIBS polymers

Stabilized styrene (Sigma-Aldrich, USA, >99%) was treated with 10% KOH solution, then washed with distilled water until neutral reaction, then dried with $CaCl_2$ and twice distilled over CaH_2 under reduced pressure. Methylene chloride and n-hexane (Ekos-1, Russia, reagent grade) were treated with concentrated H_2SO_4, a solution of soda, distilled with water until neutral reaction, then dried with $CaCl_2$, boiled twice and distilled over CaH_2. Titanium tetrachloride (Sigma-Aldrich, USA, 99.9%) was distilled over copper chips under reduced pressure. Isobutylene (Sigma-Aldrich, USA, >99%) was dried by passing it through a column filled with calcium chloride. 2,6-di-tert-butylpyridine (Sigma-Aldrich, USA, 97%) and dicumyl alcohol (Aldrich, USA, 97%) were used without prior purification.

Polymerization was carried out in a three-necked flask, which was first vacuumized and filled with argon. An initiator – freshly prepared dicumyl chloride (0.095 mmol) – was added to the flask through Kaszas technique [21]. Then it was dissolved in a "hexane: methylene chloride = 3 : 2" mixture and 0.20 mmol of 2,6-di-tert-butylpyridine was added, cooled to –60 °C in an alcohol bath, and isobutylene (34 mmol) cooled to –60 °C was added. Monomer concentration was 1.0 M. Next, the temperature was lowered to –80 °C and 1.9 mmol of titanium tetrachloride (20-fold excess) was added for commencement of polymerization.

After 25 minutes from start of polymerization, 9.2 mmol of pre-chilled styrene (2.0 M solution in a "hexane: methylene chloride = 3 : 2" mixture) was added. After 50 minutes from the start, the reaction was stopped by adding 2 mL of ice-cold methanol. The resulting sample was precipitated twice in a 10-fold excess of ice-cold ethanol. The precipitated sample was separated by centrifugation, washed with small amount of ethanol, and dried in vacuum at 55–60 °C/2 mm Hg until change in mass stopped.

Gel permeation chromatography

Molecular mass characteristics of the obtained block copolymers and efficiency of block formation were determined by gel permeation chromatography on an UltiMate 3000 instrument equipped with a PLgel precolumn (7.5×50 mm, 5 μm particle size), PLgel MIXED-C column (7.5×300 mm, 5 μm particle size) with refractometric and UV detectors (Thermo Fisher Scientific, Germany). Tetrahydrofuran was used as solvent; elution rate was 1 mL/min at a column temperature of 30 °C. Average molecular weight (M_n) and polydispersity (M_w/M_n) of polymers were calculated using the Chromeleon 7.0 software package (Thermo Scientific Dionex, Germany) using elution curves based on calibration dependences obtained applying polystyrene standards (Agilent EasiCal) with $M_w/M_n \leq 1.05$.

Polymer film production

Polymer films were obtained by irrigation from a polymer solution in chloroform (ratio of 1.6 g of substance to 8 mL of solvent) on a glass surface. The area of the obtained samples was 21 cm^2. The films were formed at 25 °C temperature for one hour and then heated to 35 °C for three hours at atmospheric pressure and 50% air humidity. Additionally, the samples were dried using a Labconco FreeZone 2.5 Benchtop vacuum dryer (USA) at –40 °C temperature and <0.133 mbar pressure.

In vitro assessment of cytotoxicity using cell cultures

The experiment was performed using the EA.hy926 cell line – hybridoma of human umbilical vein endothelial cells (HUVEC) and human lung carcinoma cells (cells provided by Dr. Cora-Jean S. Edgell, University of North Carolina, USA), which allows the cell line to reproduce the main phenotypic and functional characteristics of human microvessel endothelial cells. GORE-TEX® ePTFE vascular grafts (Gore & Associates, Inc, USA) were used as the comparison group, while culture plastic was used as the control group.

Sterile samples (n = 3 for each group; ethylene oxide sterilization) using a 0.6% agarose solution (Helicon, Russia) were fixed to the bottom of sterile 24-well culture plates. 2.0×10^5 cells were introduced into the polymer samples and cultured for 5 days in DME/F12 medium (Sigma Aldrich, USA) containing 1% HEPES buffer (Hyclone, USA), 10% fetal bovine serum (Sigma Aldrich, USA), 1% L-glutamine, 100 u/mL penicillin, 0.1 μg/mL streptomycin, 0.1 μg/mL amphotericin B, Hypoxanthine-Aminopterin-Thymidine (HAT) (Sigma Aldrich, USA) at 37 °C temperature and 5% CO_2. The medium was changed once every 2 days. Absolute cell count per 1 mm^2 of the surface and relative content of dead cells were evaluated using fluorescence microscopy (Axio Observer Z1, Carl Zeiss, Germany). For this purpose, 2 μg/mL of Hoechst 33342 nuclear fluorescent dyes (Molecular Probes, USA) and 0.03 mg/mL of ethidium bromide (AppliChem, Spain) were added 30 minutes before microscopic examination (orange staining of dead cell nuclei). To prepare the samples for microscopy, they were separated from agarose and transferred to a sterile 24-well plate. Cell count was determined in 5 different fields of view at ×200 magnification, followed by conversion to 1 mm^2 of the studied surface. The relative number of dead cells was determined from the ratio of the absolute number of dead cells per 1 mm^2 of the surface to the absolute number of cells per 1 mm^2.

Cell proliferation was evaluated on sterile samples (n = 3 for each group) using the Click-iT Plus EdU cell proliferation imaging kits (Molecular probes, USA): the nuclei of all cells were stained blue with nuclear DAPI dye, while the nuclei of proliferating cells were stained green with Alexa Fluor 488 fluorescent dye. When DNA synthesis takes place, thymidine integrates into DNA and, having an affinity for Alexa Fluor 488, selectively detects synthesized DNA, which is expressed in green fluorescent nuclei. Fluorescence microscopy was performed on an LSM 700 laser scanning microscope (Carl Zeiss, Germany). The relative number of proliferating cells was determined from the ratio of the absolute number of proliferating cells per 1 mm^2 of the surface to the absolute number of cells per 1 mm^2 of the surface.

Determination of calcification *in vitro*

Calcification resistance was determined through accelerated *in vitro* calcification. Samples 5×5 mm in size (n = 5 for each group) were placed in 2 mL of a DMEM culture medium solution (Sigma, USA) and albumin serum (FBS, Sigma, USA) containing $CaCl_2$ and Na_2HPO_4, and kept in a CO_2 incubator at a 37 °C temperature and 5% CO_2. Degree of calcification was evaluated after the third and sixth week of incubation. GORE-TEX® ePTFE vascular prosthetic material (Gore & Associates, Inc, USA) was used as the comparison group; bovine xenopericardium preserved by standard method with glutaraldehyde (GA) (NeoCor JSC, Russia) was used as positive control. Cryosections of the biomaterial and polymer samples were stained for calcium with alizarin red S (Reachim, Russia) and analyzed using an Axio Imager A1 microscope (Carl Zeiss, Germany).

In vivo biocompatibility assessment

Tissue response

In vivo inflammatory response and calcification was examined by subcutaneous implantation of 5×5 mm samples of male Wistar rats (weight 55–70 g) for a 2-week (tissue response assessment, n = 5) and 2-month (tissue response assessment, n = 5, degree of calcification, n = 5) follow-up period. After the experiment, some of the film samples were biopsied with surrounding tissues, fixed in a 4% solution of neutral formalin (MiniMed, Russia), and then restricted in paraffin (Biovitrum, Russia). Sections were stained with hematoxylin and eosin (Biovitrum, Russia) according to Van Gieson with a mixture of acid fuchsin and picric acid (Biovitrum, Russia) and alizarin red S (Reakhim, Russia). They were examined using an Axio Imager A1 microscope (Carl Zeiss, Germany).

Inflammatory response was analyzed according to the ISO 10993-6:2016 standard using a semi-quantitative evaluation system, for which the number of neovascularization foci in five fields of view was calculated for each animal at ×400 magnification. The response was also evaluated based on the thickness of the fibrous capsule (average of 10 equidistributed measurements of the width of the capsules taken over the entire dense tissue closest to the implant), fatty infiltrate and number of lymph nodes.

In vivo calcification

Another part of samples was hydrolyzed to evaluate quantitative calcium content. For this purpose, part of calcium was placed in 0.5 mL of 50% perchloric acid and kept at 150 °C until a clear solution was obtained (ePTFE was not subjected to hydrolysis, calcificates that were formed during the experiment passed into the solution). The cooled samples were diluted with distilled water. Calcium content was evaluated by inductively coupled

plasma optical emission spectrometry on an iCAP 6500 DUO spectrometer (Thermo Scientific, USA).

GORE-TEX® ePTFE vascular prosthetic material (Gore & Associates, Inc, USA) was used as the comparison groups, while bovine xenopericardium preserved by standard method with glutaraldehyde (GA) (NeoCor JSC, Russia) was used as positive control.

In vitro hemocompatibility assessment

Degree of hemolysis

The study was performed in accordance with the ISO 10993-4:2017 standard. To estimate the level of red blood cell (RBC) hemolysis, fresh donor blood was used with addition of 3.8% sodium citrate solution in a 1:9 (citrate:blood) ratio. The test samples 25 cm^2 in size (n = 5 for each type of material) were placed in containers, 10 mL of physiological saline was added and placed in a thermostat at 37 °C for 120 minutes. Saline and distilled water were used as negative and positive controls, respectively, to assess the degree of erythrocyte hemolysis. After that, 200 µl of citrated blood was added to each box, mixed and again kept in an incubator at 37 °C for 60 minutes. After incubation, the solution was taken from the boxes into tubes, followed by 10-minute centrifugation at 2800 rpm to precipitate RBCs. Optical density of the resulting solutions was measured at 545 nm wavelength on a GENESYS 6™ spectrophotometer (Thermo Scientific, USA).

The degree of hemolysis (H) in % was determined by the formula:

$$H = \frac{D_t - D_{ne}}{D_{pe} - D_{ne}} \times 100\%,$$

where D_t is the optical density of sample incubated with the studied polymer; D_{ne} is the optical density of positive control; D_{pe} is the optical density of the sample after 100% hemolysis.

For complete absence of hemolysis, the arithmetic mean value of the optical density index when measuring the positive control (saline solution with citrated blood) was taken; for 100% hemolysis, the arithmetic mean value of the optical density of the device when measuring distilled water with citrated blood was taken. Comparison groups were GORE-TEX® ePTFE (Gore & Associates, Inc, USA) and Polyethylene (LDPE) ERM – EC590 (Merck, Germany).

Platelet aggregation

The study was performed in accordance with the ISO 10993-4:2017 standard. The study used fresh donor blood with addition of 3.8% sodium citrate, in a 1:9 (citrate: blood) ratio. To obtain platelet-rich plasma (PRP), citrate blood was centrifuged at 25 °C for 10 minutes at 1000 rpm. Platelet-poor plasma (PPP) was obtained by centrifuging citrated blood for 20 minutes at room temperature and at 4000 rpm rotation speed. This speed was used to calibrate the device. An intact PRP was used as a control. ERM-EC590 polyethylene (LDPE) (Merck, Germany) was used as a comparison group for high blood platelet aggregation rates.

Measurements were taken without aggregation inducers in spontaneous mode. In the present study, a semi-automatic 4-channel platelet aggregation analyzer (photometer) "ARAST 4004" (LABiTec, Germany) was used. To initiate spontaneous aggregation in citrated blood, the level of Ca^{2+} ions was restored, and the prepared $CaCl_2$ solution with a 0.25 M molar concentration was used as reagent. The sample-to-reagent ratio was 250 µl PRP + 25 µl $CaCl_2$. The duration of contact between the studied samples and PRP was 3 minutes.

Platelet adhesion

PRP obtained from fresh citrated donor blood via 10-minute centrifugation at 1000 rpm was used for the study. Comparison groups were GORE-TEX® ePTFE (Gore & Associates, Inc, USA) and Polyethylene (LDPE) ERM – EC590 (Merck, Germany).

Samples 1 cm^2 in size were incubated with 500 µl PRP at 37 °C for 2 hours, then carefully washed with phosphate-buffered saline (PBS) to remove non-adsorbed plasma components. Then, the samples were fixed in a 2% glutaraldehyde solution prepared on 0.1 M phosphate buffer for 6 hours. Next, the samples were dehydrated in a series of alcohols of ascending concentration (50, 75, 95 and 100%) for 15 minutes each. Platelet adhesion was assessed using a S3400N scanning electron microscope (Hitachi, Japan). The adhesive capacity of the surface of the materials was evaluated in randomly selected 8 fields of view, as the platelet activation index, which was calculated by the formula:

Activation index = (number of level I platelets × 1 + number of level II platelets × 2 + number of level III platelets × 3 + number of level IV platelets × 4 + number of level V platelets × 5) / total number of platelets (Table 1).

Table 1

Platelet activation levels

Level	Characteristic
I	Disc-shaped platelet, without activation
II	Platelet is increased in size with rudiments of pseudopodia in the form of protrusions (initial stage of activation)
III	Platelet is significantly increased in size, irregular in shape, with pronounced pseudopodia, platelets accumulate
IV	Platelet spreading, cytoplasm spreads between pseudopodia
V	Platelet in the form of a spot with granules, pseudopodia cannot be identified due to cytoplasm spread

Statistical processing

Quantitative data were processed by generally accepted statistical techniques using the Statistica 6.0 application package (StatSoft, Inc., USA) for processing medical and biological information. The nature of distribution in the samples was evaluated using the Kolmogorov–Smirnov test. Non-normal distribution ($p < 0.01$) was observed in the groups, and therefore all data were presented as medians (M) and quartiles (25 and 75%). Statistical significance of differences between two independent groups was evaluated using the nonparametric Mann–Whitney U test. Differences were considered significant at a $p < 0.05$ significance level.

RESULTS

SIBS polymer synthesis

Poly(styrene-block-isobutylene-block-styrene) obtained through successive addition of monomers in controlled cationic polymerization is characterized by a number average molecular weight of 33,000 g/mol and a polydispersity index of 1.3. The polymer structure is confirmed by gel permeation chromatography.

In vitro cytotoxicity assessment using cell cultures

In studying cell adhesion (Fig. 1), there were no significant differences ($p = 0.20$) found between SIBS

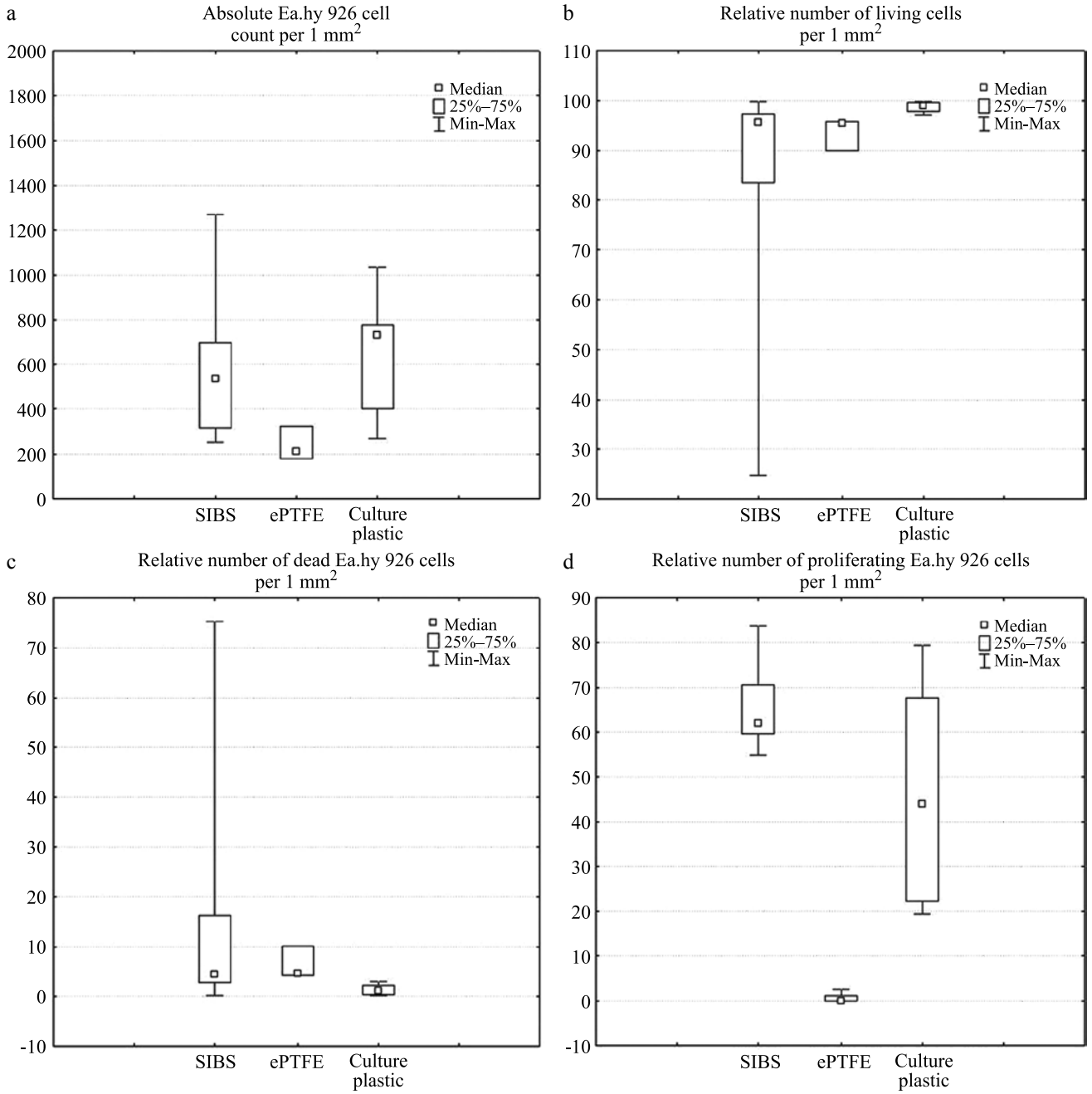

Fig. 1. Absolute number of cells (a), relative number of living cells (b), relative number of dead cells (c), relative number of proliferating cells (d). Ea.hy 926 on various polymer surfaces 5 days after cultivation

polymer properties (588 cells/mm^2) and culture plastic properties (732 cells/mm^2) that is considered the gold standard for *in vitro* cell adhesion. The absolute quantity of adherent cells for the ePTFE material (212 cells/mm^2) was 3 times less than for the SIBS polymer ($p < 0.001$).

The relative number of viable EA.hy926 cells in different polymer samples (Fig. 1, b) did not significantly differ ($p = 0.56$). Accordingly, the same situation was the case with the relative number of dead cells. In the studied polymer samples (SIBS, ePTFE), the number of dead cells did not differ among themselves ($p = 0.93$) – 4.40 and 4.72%, respectively. At the same time, this parameter had a value of 1.16% for culture plastic which turned out to be four times lower (Fig. 1, c) than on the SIBS and ePTFE surfaces ($p < 0.05$).

However, extremely low cell proliferation on the ePTFE surface (0.10%) made cell adhesion and proliferation on this material (Fig. 1, d) impossible. As can be seen from Fig. 2, maximum proliferative activity of EA.hy926 cells was detected on the SIBS polymer surface (62.04%), which was 1.4 times higher than on culture plastic (44.00%), ($p < 0.05$).

Determination of calcification *in vitro*

Polymeric materials SIBS, ePTFE (Fig. 3, b, c) were found to be resistant to calcification in an *in vitro* test, in contrast to biological tissue samples, which had already started mineralizing by the 3rd week of incubation and became slightly more pronounced by the 6th week (Fig. 3, a).

In vivo biocompatibility assessment

Tissue response

After 14 days following implantation, there was a moderate macrophage tissue response to implanted ma-

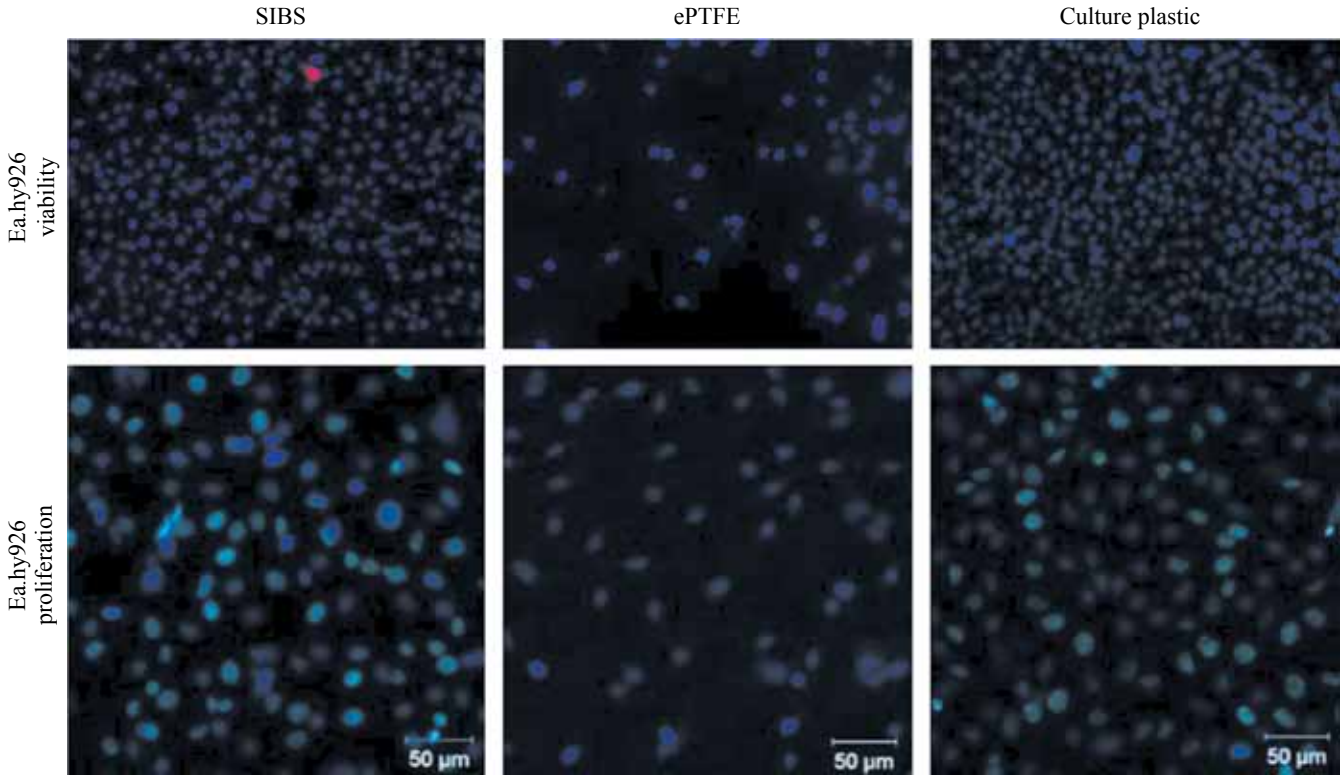

Fig. 2. Fluorescence microscopy of matrices with cells. ×200

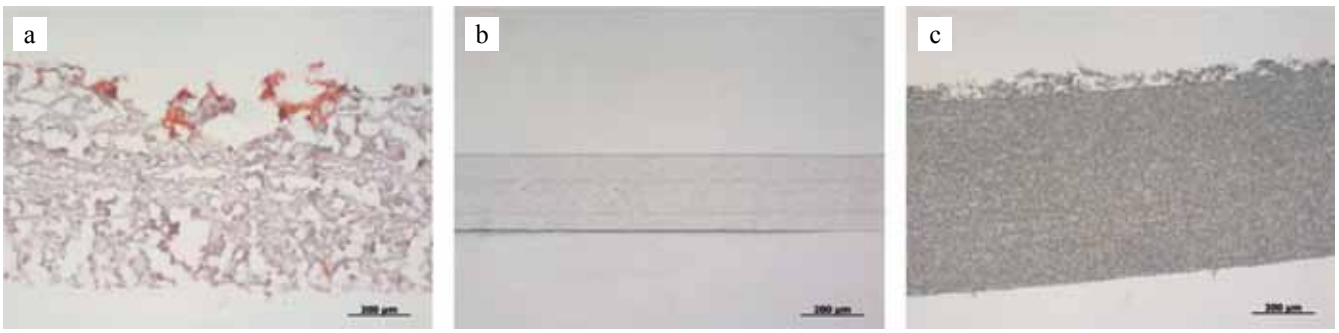

Fig. 3. Light microscopy, *in vitro* calcification test, 6 weeks incubation; samples of materials: GA-preserved bovine pericardium (a), SIBS (b), ePTFE (c). ×100

terials and no giant cells. This suggests good biocompatibility of the polymers (Fig. 4, a–c). For all types of polymers, lymph nodes were found in the tissue surrounding the implants. This indicates inflammatory processes, while lymph nodes were found in minimal number for the SIBS sample. Histological examination showed formation of loose fibrous tissue preceding the formation of a connective tissue capsule covering polymer samples with an average thickness of 69 μm (ePTFE), 50 μm (GA-xenopericardium) and 98 μm (SIBS). Fatty infiltrate associated with collagen fibers was observed in the case of biological samples. The degree of neovascularization was comparable for all types of materials – minimal capillary proliferation, 1–3 neovascularization foci with fibroblast structures in the field of view (×400 magnification). In the case of biological samples and ePTFE (due to its porous structure), cell infiltration was noted, especially active for GA-xenopericardium.

Long-term implantation (60 days) led to formation of dense connective tissue capsule in the form of collagen fibers, with an average thickness of 42 μm for ePTFE and 58 μm for SIBS (Fig. 4, e, f). In the case of GA-xenopericardium, the capsule was not clearly visualized due to active inflammatory process, which was caused by significant calcification. The presence of foreign-body giant cells two months after implantation was observed for a polymer sample of ePTFE and GA-xenopericardium; in the case of SIBS, foreign-body giant cells were not detected.

From the standpoint of a semi-quantitative assessment (ISO 10993-6:2016) based on analysis of tissue response indicators (neovascularization, fibrosis, fatty infiltrate), SIBS polymer can be classified as biocompatible relative to comparison samples (ePTFE, preserved xenopericardium, which are used today in the clinical practice of heart valve replacement).

In vivo calcification

Another biocompatibility criterion is the degree of calcification of material in contact with the body environment. Histological examinations showed active calcification of GA-xenopericardium (Fig. 4, h). The presence of calcifications in the case of ePTFE was detected on the surface of the polymer sample (Fig. 4, i).

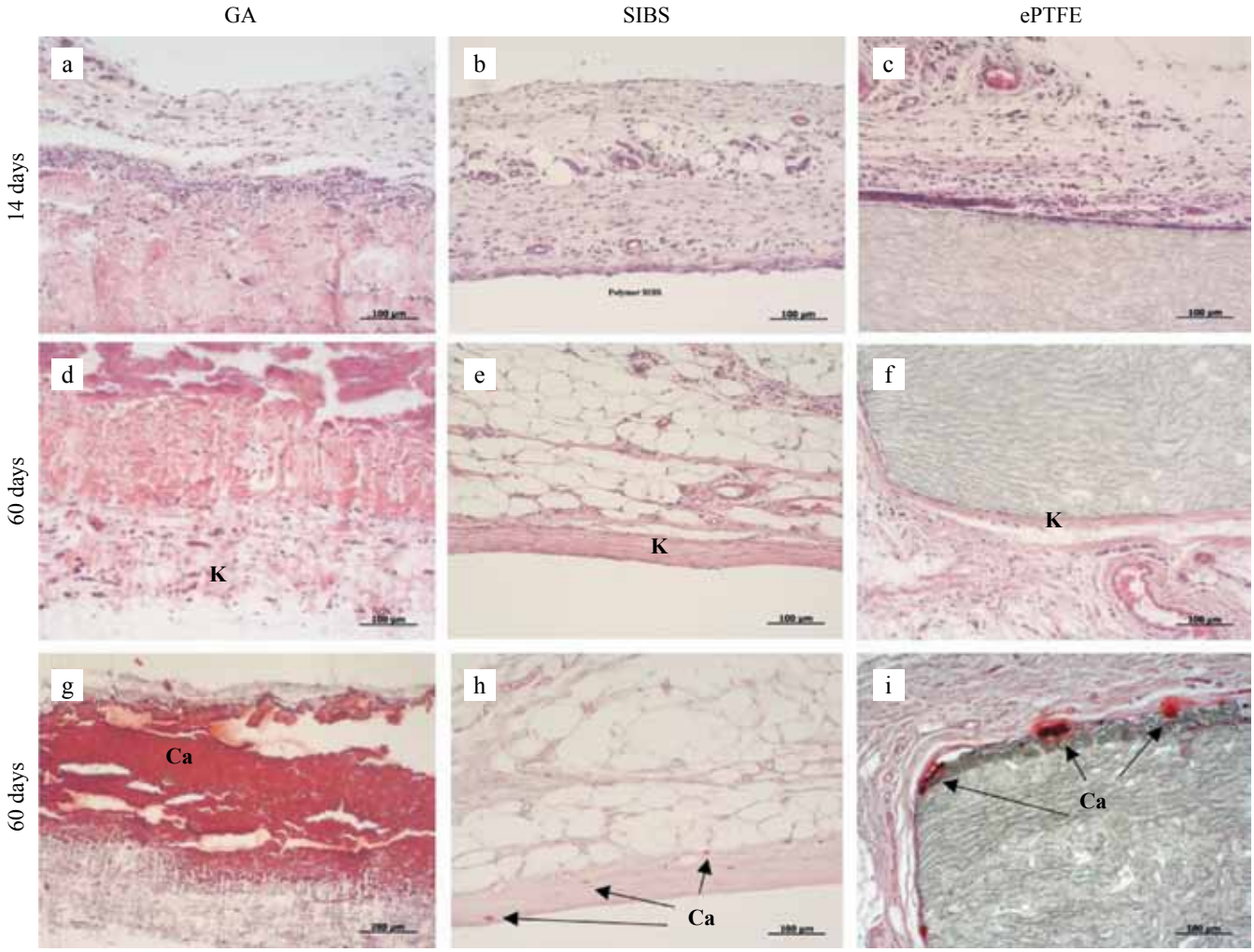

Fig. 4. Histological sections of GA-xenopericardium polymer matrices (a, d, h), SIBS (b, e, i), ePTFE (c, f, i), after implantation in rats for a period of 2 weeks (a, b, c) and 2 months (d, e, i). Stained with hematoxylin-eosin (a–f), alizarin red S (g–i). ×200

Crystalline calcium dot sites are noted in the thickness of the connective tissue capsule formed around SIBS.

Quantitative results showed statistically significant differences in the calcium content of SIBS and ePTFE samples ($p < 0.05$). Significant increase in calcium was also found in GA-xenopericardium samples ($p < 0.05$). A full description of the results is presented in Table 2.

Table 2

Calcium content in the samples (mg/g)

	Min	25%	Me	75%	Max
SIBS	0.23	0.23	**0.39**	0.51	0.55
ePTFE	0.49	0.53	**1.25**	2.70	2.95
GA-xenopericardium	1.77	2.57	**93.79**	155.30	159.80

In vitro hemocompatibility assessment

Level of hemolysis

The study showed no negative effect of SIBS polymer on the RBC membrane. Hemolysis level of RBCs after contact with SIBS was 0.35% (min: 0.03; max: 0.60; 25%: 0.11; 75%: 0.40), which is statistically less than that of negative control ($p < 0.05$).

When assessing the degree of RBC lysis after contact with ePTFE polymer, hemolysis level was 0.40% (min: 0.11; max: 2.40; 25%: 0.31; 75%: 0.67), which is also statistically significantly lower than that of the positive control ($p < 0.05$). Hemolysis level of RBCs after contact with polyethylene was 1.82% (min: 1.16; max: 2.30; 25%: 1.41; 75%: 2.10). Comparison of two polymer groups (SIBS and ePTFE) with each other found no significant differences in severity of hemolysis ($p = 0.57$). Comparison of two polymer groups (SIBS and ePTFE) with polyethylene found significant differences ($p < 0.05$).

Platelet aggregation

Maximum platelet aggregation of intact platelet-rich plasma was 8.60% (min: 7.79%; max: 15.91%; 25%: 8.02; 75%: 10.12%). Moreover, platelet-rich plasma samples in contact with polyethylene showed 73.40% platelet aggregation level (min: 67.73%; max: 82.74%; 25%: 72.35; 75%: 78.99%) Assessment of the degree of platelet aggregation after contact with SIBS and ePTFE polymers showed the following results: maximum aggregation after contact with SIBS polymer was 18.11% (min: 16.40%; max: 23.78%; 25%: 16.66; 75%: 20.42%); maximum platelet aggregation after contact with ePTFE was 22.74% (min: 18.6%; max: 28.56%; 25%: 22.45; 75%: 24.52%), which is statistically significantly lower than the maximum polyethylene-induced aggregation ($p < 0.05$).

Platelet adhesion

Platelets adherent to the surface were detected for all studied materials (Fig. 5). It is interesting to note that on one of the SIBS samples, there were individual areas with uncharacteristic accumulation of type III platelets located in the central part of the sample in a targeted manner.

In calculating the activation index of platelets adherent to polymer surface, the following indicators were obtained: activation index of platelets adherent to polyethylene surface was 3.61 (min: 2.0; max: 4.25; 25%: 2.82; 75%: 4.0). For polyethylene, level IV platelets were more characteristic – 61.29% of the total count of ad-

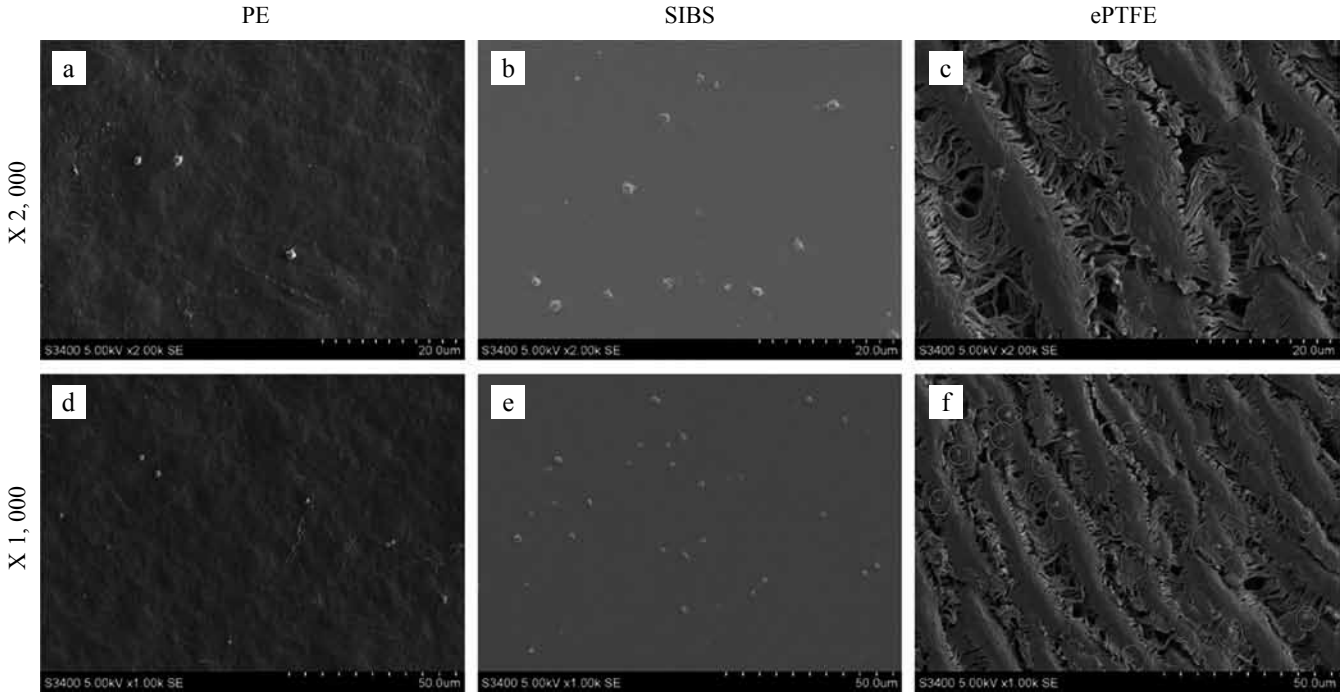

Fig. 5. SEM images of platelet adhesion on the surface of polyethylene polymer matrix (a, d), SIBS (b, e) and ePTFE (c, f)

herent platelets. Activation index of platelets adherent to SIBS polymer surface was 3.25 (min: 2.1; max: 4.0; 25%: 3.06; 75%: 3.69). Level II and III platelets were predominantly found on SIBS surface, which is 61.63% and 8.4%, respectively, as a percentage of the total number of adherent platelets. Activation index of platelets adherent to ePTFE surface was 3.76 (min: 3.0; max: 4.09; 25%: 3.29; 75%: 3.96). Level III and IV platelets were more characteristic for ePTFE surface, which is 41.54 and 26.76%, respectively.

Comparison of activation indices of platelets adherent to SIBS and ePTFE polymer surfaces with polyethylene showed no statistically significant differences ($p = 0.54$ and $p = 0.72$, respectively).

DISCUSSION

Copolymers and composites are widely used in medical practice today due to their unique properties – ability to combine structural fragments of various functionality. In particular, styrene-isobutylene-styrene block copolymers have thermoplasticity and high strength. They are resistant to hydrolytic, oxidative, and enzymatic effects due to the linear nature of carbon chain (with alternating secondary and quaternary carbon atoms) and biological inertness of side groups (Fig. 6) [15].

Assessment of the cytotoxicity of SIBS films synthesized by controlled cationic polymerization showed that they have high hemocompatibility. Proliferative potential can complement the picture of functional activity of cells in the case of short-term experiments where full dynamics of changes in cell viability does not always manage to reach maximum values. Experiments showed that the surface of the ePTFE material has low ability to cell adhesion. This is consistent with the results of other studies [22]. Ability to cell adhesion and proliferation is important in tissue engineering of heart valves [6]. Considering the results obtained during experiment, it can be concluded that SIBS polymers has a greater ability for spontaneous endothelization and greater potential in development of tissue-engineered constructs than ePTFE.

Implantation of any foreign material is inevitably accompanied by a response from the body, while formation of a stable connective tissue (fibrous) capsule indicates completion of the inflammatory process. Thickness of the fibrous capsule characterizes the degree of biocompatibility of the studied material. For the SIBS sample, the degree, after 60 days of experiment, turned out to be slightly higher than that of ePTFE sample. This correlates with the results of other experiments [17]. Formation of connective tissue on the leaflet apparatus of the implantable prosthesis creates a protective barrier, separating the synthetic material of the implant from the body environment. At the same time, this process is necessary for formation of a tight connection with the aortic root in order to prevent paravalvular regurgitation and ensure strong fixation of the implant. Foreign-body giant cells observed for explanted samples of ePTFE and GA-xenopericardium are capable of secreting reactive oxygen species and other chemical agents, potentially leading to oxidative damage and destruction of implanted devices [23]. No cells of this type were found for SIBS material after 60 days of experiment. Most likely, SIBS biocompatibility is related to the fact that the outer part of the polymer (10 nm) is a polyisobutylene block, while polystyrene blocks are located inside the polymer sample and are not in direct contact with biological tissues [24].

Calcification is the main cause of native aortic valve stenosis and bioprosthetic valve stenosis. It involves initiation and growth of calcium phosphate crystals, required for bone tissue, in atypical locations. Unlike xenomaterials, polymers do not contain phosphorus-rich cell deposits and destroyed collagen capable of promoting mineralization. They are therefore more resistant to this process. Based on results obtained from *in vitro* experiment, it can be concluded that the studied polymers do not trigger formation of crystal forms of calcium. Moreover, active calcification of GA-xenopericardium confirms the data obtained in previous studies [25].

Tendency of the test materials to calcify was also evaluated in an *in vivo* model during implantation in rats. Indicators of quantitative calcium content for the GA-xenopericardium that was selected as a positive control are consistent with published reports [26] and significantly exceed same values for the studied polymers.

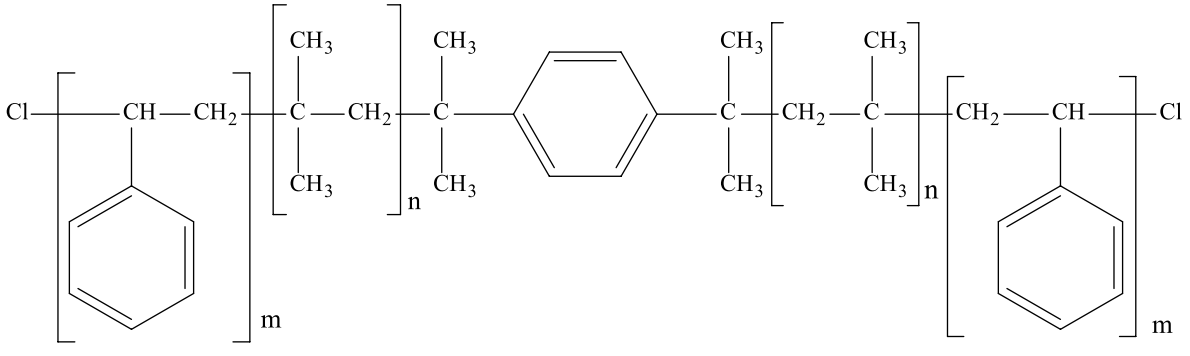

Fig. 6. Structural formula of poly(styrene-block-isobutylene-block-styrene)

There are two mineralization mechanisms arising as a result of contact between the synthetic material and the body's environment: nucleation of calcium phosphate crystals on the surface or at the interface between the connective tissue capsule and the implant; and calcification of biological tissue formed around the implant [27]. For ePTFE, the first mineralization mechanism was observed – calcifications were located on the surface of the sample; while for SIBS material, calcium was found in the fibrous capsule. According to quantitative assessment, SIBS polymer had significantly higher calcification resistance than ePTFE. This suggests that SIBS can be used as the basis for synthetic prosthetic heart valve. Literature sources also show that ePTFE materials have the tendency to mineralization in clinical experiments [8]. The high tendency of ePTFE to calcify may be due to the porous structure of its surface, resulting in accumulation of cellular elements and newly formed extracellular matrix.

In evaluating the degree of erythrocyte hemolysis induced by SIBS polymer surface, it was revealed that polymer has no toxic effect on formed elements (red blood cells). Also, ePTFE polymer, which was used as a comparison group, did not cause destruction of the cell membrane of red blood cells. At the same time, hemolysis level of RBCs after contact with polyethylene was 1.82%, which indicates that polyethylene is susceptible to thrombosis. Assessment of RBC hemolysis showed that the studied SIBS and ePTFE polymers can be considered hemocompatible, since the hemolysis level of RBCs after contact with SIBS and ePTFE polymer surfaces did not exceed 0.4% with permissible hemolysis degree of up to 2% [28]. Our findings are consistent with those of foreign authors [29].

Platelet adhesion occurs as a result of different charges existing between platelet surfaces and the surface of materials in contact with blood. Platelet adhesion is the initial stage of blood clot formation. However, adhesion alone is not enough to trigger thrombogenic reactions. Only activated platelets can release substances into the blood, which can lead to irreversible aggregation. Adhesion of platelets with regular round shape that have not yet been deformed does not carry a thrombogenic hazard, since adsorption of level I platelets is reversible and can be easily returned to the bloodstream [30]. Our results on assessment of adhesion properties suggest that platelets adhere to all polymers, regardless of type. Perhaps this is due to the potential difference between the surface of polymers and platelets [31].

A study of the effect of polymer surface on the level of platelet aggregation found that SIBS and ePTFE polymers do not increase platelet aggregation, unlike polyethylene. Going by obtained data suggesting high degree of blood aggregation after contact with polyethylene, it can be assumed that this polymer contains low molecular weight impurities that are irritating to blood platelets. This in turn leads to platelet activation and release of aggregation inducers. SIBS and ePTFE do not contain such impurities.

CONCLUSION

In terms of hemocompatibility properties, SIBS synthetic model polymer is not inferior to ePTFE used in clinical practice as a material for heart valve replacement. Moreover, experiments to determine the degree of calcification, cell adhesion and proliferation demonstrated that the studied SIBS polymer is superior. It can be concluded, therefore, that SIBS material has potential in development of new-generation polymeric prosthetic heart valves. Additional modification of the surface of the polymeric material in order to increase hydrophilicity would reduce its adhesive properties. The hemocompatibility of the model polymer can also be increased by modifying it with various antithrombotic agents, particularly heparin.

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TECHNIQUES FOR OBTAINING DERMAL EXTRACELLULAR MATRIX SCAFFOLD

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Despite advancements in modern surgery in the treatment of cutaneous injuries, the search for new methods of ensuring faster and more effective wound healing appears especially urgent today. Tissue engineering is undoubtedly of interest when it comes to developing such technologies. **Objective:** to determine the optimal protocol for obtaining a decellularized dermal matrix scaffold for subsequent development of tissue-engineered skin. **Materials and methods.** One Landrace piglet was used as the experimental animal. After preliminary skin treatment with dermatome, 0.3 cm thick samples were taken. Two decellularization protocols were considered: protocol No. 1 was based on the use of Triton X-100 and deoxycholate, protocol No. 2 was only based on deoxycholate. There were 5 processing cycles in total for the 2 protocols. After treatment, acellular matrix scaffolds were examined through histological analysis and quantitative determination of DNA concentration. Next, static recellarization of the matrix scaffolds was carried out with porcine dermal fibroblasts. After that, the matrix scaffolds were tested for cytotoxicity using XTT test and differential staining test to differentiate between live and dead cells. **Results.** Comparative analysis of the two protocols for porcine dermis decellularization showed that both protocols effectively remove cells and nuclear material, while maintaining the architectonics of the intercellular substance intact, since fibrous structures are not destroyed. But when assessing the biocompatibility of matrix scaffolds based on analysis of cell viability according to data obtained from XTT test and cell–matrix adhesion, the matrix scaffold processed under protocol No. 1, shows advantages. **Conclusion.** In this study, a decellularization protocol based on Triton X-100 and deoxycholate was noted. The results obtained mark the first stage towards developing a tissue-engineered skin.

Keywords: regenerative medicine, skin, decellularization, tissue-engineered graft.

INTRODUCTION

The skin is the largest organ in the human body and performs such several vital functions as barrier, immune, and sensory. It has the ability to self-regulation and possess a number of other features [1]. Loss of skin integrity due to injuries or diseases can lead to acute physiological imbalance and ultimately to significant disability or even death [2].

Skin damages are diverse and can be caused by burns, injuries or be caused by trophic disorders due to venous hypertension, arterial insufficiency, diabetes mellitus and other reasons that lead to ulceration of the skin. A number of hereditary diseases associated with a violation of the structural proteins of the epidermis or dermis can also cause the development of extensive skin wounds and chronic erosion [3].

As a rule, skin damages are associated with a number of complex biochemical processes aimed at wound healing. In superficial wounds, where defects are limited by the epidermis or upper dermal layer, regeneration of the epidermis only is needed, which leads to quick healing and minimal risk of scar formation. Nevertheless, in wounds that penetrate deeper through the dermal layer, with peeling the skin or subcutaneous fat, such complications as infection develop more often, and scars usually remain even after the wound has completely healed. Actually, the duration of the wound healing tends to vary in different individuals and depends on the varying severity of damage [4].

Therapeutic interventions to restore skin surface and functions are an important long-term direction of both traditional and translational medicine where several key achievements and clinical advantages have been noted in recent years [3]. Choosing the right wound healing strategy is critical to their successful closure. This choice determines the rate of healing of the wound surface, the likelihood of complications and scar formation. Currently, various methods are used to close wound defects. Cadaveric allografts, xenografts, synthetic materials are used as wound surface coatings. Autodermoplasty re-

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mains the gold standard in the treatment of most skin wounds.

Regenerative medicine is currently actively developing due to the integration of engineering disciplines with biological sciences [5]. The prospects of using cellular technologies, additional biological factors that are aimed at stimulating tissue regeneration are under consideration. Experimental work is underway to create a tissue-engineering full-fledged skin [6]. Such developments are quite exciting, and undoubtedly can be useful, especially in cases where there is a significant deficit of the skin and autodermoplasty is impossible.

The tissue-engineering approach to the creation of artificial organs involves the use of extracellular matrices, stem cells and biologically active substances. One of the methods for producing matrices is the decellularization of the native tissue or organ. The choice of the optimal method to produce an extracellular matrix is one of the main challenges for tissue engineering. In the present study, the comparative analysis of two protocols for the preparation of the acellular dermal matrix for the subsequent creation of tissue-engineering skin was performed.

MATERIALS AND METHODS

All the experiments were carried out in accordance with the Rules for the use of experimental animals (order of the USSR Ministry of Health No. 755 of 12.08.1972) and the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes (Strasbourg, 1986), after the approval of the research protocols by local ethics committee. The experiments were performed in the Central Research Laboratory of fundamental studies in the field of regenerative medicine of the Kuban State Medical University. The native dermis sampling took place under sterile operating conditions in the vivarium. The experimental animal was one Landrace breed porcine (age 12 weeks, weight 22 kg). After pretreatment of the skin with a dermatome, 0.3 cm thick dermis samples were taken.

Decellularization of the dermal matrix with subsequent assessment of the skin matrix quality

Two different processing protocols were proposed for the dermis decellularization. At the first stage, 2×1×0.3 cm samples were frozen at –80 °C, followed by 18 h treatment with Trypsin–Versene solution (1:1, Biolot, Russia) in a thermo-shaker-incubator at +37 °C. The next step was the processing of the matrix with detergent solutions on a rotating platform at 170 rpm. For protocol No. 1, an alternation of 1% solution of X100 triton (Sigma-Aldrich, USA) and 4% sodium deoxycholate solution (Sigma-Aldrich, USA) in combination with 0.002 M Na2-EDTA, 2 h each was used. There were five treatment cycles. The detergent treatment in Protocol No. 2 included only 4% sodium deoxycholate solution in combination with 0.002 M Na2-EDTA for 4 h, the number of cycles was also five. After each treatment cycle, the matrices were washed in deionized water for 30 minutes. The final processing stage in both protocols consisted of exposure to porcine pancreatic DNase I (Sigma-Aldrich, USA, 2000 IU was dissolved in 200 ml of phosphate-saline buffer Ca^{2+}/Mg^{2+}) for 4 h in a thermal shaker-incubator at +37 °C.

The obtained samples of native and decellularized dermis were fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin by the standard technique. 4 μm thick sections were obtained with a microtome. For a general histological evaluation of the preparations, the sections were hematoxylin and eosin stained (Sigma-Aldrich, USA). Cell nuclei were visualized using fluorophore (4',6-diamidino-2-phenylindole) DAPI (Sigma-Aldrich, USA). Micropreparations were studied with Olympus CX 41 microscope (Japan).

DNA content quantification was determined with NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., USA) with (Dneasy Blood and Tissue Kit, Qiagen, Sweden) reagent kit according to the manufacturer's protocol.

Recellularization of the dermal matrix followed by assessment of biocompatibility with cells *in vitro*

To assess the biocompatibility and the ability of the cells to adhere to the dermal matrix, the static recellularization of dermal fibroblasts isolated and cultured from porcine dermis was used. A standard enzymatic protocol based on 0.1% collagenase solution was used to isolate cells from the dermis. Cells were cultured using complete culture medium consisting of DMEM solution (Gibco), 10% fetal bovine serum (Gibco) and 1% solution of antibiotic-antimycotic (Gibco) in a CO_2 incubator. With the confluence achieved, up to 80% of the cells were subcultured with Trypsin solution (Biolot, Russia) and continued to cultivate until the 2nd passage. Then, the obtained number of cells was used for the XTT test and the test for differential staining of living and dead cells. An XTT reagent working solution was prepared in accordance with the manufacturer recommendations (Cell proliferation assay XTT, AphliChem GmbH, Germany). Initially, 0.3×0.3 cm samples of decellularized dermal matrices were placed in a 96-well plate, then the samples were seeded with fibroblasts suspension at the rate of 25,000 cells per scaffold with a pipettor. After 72 h of cultivation, 200 μl of XTT working solution was added to each well, with the incubation time of 4 h. The cells that were freely cultured in the plate were taken for a positive control. The results were evaluated with FilterMax F5

multifunctional reader (Molecular Devices, USA) under standard conditions at 450 nm wavelengths according to the preset protocols of the device manufacturer.

To visually assess the ability of the matrices to support cell growth and their cytotoxicity, the living and dead cells were stained differentially using fibroblasts for the decellularized scaffolds of the dermis with LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes, USA) with AM calcein and ethidium homodimer according to the manufacturer instructions. The micropreparations were studies with Olympus IX 51 fluorescence microscope (Japan).

Statistical analysis

The obtained material was statistically processed with MS Excel v6.0, GraphPadPrism version 6.04 (www.graphpad.com). The results were assessed by Student's t-test. The confidence interval was calculated according to the Student's distribution table. Significant differences correspond to $p < 0.05$.

RESULTS AND DISCUSSION

The detergent-enzymatic method was chosen for the decellularization of the dermis, which allows the most complete removal of cells from tissues, while time sparing the extracellular matrix fibers with respect to proteins. The difference in the composition of the protocols was dictated by the desire to reduce the time of exposure of tissues to sodium deoxycholate and to evaluate the difference in the effect of exposure in comparison with the protocol that uses the milder Triton X100 detergent on the extracellular matrix. At the start of finalizing the protocols, one of the reagents of choice at the preparatory stage was sodium dodecyl sulfate solution. However, the high concentration of the substance necessary for the destruction of the cells of the papillary dermis had a significant damaging effect on the extracellular matrix fibers. The inability to completely eliminate the active solution from the matrix led to significantly worse cell growth and to their death. The obtained data forced us to abandon the further use of sodium dodecyl sulfate solution, which corresponded to the literature data [7].

Also, at the preparatory stage, we encountered the dermis taken simultaneously with the epidermis was less susceptible to decellularization. Perhaps, this is due to the complexity of detergent penetration through the thick epithelial layer and, undoubtedly, the dense basement membrane [8]. To solve the arisen complexity of skin decellularization was to use Trypsin–Versene solution, which, according to the literature, effectively acts on squamous cells, removing them, while loosening the dermis itself and making it more susceptible to detergents.

Hematoxylin and eosin staining after each cycle of detergent treatment showed that the preserved cells and cell nuclei in the matrix were absent already after the 2nd treatment cycle in protocol No. 1 and after the 1st treatment cycle in protocol No. 2. However, a large number basophilically stained matrix filaments present in the sections and a high quantitative DNA content in tissues dictated the need to increase the number of treatment cycles to five in both cases (Fig. 1).

The trend was confirmed by the DAPI fluorophore staining of the nuclear material of the preparations. In the native dermis, cell nuclei actively fluoresced and were detected in large numbers. In decellularized samples, only a slight autofluorescence of extracellular matrix fibers was found after the fifth treatment cycle (the data not shown).

Determination of residual DNA content is an important stage in the evaluation of the resulting matrix after the performed decellularization. The development of adverse reactions on the part of the recipient's organism depends on the DNA presence, since many decellularized tissues are obtained from xeno- and allogeneic sources and there are reasons to concern that this DNA may be included in the recipient's cells. Quantitative

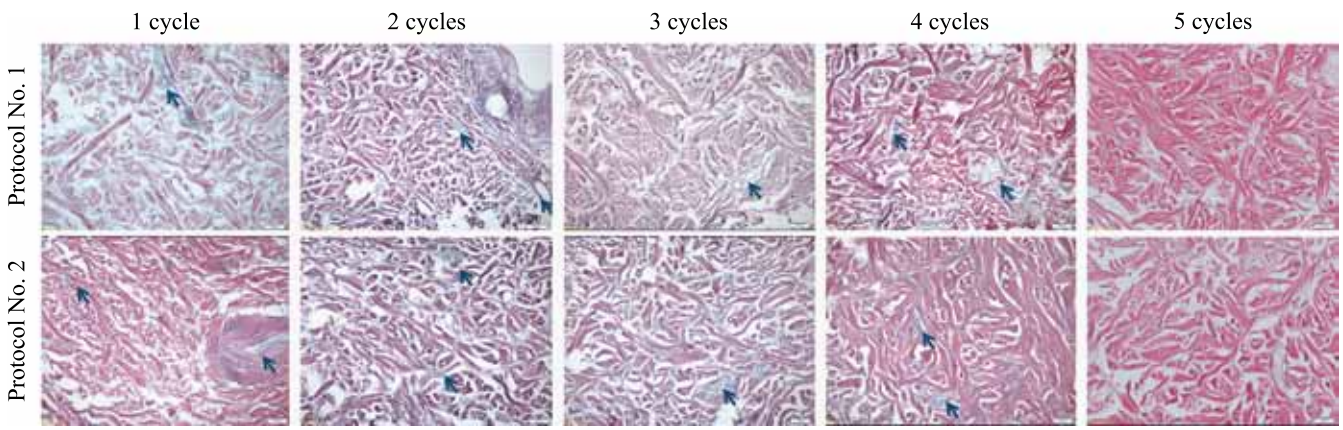

Fig. 1. Histological analysis. Dynamics of successive changes in the structure of the extracellular matrix during decellularization. Pointers indicate basophilically colored bands containing residual nuclear material. ×200

analysis showed that the DNA content in the decellularized dermis after the 5th treatment cycle decreased to 19.8% (65.4 ng/mg of the tissue) and 12.1% (40.1 ng/mg of the tissue) according to protocols Nos. 1 and 2, respectively, in comparison with the DNA content in the native dermis (330.4 ng/mg of the tissue). The obtained results testified to the effectiveness of decellularization, after which the matrix was largely ($p = 0.0011$) purified of nuclear material (Fig. 2).

After the main criteria for evaluating the effectiveness of decellularization proposed in the literature [9] were considered, a necessary condition for choosing the optimal protocol was to assess the effect of the resulting matrix on the cells after recellularization. The significance of this stage is that it allows to select the matrix that will best promote the adhesion and proliferation of cells while being the least toxic to tissues due to the possible preservation of residual detergents.

The XTT test after static matrix recellularization showed living, metabolically active cells in both test samples. However, cell viability was higher in the matrix decellularized according to the protocol No. 1 in contrast to the protocol No. 2, where viability was statistically lower ($p < 0.005$).

When comparing the samples from the experimental and control groups, it was found that the results of the XTT test in wells seeded with cells only differ from the experimental ones and significantly exceed them ($p < 0.001$), which is explained by the experimental conditions and the proven cells ability to easily attach to plastic. In general, when comparing all groups according to the XTT test results, it was found that the matrices

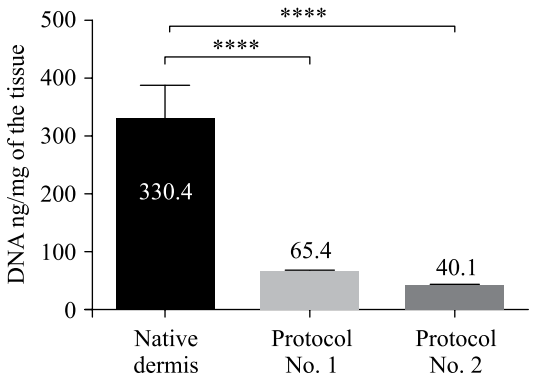

Fig. 2. Quantitative analysis of the DNA content in the native and decellularized porcine dermis

obtained according to protocols Nos. 1 and 2, seeded with cells, are biocompatible and not cytotoxic (Fig. 3).

Differential detection of living (calcein fluorescence) and dead (ethidium homodimer fluorescence) cells based on active calcein transport into living cells and passive ethidium homodimer transport into dead cells, allowed additional visualization of living cells at the static scaffold recellularization (Fig. 4). Cultured cells were found to retain their viability on both matrices obtained for 72 h. It was shown that 80 ± 10% of the cells remained viable on matrix No. 1 and 55 ± 10% on matrix No. 2. The formation of a monolayer of living cells during cultivation on the matrix obtained according to the protocol No. 1 was also noteworthy. These results showed not only the ability of cells to adhere, but also low cytotoxicity of the resulting scaffolds. The difference in results can be associated with a twofold difference in the time of treatment with sodium deoxycholate solution and, as

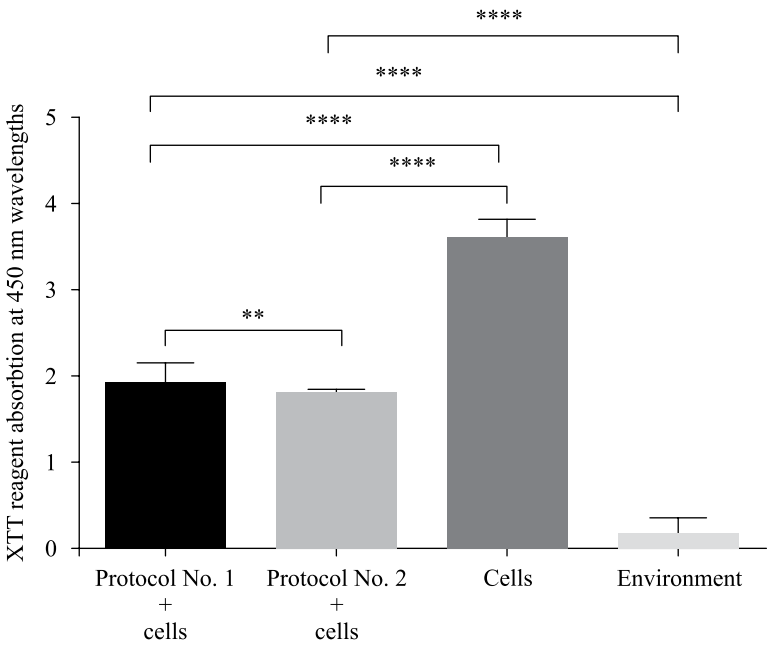

Fig. 3. Results of cytotoxic tests of biological matrix of porcine dermis. Optical density during the XTT test

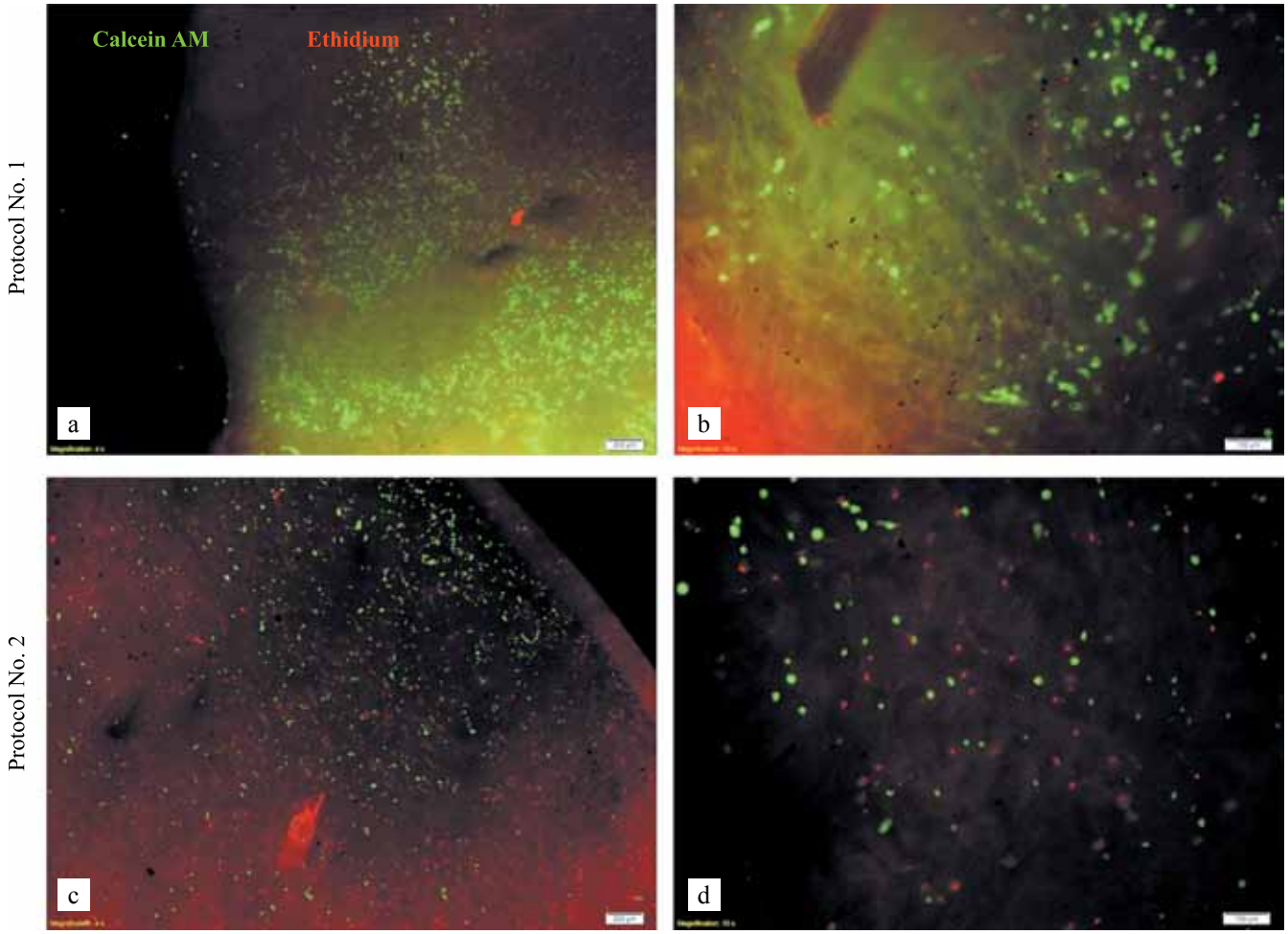

Fig. 4. Evaluation of the viability of fibroblasts settled on decellularized matrix of porcine dermis. Living cells – positive calcein AM (green) staining, dead ones – positive ethidium homodimer (red) staining. a, c – ×40; b, d – ×100

a result, its lower residual content in the matrix when the protocol No. 1 was implemented.

Thus, a comparative analysis of two protocols for porcine dermis decellularization showed that both protocols effectively remove cells and nuclear material, while the architectonics of the intercellular substance remains intact since fibrous structures are not destroyed. However, when assessing the biocompatibility of matrices based on the analysis of cell viability and their adhesion to the matrix, the matrix processed according to the protocol No. 1 had the advantages which made us note that the protocol based on the use of Triton X100 and deoxycholate is more promising for further research, considering Triton X100 is a softer detergent for decellularization.

CONCLUSION

The search for the optimal tissue decellularization protocol is fundamental in tissue engineering, which is primarily conditioned by the development of a matrix that would maximally correspond to the native extracellular matrix, at all levels biocompatible and actively functional. In the present study, the determination of the decellularization method and the initial characteristics of the resulting matrix is the first step for the further development of tissue-engineering skin.

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The authors declare no conflict of interest.

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INTERLEUKIN IL-1β STIMULATES REVITALIZATION OF CARTILAGE MATRIX *IN VITRO* WITH HUMAN NASAL CHONDROCYTES

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Revitalization of decellularized or devitalized matrix scaffolds in tracheal tissue engineering typically involves seeding the autologous recipient cells or allogeneic cells under long-term cultivation. **Objective:** to study the capability of human nasal chondrocytes for colonization of devitalized scaffolds based on native human tracheal cartilage, with proinflammatory stimulation (cytokine) by adding Interleukin-1-beta (IL-1β) to the culture medium. **Materials and methods.** Scaffolds for tracheal tissue engineering were obtained from native human tracheal cartilage through devitalization and laser etching. The scaffold was revitalized by seeding the human nasal chondrocytes. Histological examination was performed after staining with hematoxylin and safranin-O, with further microscopy using a Nikon Eclipse L200 light microscope. X-ray microtomography was performed on a Phoenix nanotom m apparatus. Electron microscopy was performed on a Nova NanoSEM 230 setup. **Results.** There was statistically significant increase in the intensity of colonization ($p = 0.0008$) with nasal chondrocytes and stimulation of their migration activity ($p < 0.0001$) in the presence of IL-1β compared with the control groups. **Conclusion.** Addition of proinflammatory cytokine IL-1β (1 μg/ml) to the culture medium enhances volumetric seeding of devitalized cartilage scaffold with human nasal chondrocytes, allowing to create highly revitalized materials for tracheal tissue engineering.

Keywords: vitalization, inflammation, interleukin-1-beta (IL-1β), cultured cells, laser etching, revitalization, tissue engineering, physiological relevance, chondrocytes, cartilage matrix.

INTRODUCTION

Obtaining tissue-engineering constructions involves the revitalization of decellularized host scaffolds before implantation through colonization with recultured cells [1, 2]. The immobilization of autologous recipient cells to the scaffold further reduces its immunogenicity and increases biocompatibility.

There are various ways to stimulate cell migration to the host scaffold, e.g. the use of chemoattractants and cell adhesion molecules at various concentrations and gradients, as well as dynamic cultivation in the bioreactor conditions [3, 4]. In this case, body reactions in response to implantation will affect the viability and differentiation of cells necessary for the maturation of physiologically compatible cartilage tissue [5].

Any surgical intervention is accompanied by aseptic inflammatory response, known as a surgical inflammation [6], and the implantation area of the finished tissue-engineering constructs is usually represented by inflamed tissues of the affected organ of the recipient. Moreover, the effect of factors and products of the inflammatory response on the behavior of populated cells after implantation of such a construct is difficult to predict; it remains an understudied area, which makes studying the effect of pro-inflammatory cytokines on the process of scaffold colonization *in vitro* quite promising.

To simulate inflammation *in vitro*, various pro-inflammatory factors, such as interleukins, tumor necrosis and interferons, can be added to the medium. Interleukin-1-beta (IL-1β) is known to inhibit the proliferation of rabbit articular chondrocytes [7]. IL-1β is also involved in the cellular regulation of chondrocytes, stimulating proliferation and inhibiting differentiation [8]. IL-1β effect on chondrogenesis of MMSC CM [9], the regulation of chondrocyte cytoskeleton components [10] and the protective physiological responses of chondrocytes during mechanical stimulation in the presence of IL-1β [11] has been shown, suggesting the effects in IL-1β that promote scaffold colonization with chondrocytes [12].

At present, it has been established that the natural IL-1β concentration in human physiological fluids reaches 200 to 300 ng/ml [13], while at inflammatory processes due to exogenous materials implantation, its value in interstitial fluid increases to 750 ng/ml or over, depending on the implantation site and individual characteristics of the recipient [13, 14]. Therefore, as a model medium, it was decided to use a solution of IL-1β at a concentration of 1 μg/ml, adopted physiologically relevant to the active inflammatory process at the implantation site.

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The ***purpose*** of the present study was to investigate the efficiency of colonization of devitalized scaffolds with nasal chondrocytes when adding IL-1β in a physiologically relevant inflammation concentration to the culture medium during scaffold revitalization in a static culture *in vitro*.

MATERIALS AND METHODS

Ethics Compliance

Donor tissues have been sampled in accordance with the scientific study protocol approved by the ethics committee of Sechenov University (decision No. 07-15 of July 15, 2015).

Preparing a tracheal scaffold based on natural human cartilage

Native human cartilage tissue devitalized through three freeze/thaw cycles and laser engraving with the formation of blind holes of 150–200 μm diameter and up to 300 μm depth was used as a scaffold. Laser engraving of material samples of 2×4 mm and 1 mm thickness was used to ensure bulk colonization.

To devitalize the native tissue, the cadaver donor trachea was separated in a laminar cabinet from the surrounding tissues, placed in a sterile PBS solution at room temperature and frozen for 15 minutes in liquid nitrogen. After freezing, the sample was thawed in a water bath at 37.5 °C for 30 minutes. After the cycle, the PBS solution was removed, the sample was washed 3 times with a sterile solution and placed in a PBS tube again. Then the freeze/thaw cycles were repeated 2 more times, and then the devitalized cartilage was separated from the perichondrium and cut into plates no more than 1 mm thick.

Next, the cartilage tissue was laser-engraved with BD-01 CO_2 laser at 10.6 μm wavelength. Laser engraving resulted in the blind holes formation of 150–200 microns diameter, up to 300 microns depth and the density of about 4 holes per 1 mm^2. The obtained samples were washed 3 times with a cold sterile PBS solution and sent for sterilization with gamma radiation by iridium-192 isotopes with 1.3 kGy absorbed dose.

Isolation and cultivation of chondrocytes for scaffold revitalization

The cartilaginous part of the human nasal septum was used as a source of chondrocytes for primary culture. The resulting cartilage of the nasoseptal septum was checked for bacterial and fungal contamination and then fermented with 0.15% Collagenase Type II solution for 12 h at 37 °C with 5% CO_2. After that, the fermented cartilage was filtered with a sterile strainer of 100 μm pore size and the filtrate was centrifuged for 4 minutes at 1,300 rpm. Cells in the obtained suspension of chondrocytes were counted, placed in a culture dish and cultured for 21 days until the first passage at 37 °C of 5% CO_2 in DMEM medium (Gibco, USA) with the addition of 5% FBS, TGF and FGF-2, changing the medium every third day. Next, the nasal chondrocytes of the first passage were cryopreserved and stored in a cryobank at –196 °C.

Scaffold revitalization with human nasal chondrocytes

To revitalize the scaffolds, human nasal chondrocytes of the second passage were used, cultured from previously cryopreserved cells of the first passage. Cell expansion was performed in DMEM medium with a high glucose content (4.5 g/L) and 5% FBS, TGF and FGF-2 at 37 °C 5% CO_2 with the medium changed every third day.

The scaffolds to be revitalized (n = 6) were laid on semipermeable membranes of Transwell plates (Corning, USA) and colonized with cells at the rate of 0.25 million nasal chondrocytes per 1 mm^2 of the scaffold surface. All samples thus revitalized were cultured under incubator conditions at 37 °C and 5% CO_2. The complete DMEM medium with a high glucose content (4.5 g/l) and the addition of 5% FBS, insulin and ascorbic acid was used as a nutrient medium.

After the first day of cultivation, all samples were divided into control (n = 3) and experimental (n = 3) groups. The culturing conditions of the control group samples remained unchanged until the end of the experiment. For the experimental group samples, the proinflammatory cytokine of human recombinant interleukin IL-1β (Sigma, art. SRP3083) was added to the culture medium at 1 μg/ml concentration. All samples were removed for histological examination after the next 6 days of cultivation.

Histological examination and X-ray microtomography

Histological examination was performed after all samples were preserved in 4% formaldehyde solution for 24 h, dehydrated with ascending ethanol concentrations and paraffinized by a standard protocol. 5 μm thick sections were sequentially hematoxylin and safranin-O stained to visualize the state of intercellular substance and populated cells. Safranin-O stains cartilaginous glycosaminoglycans in red orange, thus making it possible to qualitatively assess the saturation of cartilage tissue by color intensity [15].

For a comparative qualitative and quantitative assessment of scaffold revitalization, an original four-point scale (table) was developed based on the principles of the Bern score for evaluating pellet cultures of cartilage cells [16].

With the original scale for an independent assessment of three regions for each sample, the degree of colonization of the wells and the degree of destruction of the microarchitectonics of the scaffold were evaluated when

Table

The original four-point scale for quantitative evaluation of scaffold revitalization

Category	Value
A. Well colonization	
Complete absence of cells or single cells	0
Parietal colonization	1
Multilayer colonization with free space in wells	2
Total colonization (no visible space in wells)	3
B. Cell migration	
The shape and boundaries of the pores are clearly visible while maintaining the distance between wells	0
Single local well merging	1
Multiple well merging	2
Complete fusion of wells (no visible boundaries of individual wells)	3

the cells migrated into the scaffold thickness outside the wells.

Histological studies were performed at the Institute of Surgical Research, University of Basel, using with microtomes (Thermo Fisher Scientific, USA) and Nikon Eclipse L200 light microscope (Nikon, Japan).

Surface colonization of the samples by nasal chondrocytes was evaluated by scanning electron microscopy with gold sputtering (Nova NanoSEM 230, FEI, USA).

X-ray microtomography of tissue-engineering structures for assessing the preservation of wells in the 3D structure of the sample after revitalization was made with Phoenix nanotom (General Electric, USA).

Statistical data processing

Statistical data processing was performed using the Mann–Whitney U test with the GraphPad Prism 7 software (GraphPad Software, Inc). Differences were considered significant at $p < 0.05$.

RESULTS

Scanning electron microscopy of tracheal cartilage tissue samples after laser engraving and revitalization allowed visualizing a dense coating of the sample surface with nasal chondrocyte cells and a loose intercellular substance synthesized *de novo* (Fig. 1).

Microcomputer tomography of host scaffolds after revitalization allowed identifying the location and confirm the integrity of the wells in the sample structure in the control group; however, the microtomograph resolution was insufficient for imaging cells. The histological examination was the most informative, revealing a high degree of colonization of the wells. The results com-

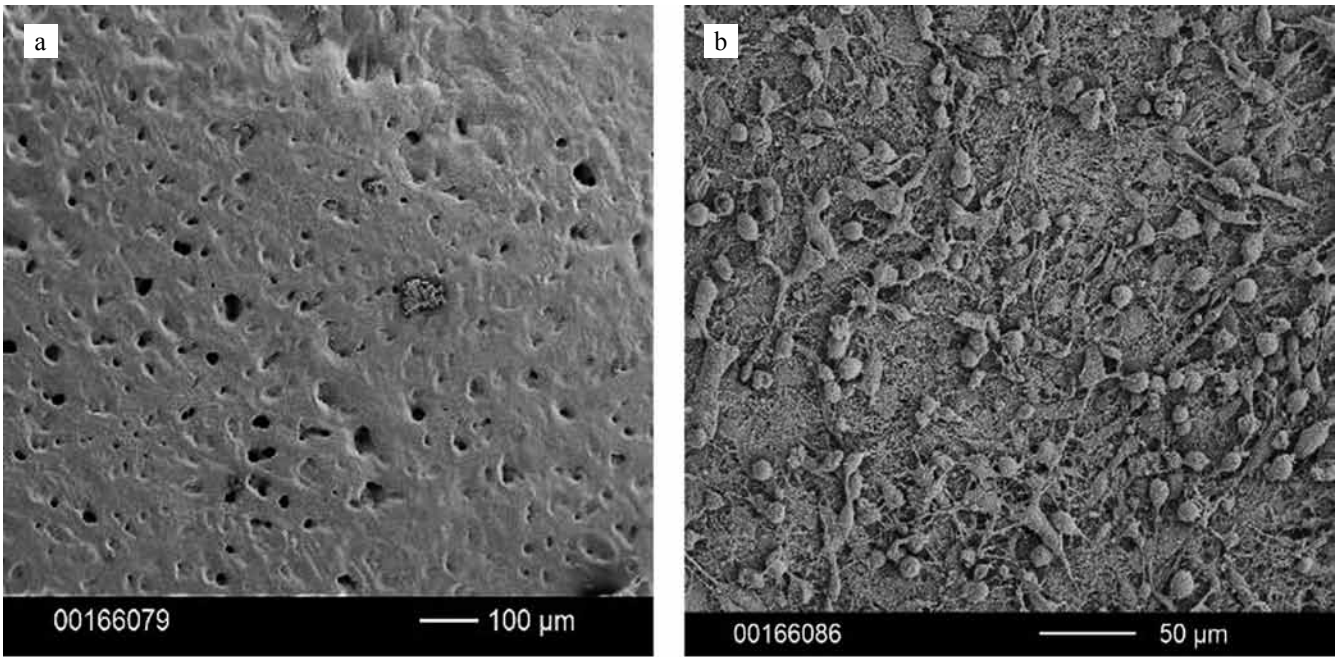

Fig. 1. High-density scaffold prior to laser engraving: a – devitalized scaffold: empty lacunas surrounded by high-dense extracellular scaffold are visible on the cartilage surface, b – laser-engraved scaffold revitalized by nasal chondrocytes, densely colonizing the surface of the material. Scanning electron microscopy

parison of histological examination and microcomputer tomography for samples of the control group is shown in Fig. 2.

The exposure to IL-1β during revitalization was shown to lead to a denser and more intensive colonization of the wells. At the same time, the migration of cells beyond the depths of the wells deep into the scaffold led to a pronounced wells and channels fusion and ultimately to the destruction of the microarchitectonics of the tissue-engineering structure (Fig. 3).

The results of a quantitative assessment of the scaffold colonization intensity and cell migration beyond the wells on the original four-point scale for structured experimental samples (when exposed to IL-1β) and the control group after colonization with nasal chondrocytes are shown in Fig. 4.

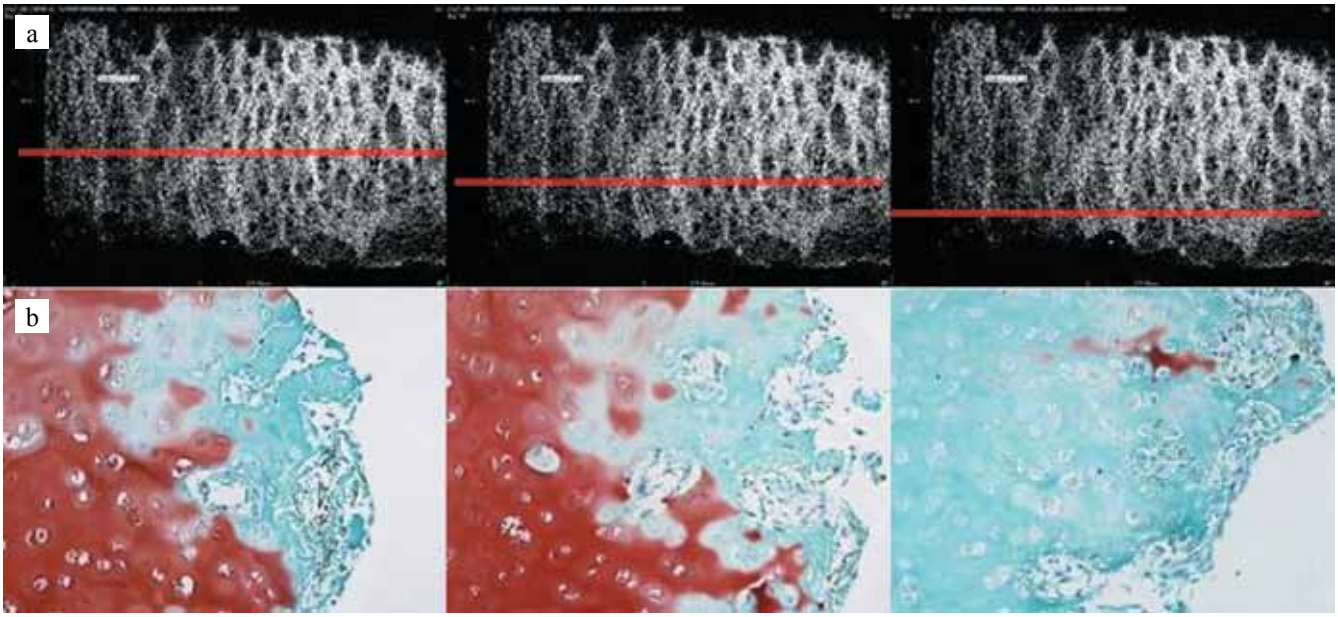

Fig. 2. Tissue-engineered graft generated by revitalization of the laser engraved tracheal cartilage with human nasal chondrocytes: a – tissue engineered graft, X-ray microtomography, red lines showing the cross-section levels; b – histological examination of transects (perpendicular to the direction of engraving). Safranin-O & Hematoxylin staining. Light microscopy, ×100

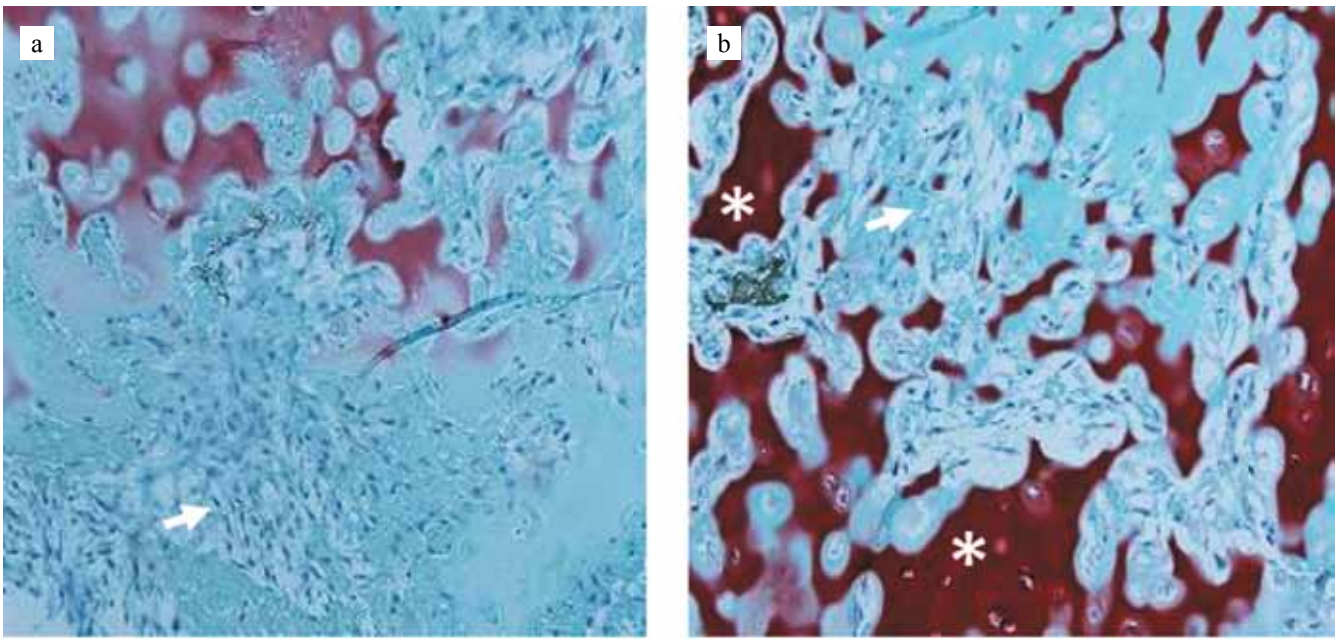

Fig. 3. Tissue-engineered graft obtained by revitalization of the laser engraved scaffold with human nasal chondrocytes under continuous exposure to IL-1β in the culture medium: the cartilage scaffold marked by asterisks; the nasal chondrocytes marked by arrows. Histological study of transverse sections (perpendicular to the direction of the engraving). Safranin-O & Hematoxylin staining. Light microscopy, ×100

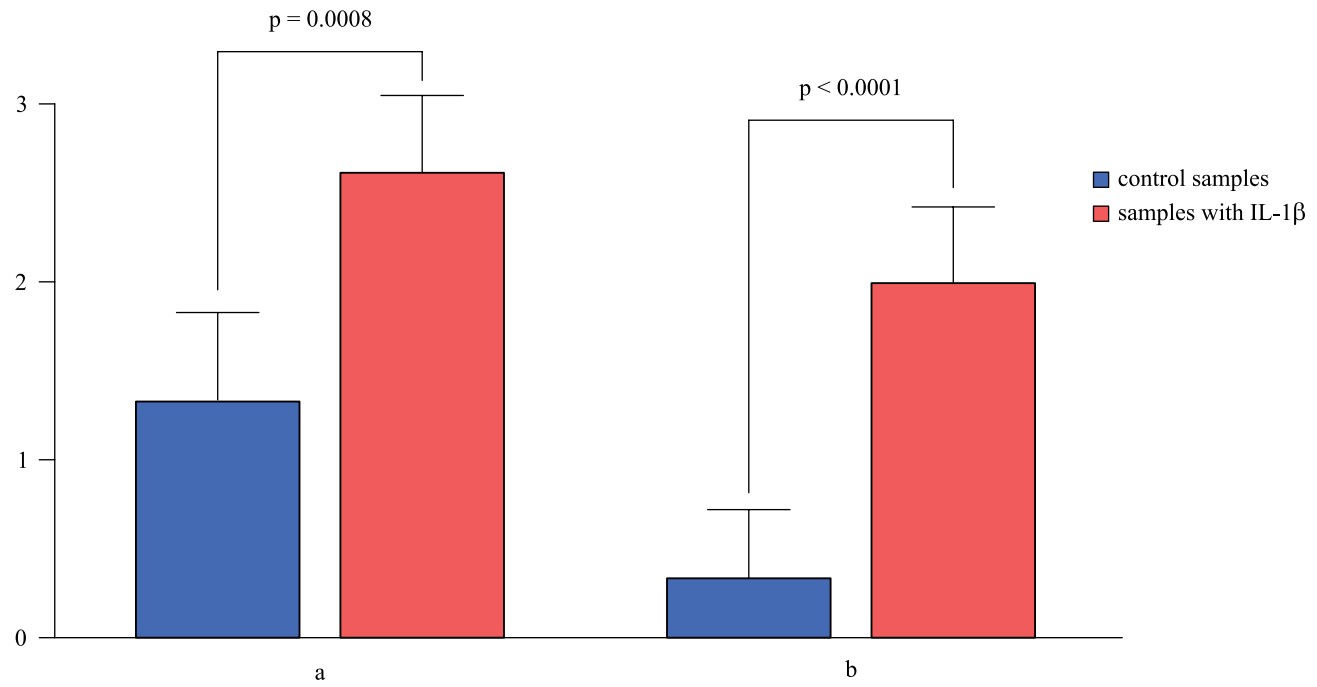

Fig. 4. Assessment of the values of intensity of scaffold colonization with human nasal chondrocytes (a) and migration of nasal chondrocytes outside the lacunae (b) under control conditions and in the presence of IL-1β. Values are means ± SD

Statistically significant differences in the intensity of colonization by nasal chondrocytes ($p = 0{,}0008$) and their migration beyond the borders of the wells ($p < 0{,}0001$) in the presence of IL-1β compared with control groups are shown.

DISCUSSION

Earlier, our experiments have showed that increasing the population density to 0.5 million cells per 1 mm^2 did not increase the density of colonization of the wells but increased the number of cells remaining on the surface of the sample. However, a lower population density does not allow to colonize all available wells in the allotted time period (up to 7 days). Besides, the use of dynamic cultivation under bioreactor conditions does not always allow volumetric population of the material *in vitro*.

It was shown that the appearance of a pro-inflammatory cytokine in the culture medium makes it possible to increase the density of the revitalization of the host scaffold, though having an ambiguous effect on the quality of the formed tissue-engineering structure. The addition of IL-1β qualitatively improves the colonization of the scaffold wells with chondrocytes, increases the population density of the construct by recipient cells, among other reasons due to increased migration of cells into the intercellular substance, which is well correlated with published data on the role of IL-1β in the regulation of cell proliferation, differentiation and apoptosis [17, 18]. A chronic aseptic proliferative (productive) inflammatory process in the field of implantation of a revitalized tissue-engineering construct can be assumed to also further contribute to the colonization of the scaffold by previously implanted recipient cells. At the same time, histological studies demonstrate partial or almost complete destruction of the microarchitectonics of the scaffold at its colonization under the influence of IL-1β: the boundaries of individual wells are erased, and the volume of intercellular substance decreases. The latter may adversely affect the mechanical characteristics of the tissue engineering structure. The effect may result from the increased synthesis of metalloproteinases by chondrocytes under the action of IL-1β [19]. Comparative studies of the mechanical characteristics of the finished structure may be the subject of further research.

Thus, the possibility of effective revitalization of laser engraved scaffolds with the colonization of scaffold wells by human nasal chondrocytes while maintaining the bulk of the initial intercellular substance of cartilage tissue was shown. It was proved that the addition of the pro-inflammatory cytokine IL-1β to the culture medium stimulates the colonization of the host scaffold with chondrocytes, being one of the necessary conditions for obtaining complete revitalized materials intended for tissue engineering of the trachea.

CONCLUSION

The addition of the pro-inflammatory cytokine IL-1β in in concentration physiologically relevant to inflammation (1 μg/ml) to a complete DMEM nutrient medium with a high glucose content of 4.5 g/l, 5% FBS, insulin and ascorbic acid, promotes volumetric colonization of the cartilage-based devitalized scaffold on nasal chondrocytes tracheal tissue, but at the same time it can cause a violation

of the complex microarchitectonics of the natural intercellular substance of cartilage. Stimulation of the population of the native scaffold cells can be used to produce highly effective revitalized materials for tissue engineering of the trachea.

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The authors declare no conflict of interest.

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EXPERIMENTAL ORTHOTOPIC IMPLANTATION OF TISSUE-ENGINEERED TRACHEAL GRAFT CREATED BASED ON DEVITALIZED SCAFFOLD SEEDED WITH MESENCHYMAL AND EPITHELIAL CELLS

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Objective: to study the viability of a tissue-engineered graft (TEG) based on a devitalized tracheal scaffold (DTS) seeded with mesenchymal stromal and epithelial cells in an experiment on rabbits with assessment of cytocompatibility and biocompatibility *in vivo*. **Materials and methods.** Syngeneic mesenchymal stromal bone marrow cells (MSBMCs) and syngeneic lung epithelial cells of rabbit were obtained. The morphology and phenotype of the MSBMC culture were confirmed via immunofluorescence staining for CD90 and CD271 markers. Pulmonary epithelial cells obtained by enzymatic treatment of minced rabbit lung tissue were stained with CKPan, CK8/18 and CK14 markers characteristic of epithelial cells. The donor trachea was devitalized in three successive freeze-thawing cycles. Double-layer cell seeding of DTS was performed under static and dynamic culturing. Orthotopic implantation of TEGs was performed at the site of the anterolateral wall defect in the rabbit that was formed as a result of tracheal resection over four rings. Results were evaluated by computed tomography, histological and immunohistochemical analyzes. **Results.** A TEG implant, based on DTS, with bilayer colonization by cell cultures of rabbit MSBMC and epithelial cells was obtained. Three months after implantation, TEG engraftment was noted, no tracheal wall stenosis was observed. However, slight narrowing of the lumen in the implantation site was noted. Six months after implantation, viability of TEG was confirmed by histological method. Epithelialization and vascularization of the tracheal wall, absence of signs of purulent inflammation and aseptic necrosis were shown. The small narrowing of the lumen of trachea was found to have been caused by chronic inflammation due to irritation of the mucous membrane with suture material. **Conclusion.** A new model for assessing the viability of a tissue engineering implant when closing a critical airway defect was created. The developed TEG – based on DTS seeded (bilayer) by lung epithelial cells and BMSCs – was successfully used to replace non-extended tracheal defects in an *in vivo* experiment. The use of tracheal tissue-engineered graft for orthotopic implantation showed biocompatibility with minimal tissue response.

Keywords: devitalization, implant, cell- and tissue-based therapy, tissue engineering, tissue therapy, thoracic surgery, transplant, trachea.

INTRODUCTION

Chronic trachea stenosis caused by oncological and non-oncological processes, is usually difficult to treat when widely spread. The frequency of stenotic tracheal lesions is up to 1% of all intubations [1] and reaches 20–25% with prolonged intubation [2]. Endoscopic stenting with porous endoprostheses does not solve the problem due to the germination of the mesh structure of the stent by the patient's own tissues or the formation of marginal restenosis. Radical surgeries like the circular resection of the affected area is significantly limited by, for example, the extent of the defect, and also carry a high risk of complications [3]. The most promising technique for treating such defects may be the use of tissue-engineering grafts (TEGs). However, the clinical use of tissue-engineering structures of the trachea is accompanied by many problems, such as the need to ensure sufficient blood supply, the formation of a respiratory epithelium and preventing softening of the tracheal wall [4, 5].

At present, an acellular scaffold of the tracheal cartilage was obtained in the experiment under the combined action of detergents and DNase I [6], the airway was recellularized by seeding mesenchymal stromal cells (MSC) [7], and the stimulating effect of the bone marrow mesenchymal stem cells (BM MSC) was shown for epithelialization and formation of cartilage tissue [8]. The combination of these techniques was used to obtain the trachea TEG.

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Go et al. (2010) evaluated the effect of epithelial cells and chondrocytes obtained during differentiation from MSC on the effectiveness of TEG in airway regeneration in porcines [9]. The scaffolds were obtained by detergent-enzymatic decellularization of allogeneic trachea. Autologous MSCs were obtained from bone marrow and differentiated into chondrocytes. Autologous epithelial cells were isolated from the trachea mucous membrane. Both types of cells were plated on a scaffold using a two-chamber bioreactor under dynamic cultivation. Substitution of a 6 cm trachea was performed by TEG engraftment. The seeding of scaffold with epithelium and chondrocytes obtained from MSC was shown to lead to tissue regeneration of the tracheal wall.

In Jungebluth et al. (2012) TEG was studied on the basis of porcine decellularized trachea seeded by autologous mononuclear cells from the bone marrow from the outside and epithelial cells from a biopsy of the oral mucosa from the inside [10]. The resulting grafts were implanted into pigs in a 6 cm tracheal defect. By the 60th day after engraftment, there were no differences in mechanical properties compared to native tissue, and the inner surface of the trachea was lined with epithelium, without signs of the graft rejection or any pronounced inflammatory response.

To repair the rabbit tracheal defect, Shin et al. (2015) used BM MSC – based TEGs and a scaffold formed from crushed decellularized articular porcine cartilage [11]. Histological examination showed that cartilage was formed with minimal inflammation or tissue granulation.

However, the viability of TEG based on a devitalized tracheal wall with a large residual content of exogenous markers at seeding of such a scaffold with a combination of two cell types was not previously evaluated in *in vivo* experiments. Besides, the literature does not describe the use of a full-fledged experimental model to assess the viability of a tissue-engineering graft when closing critical airway defects which would allow drawing conclusions about the biological and physiological compatibility and stability of the used design.

Purpose. To study the viability of a tissue engineering graft (TEG) based on devitalized tracheal scaffold (DTS) seeded by mesenchymal stromal and epithelial cells, on a model for assessing the viability of a tissue engineering graft at closing a critical airway defect in rabbits and to assess the TEG potential to maintain a stable trachea lumen in the area of engraftment.

MATERIALS AND METHODS

Laboratory animals

The experiments were performed on linear Gray Giant breed rabbits (n = 3) with 3.5–4.5 kg initial body weight, 0.5–1 years old, grown in the nursery of the Biomedical Technologies Research Center of the Federal Biomedical Agency of Russia (Svetlyie Gory settlement, Moscow Oblast). The animals were kept in the Central Vivarium of the I.M. Sechenov First Moscow State Medical University of the Ministry of Healthcare of the Russian Federation with free access to food and water.

All manipulations with laboratory animals were approved by the Local Ethics Committee of the FGAOU VO I.M. Sechenov First Moscow State Medical University of the Ministry of Healthcare of the Russian Federation (Sechenovsky University) and carried out in compliance with the Rules of bioethics approved by the European Convention for the Protection of Vertebrate Animals used for experiments or other scientific purposes (2005), and in accordance with the Laboratory Practice Rules approved by Order of the Ministry of Health of Russia No. 708 of 23.08.2010.

BM MSC culture

BM MSC was obtained in accordance with the protocol used in the CCP "Regenerative Medicine" of the Sechenovsky University. Syngeneic rabbit MSCs were isolated from bone marrow obtained by the femur perfusion. A bone marrow aspirate was placed in a sterile tube containing 50 IU/ml heparin and 0.25 mg/l gentamicin in PBS and delivered to the laboratory at +20–24 °C. Cells isolation and handling were carried out in the 5th grade clean zone according to ISO. A 10 ml bone marrow aspirate was placed in a centrifuge tube and precipitated at 350 g for 5 min. The supernatant was removed, the cell pellet was resuspended in 20 ml of lysis buffer (114 mM NH4Cl, 7.5 mM KHCO3, 0.1 mM EDTA) for 3–5 min, then centrifuged again; the cell pellet was resuspended in BM MSC culture medium based on DMEM/F12 culture medium (Invitrogen, USA) containing 10% FBS (Invitrogen, USA), 0.4 μM insulin, 20 ng/ml bFGF, 10 nM dexamethasone, 100 u/ml penicillin and 100 μg/ml streptomycin (Invitrogen, USA). The cell suspension was seeded in an amount of 1.0–1.5 × 10^6 cells/ml in culture flasks and placed in a CO_2 incubator with a 5% CO_2, atmospheric air 95% at +37 °C with high humidity. The culture medium was changed every 72 h. When 90% confluency was reached, the cells were washed with DPBS without Ca^{2+} and Mg^{2+} ions, then removed from the culture plastic with TrypLe solution (Invitrogen, USA), centrifuged at 350 g for 5 min; the supernatant was taken and the precipitate resuspended in a nutrient medium, and $^1/_3$ of the cell suspension was placed on a new culture dish.

Epithelial cell culture

Syngeneic epithelial cells were isolated from a native rabbit lung that was washed from the blood components by prolonged perfusion of the pulmonary circulation with

physiological saline with a continuous supply of the latter into the right heart ventricle of a donor rabbit with a roller pump. The purified lung tissue was cleaned to white. The lung was mobilized, separated from the surrounding tissues and, after vascular junction, was placed in a container with a transport medium of DMEM/F12 with the addition of 100 U/ml penicillin and 100 μg/ml streptomycin, and delivered to the laboratory at +20…24 °C. In a clean zone, the lung was crushed into 4 = 2 mm^3 pieces and treated with a dispase solution of 5 Г/ml for 45 min at +37 °C. The resulting suspension was filtered through a 70 μm sieve to separate lung tissue fragments from the cell suspension. The filtrate was centrifuged at 1200 rpm and the pellet was resuspended in complete KSFM culture medium (Invitrogen, USA) with the complement of Keratinocytes Supplements (Invitrogen, USA), 100 U/ ml penicillin and 100 μg/ml streptomycin (Invitrogen, USA). The resulting suspension was seeded to the bottom of a 24-well culture plate previously coated with type I collagen. The cells were cultured under conditions of CO_2 incubator with the culture medium replaced every 72 h.

Assessment of BM MSC phenotype and tracheal epithelium

The preparation of BM MSC cultures and rabbit tracheal epithelium was confirmed by TIPS with antibodies: CD90, CD271, CDH, SOX2, COL1, Vimentin for BM MSC and CK8/18, CK14, CKPan for epithelium, respectively. For this, BM MSC of the first passage and the zero-passage tracheal epithelium was washed three times in PBS and fixed in 4% paraformaldehyde solution in PBS (pH 7.4) for 10 min at 25 °C. Then, the cells were washed three times in PBS with 0.05% Tween 20 (PBS-T) for 5 min, permeabilized with methanol at –20 °C, and nonspecific antibody binding was blocked with a solution of 2% BSA in PBS-T for 20 min at +25 °C. The solution was removed and the primary monoclonal antibodies were applied at a dilution of 1:100 anti-CDH, anti-COL2, anti-CD90, anti-SOX2, anti-Vimentin for MSC culture and anti-CK8/18, anti-CK14, anti-CKPan for culture tracheal epithelial cells in a solution of 2% BSA in PBS-T and incubated for 12 h at +4 °C. Then the cells were washed three times in PBS for 5 min and secondary polyclonal antibodies at a dilution of 1:500 was applied conjugated with biotin to primary antibodies CD90, CD271, SOX2, CKPan, diluted in PBS-T with the addition of 2% BSA. Incubation was carried out for 60 min at +25 °C. Then the cells were again washed with PBS three times for 5 min and applied to PBS-T with the addition of 2% BSA: streptavidin-Alexa Fluor 488 to secondary polyclonal antibodies conjugated with biotin; secondary polyclonal antibodies Alexa Fluor 594 to primary antibodies CDH, Vimentin and biotinylated antibodies to CD90, CD271, SOX2, CKPan, CK-8/18, CK-14. As a negative control of secondary antibodies to the test cultures, no primary antibodies were added. Incubation was carried out for 30 min at +25 °C in the dark. Cells were washed with PBS three times for 5 min. The cores were stained with Hoechst-33342 at 1 μg/ml.

Rabbit trachea devitalization

A donor rabbit trachea sample isolated from the second tracheal ring to the bifurcation was used as a native material to obtain a cell-free host scaffold. By devitalization we understand the death of living cells in tissue while maintaining cellular contents and intercellular scaffold in the material. Prior to devitalization, the trachea sample was cleaned of surrounding tissues and washed with PBS buffer to remove blood clots. Then the sample was placed in a test tube with PBS buffer and frozen for 15 min in liquid nitrogen. After freezing, the samples were thawed in a water bath at +37 °C for 30 min, the PBS buffer was replaced, and then the freeze/thaw cycles were repeated twice. The process of devitalization was completed by washing the trachea samples in a 70% solution of ethyl alcohol for 1.5 h. Then the samples were placed in PBS with the addition of 100 units/ml penicillin and 100 μg/ml streptomycin for 72 h at +4 °C to remove residual amounts of ethyl alcohol and prevent the possibility of contamination.

Scaffold devitalization quality assessment

To qualitatively assess devitalization, the DTS samples were fixed in 10% formalin in PBS, washed with running water for one hour, dehydrated (Microm STR 120), and enclosed in paraffin blocks according to the standard protocol. 4 microns thick slices (Microm HM 355s) were made from the blocks, placed on uncoated glass slides, then the sections were adhered at +56 °C for 30 min. The resulting sections were dewaxed, hematoxylin and eosin stained, dehydrated and clarified in xylene (Microm HMS 70). Sections were put under coverslips in a synthetic mounting medium. The resulting preparations were digitized with Pannoramic DESK followed by a morphological study.

DTS cytotoxicity assessment

DTS cytotoxicity was evaluated by extraction on a rabbit BM MSC culture. Rabbit DTS extracts were prepared as follows: rabbit DTS samples with 3 cm^2 surface area were placed in 1 ml of BM MSC culture medium and incubated for 72 h at +37 °C. The fourth passage BM MSC were seeded in a 96-well plate at a concentration of 5×10^3 cells/well and cultured in a CO_2 incubator. Cells were counted with Countess II FL Automated Cell Counter. In 24 h, obtained DTS extracts were added to

the cells, titrated six times by successive dilutions (n = 3) in BM MSC growth medium, and cultured for 48 h.

The cytotoxicity of DTS extracts was assessed by evaluating the metabolic activity of cells with PrestoBlue reagent [12]. To conduct a cytotoxicity test, after 48 h of cultivation, the medium was changed to 90 µl/well of HBSS with Ca^{2+} and Mg^{2+} adding PrestoBlue (10 µl/well) and incubated for 30 min in a CO_2 incubator. Then, to stop the reaction, SDS solution was added to 1% final concentration in the well. The absorption was measured at 540 nm wavelength with 630 nm reference by Multiskan FC flatbed photometer. As a control, BM MSC was used in a nutrient medium without additives (n = 3). The background value was considered by adding a reagent to HBSS with Ca^{2+} and Mg^{2+} (n = 3).

TEG creation by seeding of cell cultures

TEG was produced through sequential seeding mesenchymal cells over the entire surface of rabbit DTS samples and epithelial cells of the DTS inner surface. For this, the second passage rabbit BM MSC were seeded on the surface of tracheal samples at a rotation of 3–4 rpm for 48 h at a concentration of 0.25 million cells in 10 µl of BM MSC culture medium per 1 mm^2 of DTS surface. Then, 24 h before engraftment, the inner TEG surface was seeded with epithelial cells of the rabbit trachea of the first passage at 60 × 103 cells/cm^2 and cultivated by the static method in complete KSFM nutrient medium.

Assessment of the viability of cells seeded on DTS

The confocal microscopy with TIPS of cells and the Live/Dead technique was implied to determine the viability of BM MSCs seeded on the DTS surface. For this, the cells seeded on DTS were calcein-AM and ethidium homodimer solutions stained in an incubator for 20 min in a culture medium for BM MSC cultivation. Visualization was performed by Zeiss LSM 710 confocal microscope with Plan-Apochromat 10x/0.45 M27 lens.

TEG orthotopic engraftment

Surgery was performed in aseptic conditions under combined anesthesia (intramuscular injection of a combination of Xila preparations (0.1 ml/kg) and Zoletil-100 (0.1 ml/kg) supplemented with local anesthesia of the surgical field with a 0.5% novocaine solution. For orthotopic engraftment, TEGs received access to the cervical trachea through a longitudinal section along the midline of the neck up to 1 cm long dividing the muscles and fascia of the neck by blunt and sharp dissections. The front and side walls of the trachea were mobilized by blunt dissection. The anterolateral tracheal wall was resected over four rings below the second tracheal ring to form a window. The TEG sizes were corrected intraoperatively for the defect size, fixed in the engraftment area with the inert non-resorbable Proline 6-0 material with the closure of the anterolateral wall defect, the tightness of the tracheal suture being checked, and the wound sutured in layers. The animal was monitored until anesthesia withdrawal and antibiotic therapy for 5 days after the operation. Ketanov (30 mg/ml) was administered 0.5 ml per day.

MSCT

In three months after TEG engraftment, multispiral computed tomography (MSCT) under sedation (intramuscular injection of tiletamine and zolazepam solutions) was performed for an intermediate assessment of TEG engraftment and tracheal lumen in the engraftment area. CT was performed on Siemens Magnetom Verio tomograph with DICOM imaging. To visually assess the condition of the airways and the trachea lumen, a 3D model was created of the airways in STL format by the volumetric segmentation in 3D Slicer.

Morphological analysis

Animals were removed from the experiment after 6 months with Xila (1.0 ml per 1 kg of animal body weight). Autopsy material was taken within the boundaries of the intact trachea, at least 5 mm from the edges of the implant. The trachea with the TEG area was removed and cut into two parts lengthwise in the sagittal projection.

To morphologically evaluate the results of TEG engraftment, the trachea samples were fixed in a 10% neutral buffered formalin solution for 24 h, histologically prepared and enclosed in paraffin blocks by the standard protocol. 4 µm thick slices (Microm HM 355s) were placed on slides and glasses coated with poly-L-lysine, and then adhered at +56 °C for 30 min. The obtained sections were deparaffinized, hematoxylin and eosin stained and placed under coverslips in a mounting medium (Shandon-Mount). The resulting preparations were digitized with Pannoramic DESK, and then analyzed morphologically.

The sections coated with poly-L-lysine were prepared for TIPS to further confirm TEG epithelization. For this, dewaxing was first performed and then the antigen was unmasked in Tris-EDTA (pH 10.0) buffer in a microwave oven at 100 W for 10 min, cooled to room temperature and washed in PBS. Blocked the non-specific binding of antibodies with a solution of 2% BSA in PBS-T for 20 min at +25 °C. The solution was removed and the primary anti-CDH, anti-CKPan and anti-Ki67 monoclonal antibodies were applied in 2% BSA solution in PBS-T and incubated for 12 h at +4 °C. Then the cells

were washed three times in PBS for 5 min and secondary polyclonal antibodies applied with biotin to primary CDH, CKPan antibodies, diluted in PBS-T with the addition of 2% BSA. Incubation was carried out for 30 min at +3 °C. The cells were again washed with PBS three times for 5 min and PBS-T was applied with the addition of 2% BSA: streptavidin-Alexa Fluor 488 to secondary polyclonal antibodies with biotin; secondary polyclonal antibodies Alexa Fluor 594 to Ki67 primary antibodies. The sections without addition of primary antibodies were used as a negative control. Incubation was carried out for 30 min at +32 °C. the sections were washed with PBS three times for 5 min. The cores were Hoechst-33342 stained in 1 μg/ml. The preparations were enclosed under coverslip in 80% glycerol in PBS. Microscopy was performed with Nikon Eclipse TE-2000 fluorescence microscope.

Statistical analysis of results

Statistical analysis was performed in GraphPad Prism version 8.0 with a Gaussian distribution test with Shapiro–Wilk's test and the D'Agostino and Pearson omnibus normality test. The differences between the control group without the extract addition and the analyzed dilutions were checked by One-way ANOVA test and post hoc Dunnett test. Differences was considered significant at $p < 0.05$.

RESULTS

Phenotyping primary cell culture

At BM MSC TIPS it was shown that according to cell morphology, the cells were large, flattened, fibroblast-like. Cells expressed CD90 and CD271surface markers; their concurrent presence is characteristic of MSC. Cells also expressed type II collagen, which is necessary for the synthesis of extracellular scaffold, especially cartilage.

Morphology showed small lung cells with a large number of intercellular contacts, densely located on the plastic. All cells expressed a cytoskeleton protein, total cytokeratin (CKPan), the main epithelial cells marker. At this, CKPan-negative and Vimentin-positive cells were not observed in the culture, indicating the purity of the obtained culture from fibroblast-like cells. Cells were also stained for cytokeratins CK8/18 and CK14, which confirmed the epithelial phenotype [13].

Tracheal tissue devitalization

DTS was compared with native tracheal tissue histologically and with hematoxylin and eosin staining (Fig. 1). In Fig. 1, b, the tissue of the cartilaginous ring of the trachea is seen, with the destroyed tissues of the perichondria, submucosal membrane and epithelium around, especially clear in comparison with native tissue (Fig. 1, a). The structure of the cartilage tissue is preserved. In the depths of the tissue, the remaining chondrocytes are observed in gaps. The architectonics of the cartilaginous tissue has not altered, meaning the preservation of its mechanical strength.

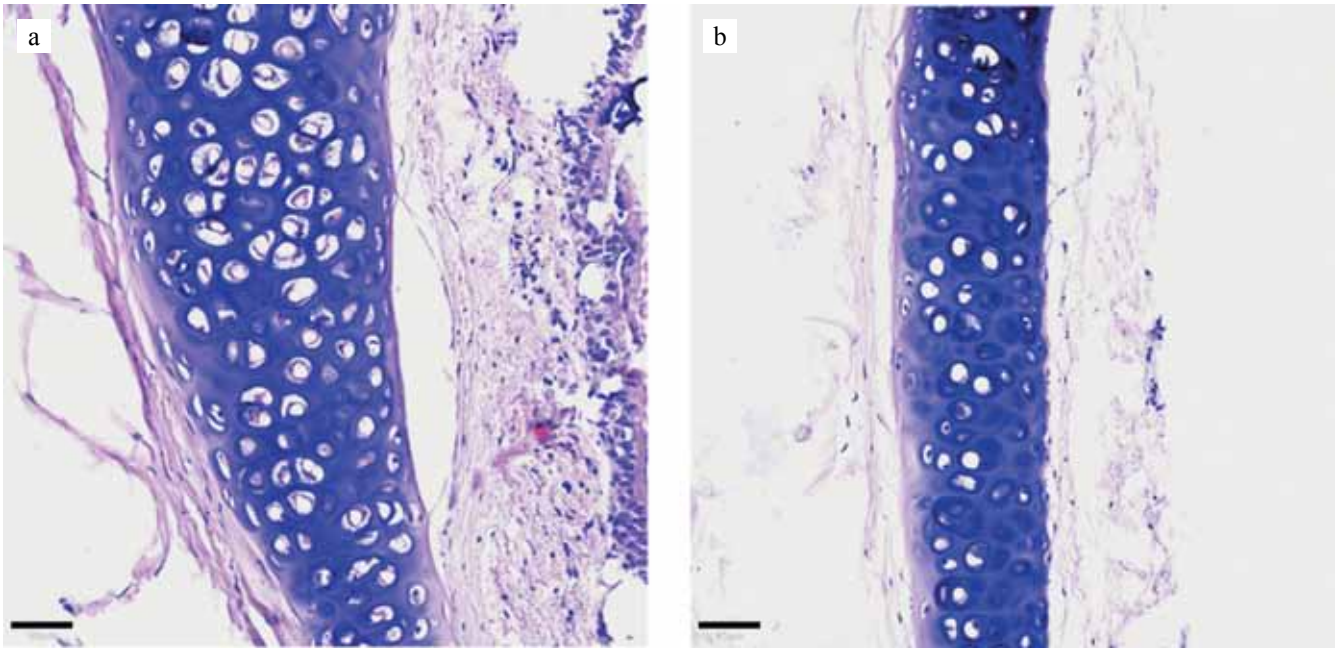

Fig. 1. Tracheal cartilage sample after devitalization: a – native tracheal tissue; b – devitalized tracheal scaffold. Hematoxylin-eosin staining. The scale bar is 50 μm. ×100

DTS cytotoxicity and seeded cell viability

BM MSC cellular activity after DTS extract addition in comparison with the control group was 144.3 = 3.8% ($p < 0.05$).

DTS vitalization created the trachea TEG with completely seeded BM MSC on the surface, most of which were calcein-AM-stained viable cells.

TEG engraftment after implantation

In the 3rd month after implantation, for an interim assessment of wound healing and the condition of the trachea lumen, CT was performed under general anesthesia. The set of slices was converted into 3D image where images were taken in three planes: from above (Fig. 2, a), from the side (Fig. 2, b) and from the front (Fig. 2, c).

A slight narrowing was noted in the area of implantation of not more than $^1/_3$ of the lumen, which is seen above and below the green line (plane of cut sections of photographs) in Fig. 2, a. To fully assess the magnitude of the narrowing relative to the entire lumen of the trachea, a volume model was created, the segmentation area is shown in green in Fig. 2, c.

Fig. 3 shows a calculated model of the respiratory tract which includes soft tissue of the rabbit's pharynx and nasopharynx, larynx, trachea before bifurcation, bronchi and soft lung tissue.

Fig. 3, d shows the area of engraftment is shown; a slight narrowing of the lumen is seen. No stenosis was observed in the area of engraftment.

Morphological analysis

In the areas of TEG engraftment neither granular leukocytes nor lymphocytes were detected, which meant the absence of signs of purulent inflammation or aseptic necrosis around TEG material (Fig. 4, a, c). This indicates the absence of complications in the animal during the experiment associated with tissue response to the graft and the development of infectious diseases of the respiratory tract.

The reason for the narrowing of the lumen revealed in the 3rd month was found by CT. On the right side of the trachea (Fig. 4, a), a chronic inflammatory reaction developed around the suture material which led to partial destruction of the graft in this area, replacement by connective tissue and hypertrophy of the epithelial layer with an increase in the content of goblet cells producing mucus. This is clearly visible on the posterior wall of the trachea (Fig. 4, b), where the epithelium became thickened and polyps formed. On the opposite side of the incision, the area of inflammation decreased in size, which implies that the reaction to the suture material was local in nature. The tissue at a distance from the described suture material had normal morphology. As can be seen in Fig. 4, d, the epithelium on the implant

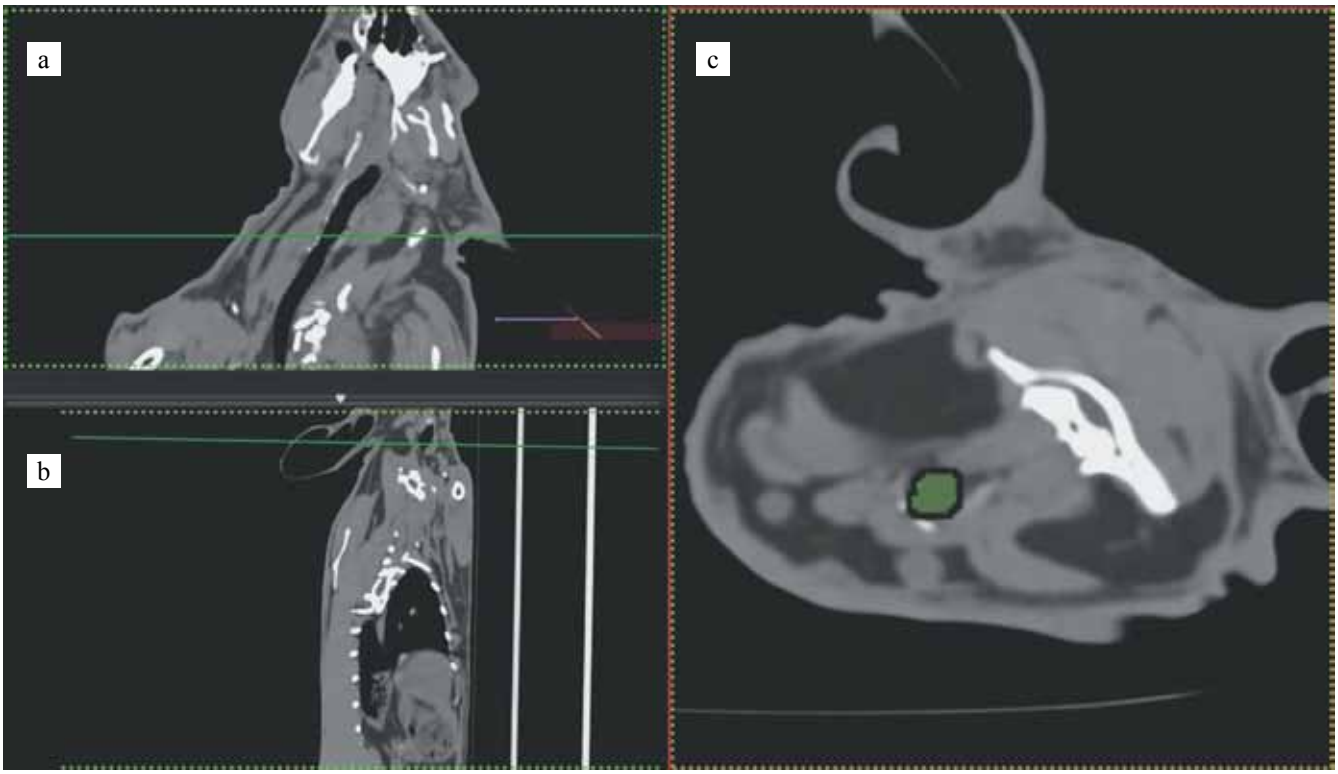

Fig. 2. CT scan of the rabbit neck and thorax, the image planes: a – from above, b – from the side and c – from the front. The green line (a, b) – is the image cut-off plane. The green area (c) – the area of automatic segmentation of the respiratory tract

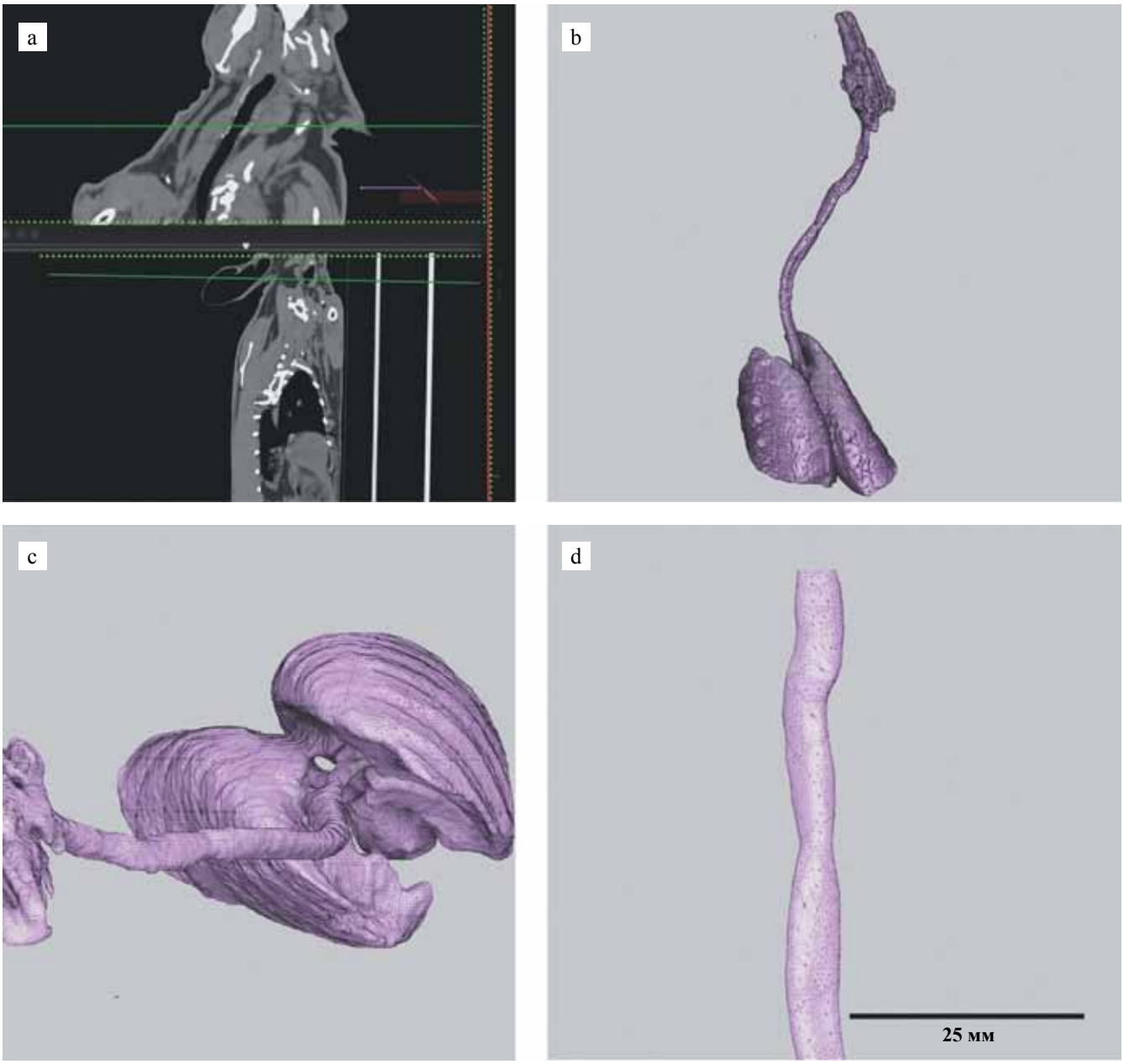

Fig. 3. The rabbit respiratory tract model: a – CT scan of the tissue-engineered graft engraftment area; b–c – general view of the respiratory tract model; d – tracheal lumen model in the area of engraftment

was not hyperplastic; tracheal and implant tissues fused, two rows of cartilaginous rings were observed: large – native, smaller – TEG sections. The submucosal layer was represented by multidirectional bundles of collagen fibers that surrounded the sites of resorption of the cartilage structures of the implant destroyed by macrophages.

Fig. 5 shows the results of immunofluorescence staining for CKPan (Fig. 5, a–c), intercellular interactions – cadherins (CDH) (Fig. 5, d), and Ki-67 cell proliferation marker (Fig. 5, a, b).

The TEG area without signs of inflammation from the suture material is shown in Fig. 5, a. The epithelium of the normal morphology is seen, while the proliferating cells (Ki-67) are located mainly in the submucosal layer. In the area of inflammation (Fig. 5, b) Ki-67 positive cells are observed both in the submucosal and epithelial layers, which indicates continuous cell proliferation. The epithelium in the area of inflammation is also hypertrophied, as can be seen in Fig. 5, c and d.

DISCUSSION

Evaluation of the quality of obtaining biologically and physiologically compatible scaffolds for creating TEG is based on experiments both *in vitro* and *in vivo*. At the native trachea devitalization, most of the cells from the volume of the tracheal scaffold were removed in the freeze/thaw cycles, which, most likely, favorably affected the decrease in the immune response to the

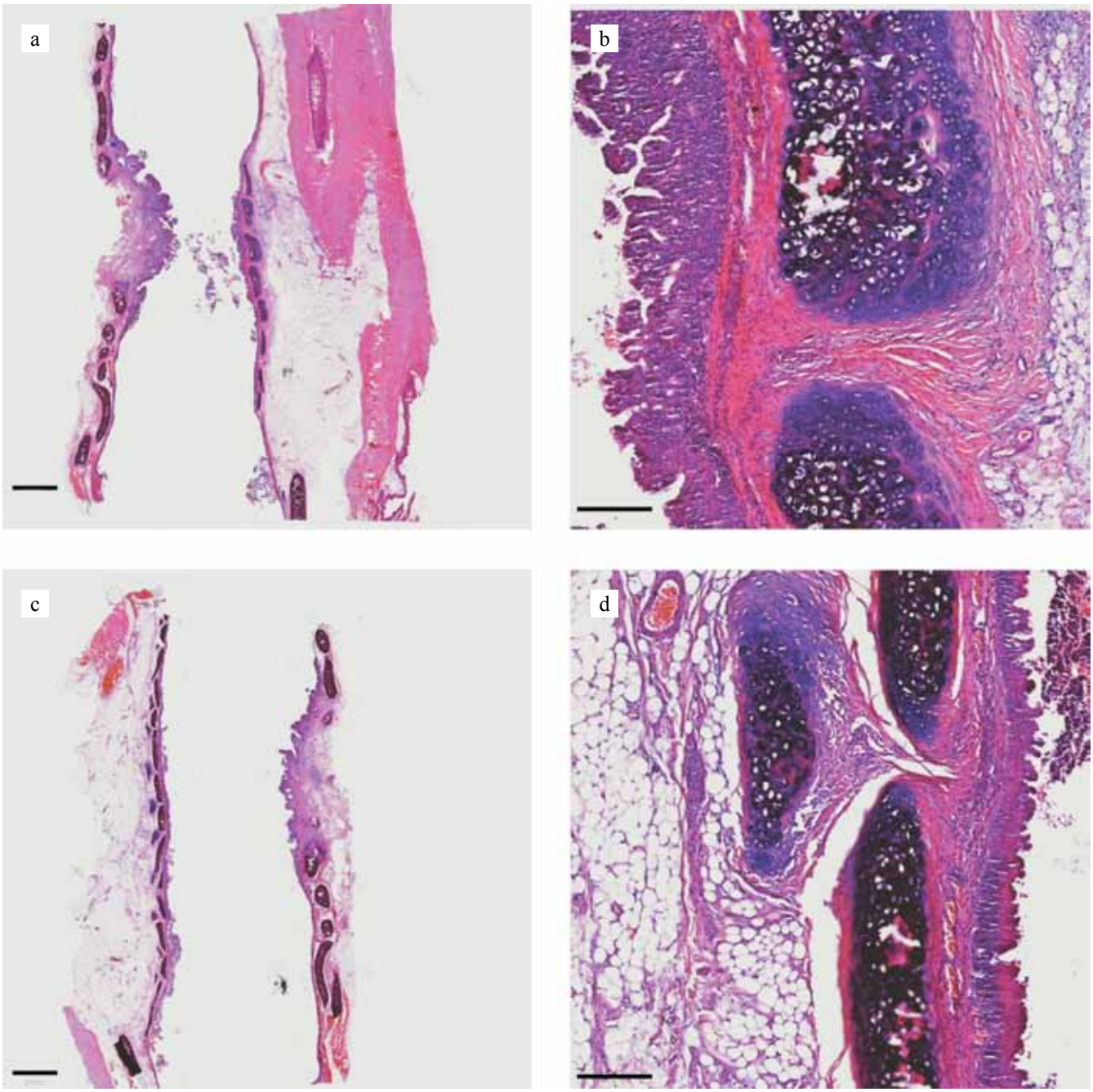

Fig. 4. Sample of rabbit trachea tissue at 6 months after the graft engraftment, hematoxylin-eosin staining: a, c – overview of tracheal tissues from two sides relative to the incision, the scale bar is 2 mm; b, d – areas of the mucous membrane at a distance from the source of inflammation, the scale bar is 200 μm

implant. Moreover, if previously protocols with 5-fold freeze-thaw cycles were used to get the devitalized TEG scaffold [14, 15], then our use of a 3-fold cycle was also suitable for obtaining DTS. Moreover, the method demonstrated satisfactory results of DTS vitalization with a known high level of presence of syngeneic cells preserved after freezing and thawing cycles in the material. Accordingly, this devitalization method may be suitable for producing TEG scaffolds.

Earlier techniques for the efficient seeding of TEG scaffolds in the regimes of static and dynamic cultivation [16] have shown their suitability for creating TEG trachea with a two-layer cell coating.

A model was proposed and developed for assessing the viability of a tissue-engineering graft when closing a critical airway defect using MSCT, histology, and immunohistochemistry.

It can be concluded, by the absence of neutrophils and eosinophils in the areas of the epithelium and submucosal layer of the surrounding TEG tissues, that the graft did not have toxic effects on the recipient's tissue. In the absence of chronic inflammatory reaction, a specific

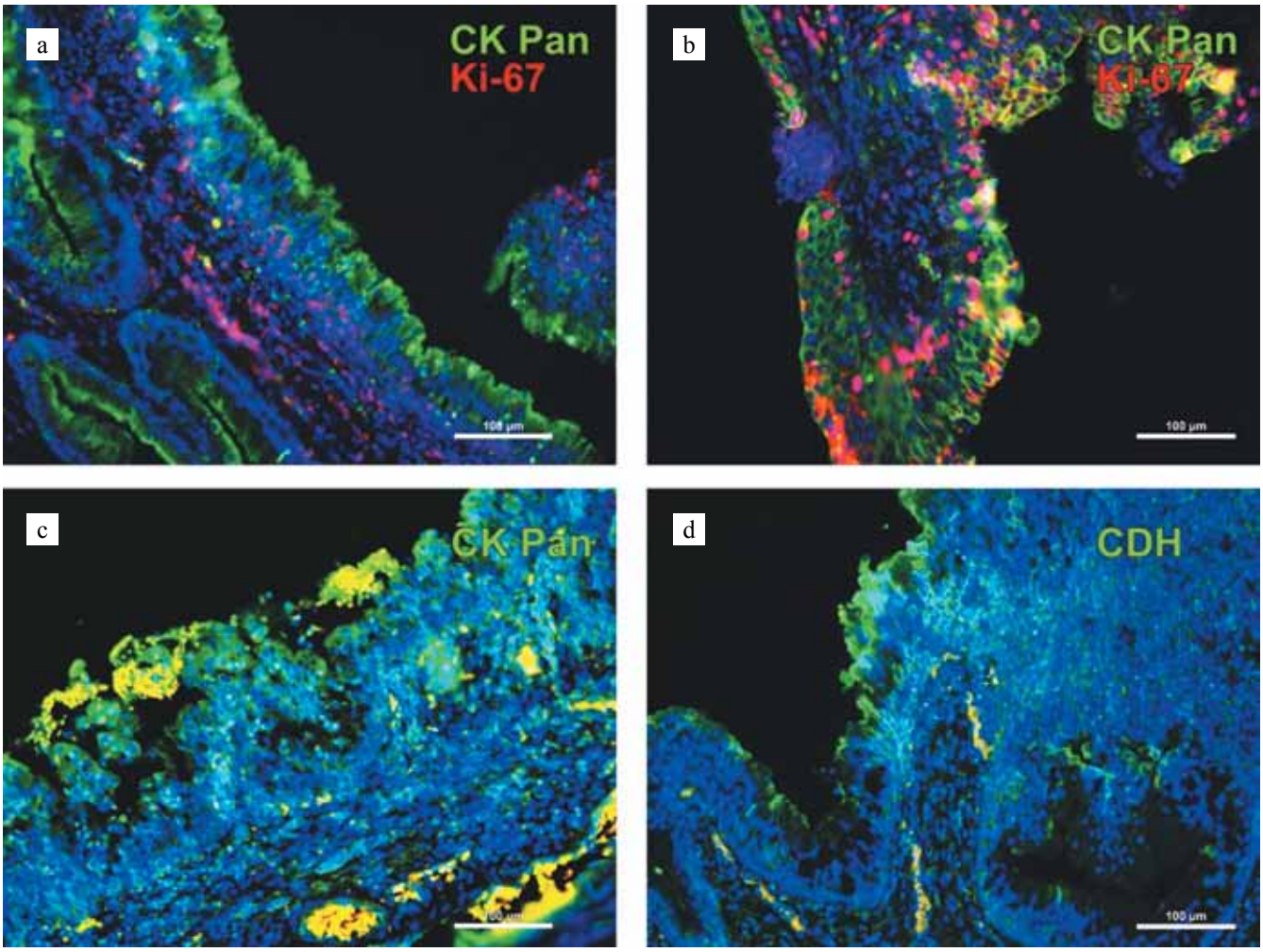

Fig. 5. The wall of the rabbit trachea in the engraftment area. Immunofluorescent staining of cell nuclei for Hoechst 33342 (blue fluorescence). The scale bar is 100 μm. ×200

immune response to TEG also did not develop. A large number of vessels and capillaries were observed in the submucosal layer, which indicates good vascularization of the structure.

Based on the results of a histological examination of TEG and the tissues surrounding the implant, it can be concluded that the graft is viable, well epithelized, vascularized and integrated into the structure of the recipient's trachea, despite the development of complications associated with inflammation due to suture material.

CONCLUSION

The creation and evaluation in *in vitro* and *in vivo* surgical experiment of a tissue-engineering graft on the basis of the devitalized rabbit tracheal scaffold seeded by bone marrow mesenchymal stromal cells and epithelial cells confirmed the cytological and biological compatibility of the graft. The minimal narrowing of the trachea in the engraftment area in the absence of stenosis indicates the TEG ability to maintain a constant lumen of the recipient's trachea. Further research is needed on improving the biologically and physiologically compatible tissue-engineering trachea based on the devitalized tracheal scaffold retaining tissue microarchitectonics.

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The authors declare no conflict of interest.

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NEW TRENDS IN THE STUDY OF POST-TRANSPLANT ACUTE KIDNEY INJURY AFTER LIVER TRANSPLANTATION

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Acute kidney injury (AKI) after liver transplantation (LT) is a pressing issue and remains the focus of many researchers. The etiology of AKI is multifactorial, but the main one is ischemia-reperfusion injury to the liver transplant. Numerous preoperative, intraoperative and postoperative risk factors contribute to the development of AKI. The use of standard classifications, such as AKIN, RIFLE and KDIGO, has improved post-transplant AKI diagnosis. However, determination of creatinine levels in the blood enables AKI diagnosis only in the later stages of this syndrome. Therefore, studies are currently underway to find ways of early diagnosis of AKI using biomarkers. Transition to a molecular level not only improves accuracy but also facilitates early diagnosis of AKI. Currently, the diagnostic capabilities of neutrophil gelatinase-associated lipocalin (NGAL) are the most investigated. To date, there are no known measures of preventing post-transplant AKI. Moreover, treatment of this condition cannot be considered satisfactory. Even a mild post-transplant AKI can be fatal. In severe AKI, where renal replacement therapy is used, there is a risk of death in the intensive care unit. More than half of AKI patients develop chronic kidney disease requiring chronic hemodialysis.

Keywords: acute kidney injury, liver transplantation, risk factors, predictors, biomarkers.

INTRODUCTION

Acute kidney injury (AKI) following liver transplantation remains a pressing issue in modern medicine. It concerns both severe therapeutic and surgical patients. Patients with cardiovascular disease and sepsis have a particularly high risk of developing AKI. About 40% of patients with acute decompensated heart failure have AKI. With increased incidence of heart failure, AKI prevalence is predicted to rise [1, 2]. Sepsis is the most common cause of AKI in critically ill patients. Differences in patient characteristics, pathophysiology and outcomes distinguish septic AKI as a separate clinical entity from non-septic AKI [3]. About 40% of patients who underwent surgical interventions develop AKI after cardiac (18.7%), general (13.2%), and thoracic (12.0%) surgeries [1, 2, 4, 5].

The AKI problem has not spared the transplantology sector. After transplantation of non-kidney solid organs, most patients develop acute reduction in renal function [1, 2]. Moreover, some patients develop end-stage renal disease requiring renal replacement therapy (RRT). Many patients also develop CKD. AKI incidence varies depending on the organ to be transplanted. AKI after transplantation of non-kidney solid organs leads to longer hospital stay, higher cost of treatment, increased risk of death, and more common *de novo* CKD [6, 7]. AKI is a common and severe complication developing after liver transplantation (LT) [8–12]. It is more often common for livers retrieved from asystolic donors. It usually develops in the early stages following a LT – from six hours to the end of the first day after reperfusion [13]. Late onset of AKI is observed in fewer patients [14]. Post-LT AKI develops not only from deceased donor LT, but also from living donor LT [15–17]. After living donor LT, 6.3% of patients (34/538) required postoperative RRT [15]. This complication is less common after LT (29%) than after abdominal surgery (47%). However, the number of cases requiring RRT is higher after LT than after abdominal surgery (71% and 53% of patients respectively) [1, 2].

INCIDENCE OF ACUTE KIDNEY INJURY

Since the Model for End-Stage Liver Disease (MELD), which uses serum creatinine levels to predict survival for liver disease, was introduced in 2002, incidence of kidney dysfunction among potential liver recipients has increased significantly. This has led to increased incidence of simultaneous liver-kidney transplantation. A decision to conduct simultaneous liver-kidney transplant surgery is difficult and must be strictly balanced. The severity and duration of pre-LT renal dysfunction, hepatitis C, diabetes, and other risk factors for kidney disease are associated with higher risk of post-transplant renal failure. However, there are currently no clinical findings that would accurately predict renal recovery after LT [18].

Incidence of post-LT AKI ranges from 17% to 94% [7]. According to E.A.J. Hoste et al. [19], AKI prevalence ranges from 1% to 66%. Table 1 shows information on

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Table 1

Incidence of acute kidney injury after liver transplantation

Authors	Year of publication	Country of publication	AKI incidence in %	Remarks
A.G. Barreto et al. [14]	2015	Brazil	46.7	
I.A. Hilmi et al. [25]	2015	USA	52	
M.H. Park et al. [15]	2015	South Korea	27.3	Living liver donors
P. Wiesen et al. [26]	2016	Belgium	58.3	
M. Hamada et al. [27]	2017	Japan	46.2	Pediatric LT
E.C. de Ataide [28]	2017	Brazil	46.84	
T. Mizota et al. [17]	2017	Japan	30.7	Living liver donors
Z.Q. Zhou et al. [29]	2017	China	40.8	
Y. Zongyi et al. [20]	2017	China	3.97	
I. Jocmans et al. [13]	2017	Belgium	26	
M.S. Chae et al. [16]	2017	South Korea	22.7	Living liver donors
E. Trinh et al. [7]	2017	Canada	56.6	
Y. Cheng et al. [30]	2018	China	64.2	
M. Kalisvaart et al. [21]	2018	Netherlands	65	Cardiac death liver donors
C. Pulitano et al. [31]	2018	Australia	32	

post-LT AKI occurrence in studies of other authors. The incidence of post-LT AKI ranges from 3.97% [20] to 65% [21] of liver recipients. Such wide variations in AKI prevalence can be explained by not only population differences, but also inconsistent use of standardized AKI classification criteria. AKI etiology and incidence also vary between high- and low-income countries. The incidence is lower in high-income countries than in low-to-middle-income countries, where contaminated water and endemic diseases, such as malaria contribute to high burden of AKI. Outcomes of AKI are similar to or more severe in low-income patients. Later detection of AKI hinders recovery and leads to high mortality [19].

AKI is often observed not only after LT from donors with advanced criteria, but also from "standard" donors and can lead to CKD and/or death [22].

I.G. Jun et al. [23] retrospectively analyzed the data of 1617 patients who underwent living donor liver transplantation. 271 of the patients received ABO-incompatible (ABOi) living donor liver transplantation (LDLT). AKI incidence was significantly higher after ABOi LDLT than with ABO-compatible LDLT (67.0% versus 48.2%; $p < 0.001$). Besides, length of ICU stay ($p = 0.01$) was significantly prolonged, but there were no significant differences in mortality ($p = 0.74$), graft failure ($p = 0.32$) and postoperative dialysis ($p = 0.74$) between the two groups of patients. Hemoglobin level and duration of surgery were independent risk factors for AKI after ABOi LDLT [23].

A meta-analysis of databases (MEDLINE, EMBASE and Cochrane Databases) from inception until December 2018 showed that the incidence rates of post-LT AKI and severe AKI requiring RRT are 40.8% and 7.0%, respectively are 40.8% and 7.0%, respectively. There is reliable association of AKI with increased mortality and graft failure. Incidence of AKI after LT has remained stable over the last 10 years of the study [24].

ORIGIN AND PATHOGENESIS OF ACUTE KIDNEY INJURY

Acute kidney injury is a syndrome with various etiologies and pathophysiological processes leading to impaired kidney function [1–3, 32]. In addition to retention of waste products, impaired electrolyte homeostasis and altered drug concentrations, AKI induces a generalized inflammatory response that affects many internal organs [19].

According to most researchers, post-LT AKI is multifactorial in origin [1, 2, 19]. It has been causally associated with exposure to high levels of toxic free-radicals, renal ischaemia with hemodynamic instability, effects of end-stage liver disease on the kidney and infectious complications after LT. In addition, AKI has been associated with the severity of native liver disease according to MELD [30], pre-LT renal dysfunction, graft quality, perioperative factors, particularly calcineurin inhibitor nephrotoxicity [11].

One of the main etiological factors of AKI in LT is hepatic IRI [11, 13, 33]. M. Kalisvaart et al. [21] studied the effect of warm ischemia duration on AKI development in 368 recipients who received liver from cardiac death donors. AKI severity significantly increased with longer duration of warm ischemia: from 61 minutes in recipients without AKI up to 69 minutes in recipients with the most severe form of AKI ($p < 0.001$). The length of warm ischemia should ideally not exceed 60 minutes because a longer time will increase the severity of post-LT AKI. It is known that cold storage of donor organs leads to increased ischemic damage. However, M. Kalisvaart et al. [21] found no relationship between length of cold ischemia and severity of AKI.

Vascular pathology can be an etiological factor of AKI. W. Beaubien-Souligny et al. [34] presented a rare observation in which inferior vena cava stenosis was the cause of post-LT AKI. A month after undergoing LT, a 25-year-old man with cirrhosis caused by sclerosing cholangitis and autoimmune hepatitis developed severe AKI in combination with recurrent ascites and lower extremity edema. An ultrasound scan revealed inferior vena cava stenosis. There was rapid improvement in renal function after angioplasty with stent installation.

AKI pathogenesis is still not clear [11]. In the pathogenesis of AKI in hepatic IRI, I. Jochmans et al. [13] distinguish four components. First, the main role is played by systemic inflammatory response as activated Kupffer cells initiate the release of circulating inflammatory and pro-inflammatory cytokines and transcription factors. Increased level of tumor necrosis factor α and other interleukins disrupts regulation of endothelial adhesion molecules in distant organs, particularly in the kidneys, and with that leukocyte recruitment and increased vascular wall permeability occur in them. Activated neutrophils release enzymes and cytokines into the subendothelial space, directly causing kidney injury and recruitment of monocytes and macrophages. Second, hepatic IRI leads to increased endothelial apoptosis, which further promotes leukocyte infiltration of the vessel walls. Third, oxidative stress and reactive oxygen species also contribute to kidney injury. Fourth, damage to the actin cytoskeleton of the tubular and renal endothelial cells can lead to increased apoptosis. According to I. Jochmans et al. [13], reducing hepatic IRI, for example, by using machine perfusion technology, might not only improve graft function but also limit the effect of the injury on the kidney and reduce AKI incidence.

MORPHOLOGY OF ACUTE KIDNEY INJURY

Morphology of kidney in acute injury is poorly studied because biopsies are not performed in AKI patients. In the study of autopsy material in AKI, there are various degrees of dystrophy and necrosis of renal tubular epithelial cells, up to acute tubular necrosis. The glomerular capillaries are anemic, with collapsed lumen.

RISK FACTORS FOR ACUTE KIDNEY INJURY

In a study by P. Wiesen et al. [26] in a univariate analysis, the severity of renal dysfunction was correlated with the presence of ascites and prior bacterial infection, preoperative bilirubin, urea and creatinine levels, use of vasopressors, need for postoperative mechanical ventilation, postoperative bilirubin and urea, aspartate aminotransferase, and hemoglobin levels and the need for transfusion. Multivariate analysis showed that body mass index [BMI] ($p = 0.004$), preoperative creatinine level ($p < 0.0001$), use of vasopressor ($p = 0.0002$), maximal postoperative bilirubin level ($p = 0.044$) and minimal postoperative hemoglobin level ($p = 0.0005$) were independent predictors of early post-LT AKI. In multivariate analysis, neither donor status nor aspartate aminotransferase levels had significant effect on early postoperative renal dysfunction [26].

In another study, univariate analysis showed that preoperative factors (BMI, diabetes mellitus, C-reactive protein), intraoperative factors (packed red blood cell transfusion, furosemide, and oxygen content at the anhepatic phase, five minutes and one hour after graft reperfusion, and at peritoneal closure) and postoperative factors (severe postreperfusion syndrome) were significant AKI risk factors. Multivariate analysis showed that oxygen content 5 minutes after graft reperfusion, BMI, and furosemide administration were independently associated with postoperative AKI. Thus, postoperative AKI was independently associated with oxygen content 5 minutes after graft reperfusion, BMI, and furosemide administration [16].

In a multivariate analysis after living donor liver transplantation, independent risk factors for AKI were: BMI >27.5 kg/m^2; serum albumin <3.5 mg/dl; MELD score >20; operation time >600 min; warm ischemia time >40 min; postreperfusion syndrome; mean blood glucose during the day of surgery >150 mg/dl; cryoprecipitate >6 units; blood loss/body weight >60 ml/kg; calcineurin inhibitor use without combined mycophenolate mofetil [15]. The authors argue that doses of calcineurin inhibitor should be reduced by combined use of mycophenolate mofetil to reduce incidence of postoperative AKI.

Increased preoperative total bilirubin level and increased intraoperative blood loss, as well as prolonged hospitalization were independently associated with the risk of developing AKI after pediatric liver transplantation [27].

Recently, increased intake of serum phosphate was found to be associated with increased risk of AKI at all stages of hospital stay [35].

As can be seen from the above recent reports, there are numerous preoperative, intraoperative and postoperative risk factors for AKI. We will look deeper into AKI risk factors at each of these stages.

Preoperative risk factors

The greatest number of risk factors for post-LT AKI exists in patients before surgery. Reports focus on various factors. Research by H. Aksu Erdost et al. [36] showed that with MELD score >20, the recipient has an increased risk of post-LT AKI. Viral hepatitis in the recipient, longer warm ischemia time (WIT) and high levels of serum lactate are risk factors for AKI before LT in A.G. Barreto et al. [14]. In addition, predisposing factors for development of AKI were female sex, weight (>100 kg), non-alcoholic steatohepatitis, and severity of native liver disease [25]. Post-LT renal dysfunction prevails in patients with decompensated native cirrho-

sis [37]. In the study of H.P. Chen et al. [9], the most significant risk factor for post-LT AKI was preoperative cerebrovascular disease.

An important predisposing factor for development of AKI is diabetes mellitus, which has existed before LT [25]. After LT, progression of kidney injury to end-stage renal disease was particularly pronounced in patients with diabetes mellitus [38]. The authors suggest that diabetes mellitus can be considered as a criterion in making decisions regarding simultaneous liver-kidney transplantation. Every year the number of such operations increases both in CKD and in AKI [39]. Only in a study by O. Komurcu et al. [40], no effect of hyperglycemia (blood glucose >200 mg/dl) on increased risk of AKI, increased postoperative infections, or increased post-LT mortality.

Independent and reliable ($p < 0.05$) risk factors for AKI include high preoperative serum creatinine levels and a long period of treatment with dopamine [20]. Acute kidney damage is associated with high BMI, low urine output [29], low serum albumin and elevated levels of direct bilirubin, alkaline phosphatase and gamma-glutamyltransferase [41].

Inflammatory and anti-inflammatory cytokines play critical roles in the development of AKI. Based on this, a study was undertaken of the role of cytokine gene polymorphisms in kidney deterioration after LT. It was found that the IL4-33 T/T genotype was significantly associated with higher incidence of AKI compared with the other two genotypes ($p = 0.03$). Therefore, the IL4-33 T/T genotype might be a risk factor for post-LT AKI [42].

Donor risk factors

Warm and cold donor liver ischemia is considered as an independent and significant ($p < 0.05$) risk factor for post-LT AKI. The degree of ischemia increases for organs from high-risk donors, especially from asystolic donors [20]. In a study by M.B. Doyle et al. [43], AKI incidence depending on the nature of the donor liver – from a donor with cardiac death or with brain death – was investigated. Although cardiac death liver donors were younger than brain death donors ($p < 0.0001$) and had lower MELDs ($p = 0.03$), AKI was more common in cardiac death livers than in brain death livers (16.3% of recipients required dialysis – against 4.1%, $p = 0.01$).

AKI does not depend directly on high-risk liver donors (asystolic donors or donors older than 65 years) but is associated with the severity of hepatic IRI [13, 25]. J. Roller and M. Glanemann [44] point out that to reduce the likelihood of developing AKI in LT, warm and cold ischemia time of the donor liver should be minimized.

It is difficult to identify which LT candidates with severe kidney injury will have full restoration of renal function after LT alone. H.L. Laskey et al. [45] found that in such recipients, full restoration was the median WIT – 31 minutes (24–46 minutes), and there was no recovery with a WIT of 39 minutes (34–49 minutes; $p = 0.02$). For each minute of increased WIT, there was an 8–9% increase in the risk of lack of renal recovery after LT.

Intraoperative risk factors

Hemodynamic instability during LT is crucial in development of AKI and deserves closer attention. Severe hypotension, even for less than 10 minutes, was significantly associated with severe AKI [17].

Univariate analysis showed that intraoperative factors (red blood cell transfusion, furosemide, and oxygen content at the anhepatic phase, five minutes and one hour after graft reperfusion, and peritoneal closure) were significant AKI risk factors [16].

During LT, an independent and significant ($p < 0.05$) risk factor for AKI was too much blood loss [20], and accordingly, volume of transfused blood and/or its components (red blood cells, freshly frozen plasma) [25, 30, 36]. According to H. Aksu Erdost et al. [36], normalizing hemoglobin levels without transfusion of blood components can prevent AKI.

It is believed that blood transfusion is a risk factor for AKI only if the quantity is large [13, 30]. Besides, transfusion of long-stored red blood cells significantly increases the risk of postoperative AKI in patients after LT. In one study [46], patients who underwent LT were divided into two groups. The first group consisted of patients who received transfused red blood cells that had been stored for less than 14 days, and the second group consisted of patients who received red blood cells that had been stored for 14 days or more. Postoperative AKI was observed in 40.5% of patients of the first group and in 65.1% of the second ($p < 0.01$). The incidence of severe post-LT AKI was significantly higher, and the length of stay in the ICU was much longer in the second group [46]. The risk of developing AKI increases with surgery lasting for more than 480 minutes [29].

In patients after LT, progression of kidney injury to the end-stage renal disease was especially pronounced at glomerular filtration rate (GFR) <60 ml/min during surgery [47]. According to I.A. Hilmi et al. [25], the presence of severe unstable hemodynamics during reperfusion does not affect the incidence of post-LT AKI. In contrast, J. Roller and M. Glanemann [44] emphasize the need to maintain adequate blood pressure during reperfusion to reduce the likelihood of AKI in LT.

Recently, it has been found that indicators such as elevated baseline central venous pressure (CVP), elevated baseline right ventricular end-diastolic volume (RVEDV) after anesthesia induction and decreased mixed venous oxygen saturation (SvO_2) during anhepatic phase in LT, are risk factors for postoperative AKI [48]. Intraoperative oliguria, combined with decreased SvO_2, is a more

accurate predictor of post-LT AKI than just one of these indicators [48].

Postoperative risk factors

Calcineurin inhibitor nephrotoxicity and postoperative infections are independent and significant ($p < 0.05$) risk factors for post-LT AKI [20]. Development of AKI is facilitated by the non-optimal function of the liver graft [29, 49] and use of vasopressors [29]. Peak aspartate aminotransferase, occurring at 6 hours after reperfusion, was the only independent risk factor for AKI. Early liver graft dysfunction occurred more frequently in AKI patients [13]. Postoperative risk factors for AKI also include high preoperative MELD score and native liver cirrhosis [30]. A study by S. Yoo et al. [50] suggested that increased perioperative glucose variability, but not hyperglycemia, was independently associated with increased risk of post-LT AKI.

DIAGNOSTIC CLASSIFICATIONS FOR ACUTE KIDNEY INJURY

The use of standard classifications for AKI diagnosis and stratification has increased detection rates for this syndrome in clinical practice and epidemiological studies [5]. R. Caragata et al. [51] point to gradual changes in the concept of AKI and emphasize the need for standardized definition in subsequent studies. AKI classifications based on the AKIN, RIFLE, and KDIGO criteria enable assessment of the severity of post-LT renal dysfunction in patients [52].

The RIFLE and KDIGO criteria identify more AKI cases than do AKIN criteria (RIFLE 84.2% vs. KDIGO 87.5% vs. AKIN 72.8%, $p < 0.001$), although the prediction of in-hospital mortality was similar between the three classifications. In septic patients, AKI, defined only by a decrease in urine output, was a better predictor of in-hospital mortality than was AKI, determined either by serum creatinine (SCr) itself or by both SCr and urine output ($p < 0.001$), indicating the diagnostic and prognostic importance of diuresis in patients with septic AKI [53]. Other authors have also noted the advantage of RIFLE and KDIGO classifications over the AKIN classification in the diagnosis of AKI in critically ill patients [1, 2, 54]. Most authors prefer the KDIGO classification in diagnosing AKI and predicting in-hospital mortality [55–58].

AKI is defined as an increase in serum creatinine by 50% or more from its preoperative baseline level. Stage 1 AKI is characterized by 0.3 mg/dl of serum creatinine or a 50% increase after LT. Stages 2 and 3 are defined by a two-fold and three-fold increase in serum creatinine levels, respectively [59]. Determination of serum creatinine level is a sensitive and specific method in diagnosis and classification of post-LT AKI [37].

Inclusion of oliguria, which is common after LT, into the diagnostic criteria, dramatically increases the measured incidence of AKI. Oliguria without serum creatinine increase was significantly associated with adverse postoperative outcomes [60].

Post-reperfusion syndrome, which reflects severe IRI, is a predictor of AKI following donation after brain death liver transplantation [22]. At the same time, increased plasma levels of aspartate aminotransferase (AST) is the only reliable predictor of hepatic IRI. Therefore, elevated AST blood levels should also be considered as a predictor of AKI [13, 33].

NEW DIAGNOSTIC APPROACHES IN ACUTE KIDNEY INJURY

A new trend in early diagnosis of various diseases, including in patients after solid organ transplantation, is the search and study of various biomarkers for this purpose [61, 62]. Although serum creatinine remains the gold standard for assessing kidney function, this test has low specificity and sensitivity for ***early detection*** of AKI [63]. Therefore, as well as the fact that modern AKI therapy leaves much to be desired, researchers are currently focusing not on treatment methods, but on prevention and early detection of AKI in critically ill patients, including LT recipients [64, 65]. New biomarkers for predicting or detecting AKI early can potentially increase the possibility of treating this condition in donor liver recipients [66]. Transition to molecular level, particularly to identification of tubular injury biomarkers, permits earlier and more accurate detection of AKI [67].

The diagnostic capabilities of neutrophil gelatinase-associated lipocalin (NGAL) and G1 cell cycle arrest biomarker as biomarkers have been confirmed in many clinical trials involving cardiac patients (B. Wu et al., 2019) [63]. To predict post-LT AKI, it was also proposed to determine NGAL (A.C.Y. Yeung et al., 2018) [68].

Serum and urinary systemic macrophage migration inhibitory factor (MIF) and NGAL levels were used as early predictors of severe post-LT AKI in 45 patients (mean age 55 ± 8 years). Of these, 19 patients (38%) developed severe AKI within 48 hours after reperfusion. At the end of LT operation, serum MIF was predictive of severe AKI ($p = 0.03$), whereas urinary MIF, serum and urinary NGAL were uninformative. On the first postoperative day, serum MIF ($p = 0.006$), urinary MIF ($p = 0.03$) and urinary NGAL ($p = 0.02$) levels predicted severe AKI, while serum NGAL was not indicative [69]. M.A. Kandil et al. [70] also believe that serum NGAL levels are not a predictor of AKI. Nevertheless, A.C.Y. Yeung et al. [68] consider it necessary to conduct further studies to standardize the method for determining NGAL and confirm its clinical usefulness. This was carried out in a recent study [71], which showed that whole-blood NGAL concentration at ICU admission is a good

stratifier of AKI in critically ill patients (Table 2). However, in septic patients, NGAL concentration is higher regardless of the presence or absence of AKI: an average of 481 (247–687) μg/L in those with sepsis and 623.5 (361–798) μg/L in the subgroup of septic shock.

Table 2

Whole-blood NGAL concentration in critically ill patients, depending on the AKI stage based on KDIGO Classification [71]

AKI stage	NGAL average concentration, μg/L	Variation range of NGAL concentration, μg/L
0	78	60–187
1	263	89–314
2	484	333–708
3	623	231–911

Hyperuricemia often occurs after organ transplants and is an independent predictor of renal failure. Hyperuricemia may accompany decreased renal function in this category of patients [38, 47]. In addition, hyperuricemia is an independent predictor of post-LT mortality, especially in patients with an estimated GFR <60, and a predictor of a doubling of creatinine in patients with diabetes mellitus. Treatment of hyperuricemia leads to improved renal function in liver recipients [38].

Determining the concentration of tissue inhibitor of metalloproteinase-2 (TIMP-2) and insulin-like growth factor-binding protein 7 (IGFBP7) in urine is proposed as biomarkers for early detection of AKI in various clinical situations [72]. They have been recognized in many countries of Europe and the USA as a test in assessing the risk of AKI in patients after major surgery, with hemodynamic instability or sepsis [73]. However, these biomarkers have proved ineffective in predicting post-LT AKI, and should not be recommended for use in clinical practice [74].

C. Pulitano et al. [31] conducted a prospective study of the potential relationship between gene expression, serum mediators, and onset of post-LT AKI. Reperfusion liver biopsy specimens in the AKI group showed higher expression of several genes involved in IRI compared with the non-AKI group. Changes in gene expression of ET-1, interleukin (IL) 18, and tumor necrosis factor α (TNF-α) were associated with creatinine peak value. AKI patients also had significantly higher ET-1, IL18, and TNF-α serum levels on the first day after surgery. Multivariate analysis showed that ET-1 and IL18 serum levels are independent predictors of AKI [31].

There were attempts to use preoperative serum D-dopachrome tautomerase concentrations as a predictor of AKI in patients after LT. However, this biomarker was useful only as a predictor of the outcome of operation, but not development of AKI [75].

PREVENTION AND MANAGEMENT OF ACUTE KIDNEY INJURY

There are still no ways for preventing post-LT AKI [11]. Use of continuous veno-venous hemofiltration in LT for patients who need renal replacement therapy before surgery does not reduce the length of ICU or hospital stay, but increases survival rates [76]. Another large retrospective study demonstrated that the use of veno-venous bypass during LT was associated with a significantly lower incidence of posttransplant AKI in patients with compromised pretransplant renal function but did not require renal replacement therapy [77].

In order to reduce the risk of AKI, it is necessary to maintain sufficient oxygen content immediately after graft reperfusion in patients undergoing LT by thorough mechanical ventilation and blood transfusion [16]. Targeting perioperative systemic therapy reduces the risk of AKI [78]. The authors believe that systemic oxygen delivery, by means of fluids and inotropes, can be the best way to increase kidney perfusion and oxygenation in high-risk patients undergoing major surgery.

Analysis of recent reports has shown that there are no major breakthroughs in the treatment of AKI. Early intervention with this formidable complication has both short-term and long-term positive effects. AKI treatment is usually performed in the ICU. First of all, it is aimed at preventing or eliminating pulmonary edema and hyperkalemia. To date, renal replacement therapy remains the gold standard for the treatment of severe AKI, although the ideal timing and technique of this therapy remain under debate [79]. The question of using loop diuretics, which are widely used in emergency and intensive care medicine, in patients with AKI with preserved euvolemia, needs to be determined [80].

OUTCOMES OF ACUTE KIDNEY INJURY

The outcome of post-AKI can vary from complete recovery to death. Acute renal failure causes serious difficulties in the management of these patients, affects the outcome of operation and is an independent risk factor for death [9, 14, 81]. AKI patients require much longer artificial lung ventilation [14], they stay longer in the ICU and hospital [9]. There are also so many complications, such as frequent postoperative bleeding, infections (bacteremia, pneumonia) [9] and early development of LT dysfunction [31] with decreased survival rates in the first few months [25]. Even a mild or transient post-LT AKI can lead to severe complications, prolonged stay in the ICU and hospital, as well as increased morbidity and mortality [13, 25, 59].

One study showed that between 2002 and 2013, there was an increase in the number of patients with severe AKI after LT requiring programmed hemodialysis [12]. High MELD-Na score (≥22) is a predictor of hemodialysis need [14].

Severe AKI requiring renal replacement therapy is a known risk factor for death in the ICU [25]. Of 177 patients who underwent liver transplant, 35 patients (19%) required renal replacement therapy in the early post-transplantation period. The mean patient age was 31.1 ± 20.0 years. The MELD score was 16.7 ± 12.3. In-hospital mortality in the AKI patients who underwent renal replacement therapy was 23.3%, and 40% of patients remained on hemodialysis [82].

IRI is responsible for occurrence of post-reperfusion syndrome, which is the first manifestation of severe AKI [22] and which affects morbidity and mortality after LT [83–85]. Development of moderate or severe hepatic IRI in conjunction with AKI has the greatest negative impact on treatment outcomes in these patients. The 90-day survival of patients sustaining both complications was 89%, compared to 100% in patients with either or neither complication [33].

Late survival rates of patients with post-LT AKI, according to various clinics, varies widely. The 1- and 5-year cumulative survival rates of patients with AKI were 33.95% and 25.24%, respectively, compared with 86.34% and 70.05% in non-AKI patients ($p < 0.001$) [20]. In another study, patient survival one year after surgery was 90% in AKI patients versus 98% in non-AKI patients [13].

More than half and even most AKI patients develop CKD. The risk of death increases exponentially with GFR <30 ml/min/1.73m^2 [12, 14, 22]. Incidence of *de novo* CKD and the need for dialysis three months and one year after liver transplantation were significantly higher among patients who developed AKI [25]. This complication is also an important risk factor for long-term postoperative *de novo* CKD [31, 85].

CONCLUSION

Even though post-liver transplant acute kidney injury is less common than in heavy therapeutic and surgical patients, including after transplantation of other solid organs, the urgency of this problem remains to this day and still attracts major attention from many researchers. Incidence of post-LT AKI varies widely. The origin of AKI is multifactorial, but the main cause is hepatic IRI. Acute tubular necrosis is observed in severe AKI. Acute kidney injury has numerous preoperative, intraoperative and postoperative risk factors. The use of standard classifications, such as AKIN, RIFLE and, to a greater extent, KDIGO has improved post-LT AKI diagnosis. However, serum creatinine levels are used to diagnose AKI only in the later stages of development of this syndrome and this does not meet hospital's needs. Therefore, research is currently underway to find ways of detecting early AKI using biomarkers. Transition to molecular level, particularly to identification of tubular injury biomarkers, permits earlier and more accurate detection of AKI. Currently, the diagnostic capabilities of NGAL are the most studied. To date, there are no known measures of preventing post-LT AKI. Moreover, there is no effective treatment for this condition. Even mild post-LT AKI can be devastating. In severe AKI requiring renal replacement therapy, there is a risk of death in the ICU. Over half of AKI patients develop CKD requiring chronic hemodialysis.

The authors declare no conflict of interest.

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INTRAVASCULAR IMAGING OF ATHEROSCLEROTIC PLAQUES IN PATIENTS WITH CARDIORENAL SYNDROME: POTENTIAL USE OF OPTICAL COHERENCE TOMOGRAPHY

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Currently, kidney transplantation and hemodialysis are the primary therapies for end-stage renal disease. High mortality, mostly caused by cardiovascular disease, remains the main challenge in the treatment of this category of patients. It has been shown that in patients with end-stage chronic kidney disease undergoing hemodialysis, the risk of mortality due to cardiovascular disease is up to 20 times higher than in the sex- and age-matched general population. The indicated data determined the appropriateness of isolating cardiorenal relationships into a single cardiorenal syndrome (CRS). Due to the facts mentioned above, intravascular imaging methods, notably optical coherence tomography (OCT), are particularly important in diagnosing coronary artery lesions. This review analyses the data published to date on the features and capabilities of OCT in CRS patients.

Keywords: cardiorenal syndrome, optical coherence tomography, intravascular imaging.

Cardiovascular disease remains a leading cause of morbidity and mortality in patients with chronic kidney disease (CKD). In this case, the risk of mortality increases as estimated glomerular filtration rate (eGFR) falls: in patients with end-stage CKD undergoing hemodialysis, the risk of mortality from cardiovascular diseases is up to 20 times higher than in the sex- and age-matched general population [1]. Symbiosis of the mechanisms of regulation and functioning of the heart and kidney, and dysfunctions of both organs served as the basis for distinguishing cardiorenal relationships into a single syndrome. As defined by Ronco et al. (2008), cardiorenal syndrome is a simultaneous dysfunction of both the heart and the kidney, in which acute or chronic injury to one organ can cause acute or chronic injury to the other [2].

Coronary heart disease (CHD) is a leading cause of morbidity and mortality in CRS patients. CKD is a risk factor for acute coronary syndrome (ACS) [3].

Postmortem and life-time computed tomography imaging and intravascular ultrasound (IVUS) showed a reliable correlation between CKD and severity of CHD and atherosclerotic plaque calcification in coronary arteries.

Optical coherence tomography (OCT) permits visualization of atherosclerotic plaques in coronary arteries with higher resolution; better than with IVUS, calcium penetration is determined. However, the use of OCT in CKD patients is limited due to the need for additional use of contrast agents required to create an optically clear medium.

This review presents an analysis of reports published to date on the features and capabilities of OCT in CRS patients. Our own examples of visualization of atherosclerotic lesions in coronary arteries are given as illustrations [4].

OCT is an intravascular imaging method based on reflection of infrared rays from vessel wall structures [5, 6]. OCT was developed in the late 1980s and early 1990s [7]. In the early 2000s, there were studies showing that OCT is a safe diagnostic method and is not inferior in efficiency to IVUS [8]. This led to more studies with OCT and increased the need to unify image analysis techniques. The first part of a review document on the methodology, terminology and clinical use of OCT, prepared by an international team of experts, was published in 2010 [9]. The document covered the physical principles of OCT, method for obtaining OCT images, and safety and effectiveness of OCT. Data on normal morphology of coronary arteries and assessment of atherosclerotic lesions in coronary arteries were presented. Some conflicting aspects, as well as advantages and disadvantages of OCT over IVUS were analyzed. In 2012, the second part of the review document was published, which was devoted to clarifying some issues not covered in the first part, as well as describing the method of installing stents under OCT [10]. In 2018, the first part of the approval document of the European Association of Percutaneous Cardiovascular Interventions was published. The document analyzed the advantages and disadvantages of

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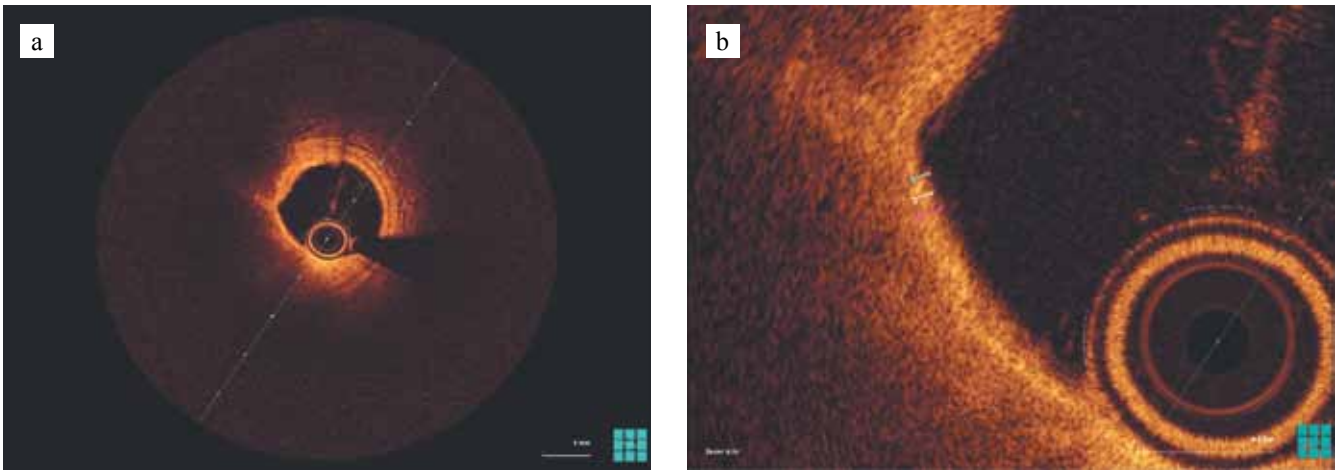

Fig. 1. Thin-cap atherosclerotic plaques (OCT): a – lipid pool at 6–11 hours of conditional dial (lipids occupy more than 1 quadrant, which means that this plaque can be classified as lipid-rich); b – 3-times measured fibrous cap thickness in the thinnest part (60 μm; 60 μm; 70 μm; average: (60 + 60 + 70) / 3 = 63.3 μm)

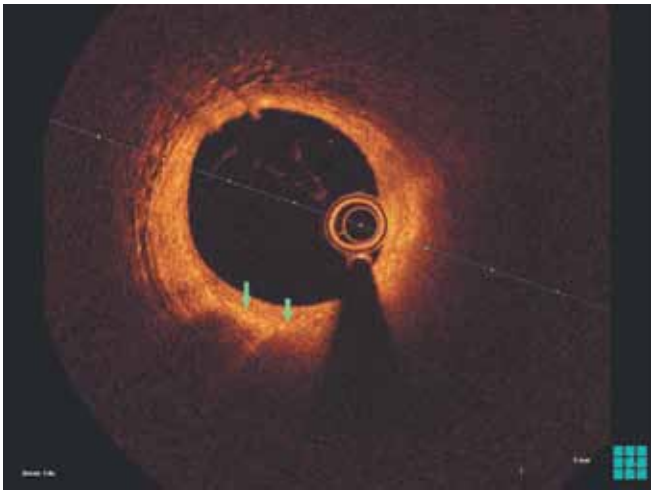

Fig. 2. Cluster of macrophage foam cells (OCT) marked with green arrows: linear areas of high intensity with a "shadow" behind

IVUS and OCT, presented the evidence base for the use of intravascular imaging methods, described in detail the technique of interventions, obtaining and analyzing images, as well as indications and contraindications for IVUS and OCT [11]. The second part of the conciliation document is likely to be published, since OCT is now actively developing and allows one to evaluate many parameters of the morphology of coronary arteries before and after installation of stents and scaffolds. Of all the currently existing intravascular imaging techniques – near-infrared spectroscopy (NIRS), IVUS, IVUS with virtual histology, iMAP-IVUS – OCT has the highest diagnostic value [4].

One of the main problems with cardiorenal syndrome is high mortality from cardiovascular diseases in which ischemic heart disease leads [12, 13]. The above facts justify the use of OCT in CRS patients in detecting vulnerable plaques that are susceptible to compromised fibrous cap, leading to thrombosis, and as a result ACS. Such plaques should be detected in a patient's stable condition.

OCT is the only intravascular imaging technique whose axial resolution allows one to estimate the thickness of the plaque cap (Fig. 1, b) [14], which can be affected by statin administration [15]. Statins also have pleiotropic effect, which manifests itself particularly through decreased severity of macrophage inflammation (Fig. 2) [16, 17].

However, estimating the fibrous cap thickness is not enough to classify the plaque as a thin-cap plaque, since there is also the need to evaluate the lipid core volume (Fig. 1, a). Xing et al. demonstrated that the presence of lipid-rich plaques doubles the risk for adverse cardiac events [18].

The presence of vasa vasorum (Fig. 3) and cholesterol crystals (Fig. 4) in the plaque increases the likelihood of plaque disruption. The sensitivity and specificity of OCT to detect plaque neovascularization compared with pathological data are 52% and 68%, respectively [19]. Nakamura et al. showed that lipid-rich plaque is significantly higher in patients with cholesterol crystals [20]. Dai et al. demonstrated that cholesterol crystals are more common in patients with acute ST-elevation myocardial infarction as compared with patients with non-ST elevation acute coronary syndrome (50.8% vs. 34.7%, respectively) [21].

Plaque erosion is the cause of sudden cardiac death in about 30–40% of cases [22, 23]. It has been shown that there is higher prevalence of plaque erosion in younger patients (<50 years old) and it is more often detected in the left coronary artery (LCA). The study revealed a relationship between plaque erosion and CKD. A classification of risk factors for erosion has also been proposed. The classification takes into account both clinical data and data that can only be obtained via OCT, such as presence of a thin cap [24].

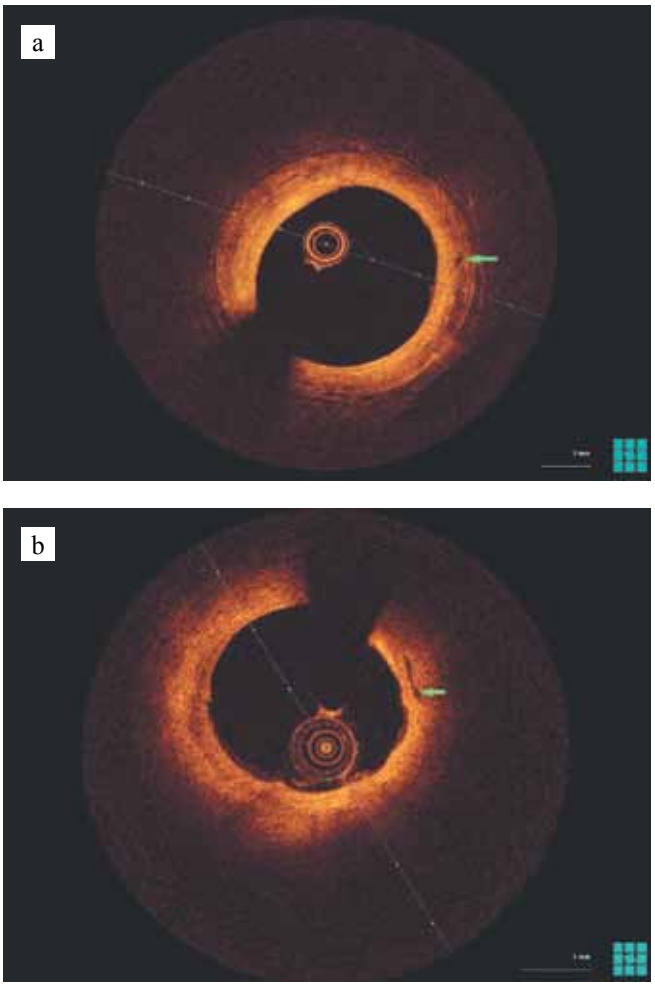

Fig. 3. Microchannels inside the atherosclerotic plaque (marked with a green arrow). OCT

Fig. 4. Cholesterol crystals inside the plaque (marked with a green arrow). OCT

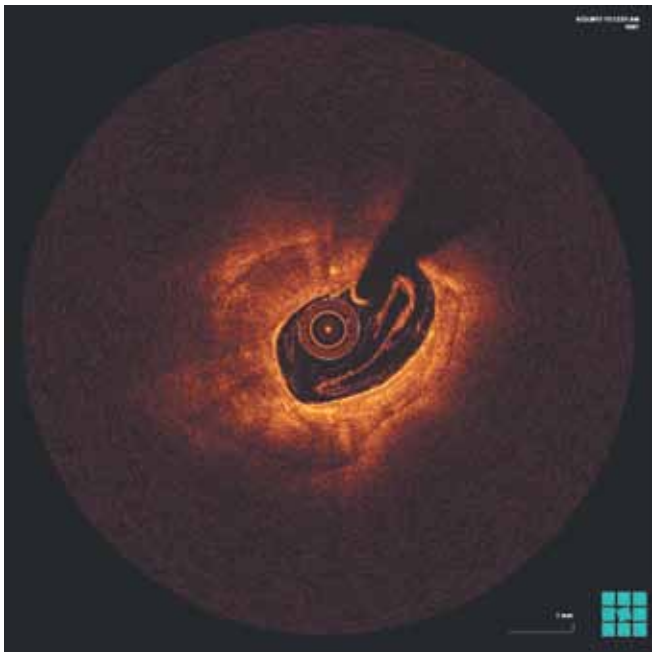

Fig. 5. Calcified nodule inside the atherosclerotic plaque (OCT). Calcifications are determined along the entire circumference of the vessel lumen (4 quadrants)

Calcification is one of the ACS development mechanisms (Fig. 5). In patients with even initial signs of CKD, calcifications are more likely to be detected as compared to the general population. Calcification is becoming more pronounced as renal dysfunction progresses. It is independently associated with cardiovascular mortality. Calcification is most pronounced in patients with end-stage renal disease (ESRD) [25]. It has been demonstrated that the number of calcifications distorting the vessel lumen is higher in patients with acute ST-elevation myocardial infarction [26].

There are few studies on coronary artery atherosclerosis in CKD patients. These studies are heterogeneous. Reports published to date significantly differ in methodology and criteria for inclusion of patients. Therefore, the results of these studies also differ [11, 27, 28].

Kato et al. conducted a study of the morphological characteristics of coronary atherosclerotic plaques using OCT among CKD and non-CKD patients. CKD was defined as eGFR <60 mL/min per 1.73 m^2 calculated using the Modification of Diet in Renal Disease (MDRD) equation. When lipid was present in ≥90° in any of the cross-sectional images within the plaque, it was considered to be a lipid-rich plaque. In lipid-rich plaques, the lipid arc was measured on the cross-sectional view at 1-mm intervals over the entire length, and the values were all averaged. In addition, the lipid length, lipid index (mean lipid arc multiplied by lipid length, mm°), and the fibrous cap thickness were measured. The authors also studied the presence of thin-cap fibroatheroma (a lipid-rich plaque with fibrous cap thickness ≤65 μm at the thinnest part), calcifications, macrophage accumulations, cholesterol crystals, microchannels, plaque disruption,

and intracoronary thrombus (divided into "white" and "red"). It was shown that compared with non-CKD patients, plaques in the CKD patients had a wider lipid arc, longer lipid length and a larger lipid index. In addition, calcifications, cholesterol crystals, and plaque disruption were more prevalent in CKD patients. Prevalence of lipid-rich plaques, thin-cap fibroatheroma, macrophage accumulations, microchannels, and thrombus was similar between the groups. Lower GFR and the presence of diabetes were independently associated with a larger lipid index [29].

In a study by Dai et al. decreased renal function was determined by changes in GFR levels. Creatinine level, cystatin C level and both indicators were used in calculating the eGFR. Patients were divided into 3 groups according to the eGFR calculated: the 1st group consisted of patients with GFR ≥90 mL/min/1.73 m^2, second – GFR 60–89 mL/min/1.73 m^2, third – GFR <60 mL/min/1.73 m^2. The fibrous cap thickness varied in all patient groups. The average lipid arc, lipid length and lipid index were different only when comparing groups 1 and 2, and groups 1 and 3. The number of plaques with cholesterol crystals significantly differed only when comparing groups 1 and 2. The number of plaques with calcification significantly differed only when comparing groups 1 and 3 [30].

In a study by Chin et al., hemodialysis patients who underwent OCT imaging were compared 1:1 with non-CKD patients. CKD absence was defined as eGFR >60 mL/min per 1.73 m^2, as well as absence of any signs of kidney injury. Analysis of the culprit plaques showed that the main and control groups of patients differed in the mean calcium arc and maximum calcium arc. Analysis of non-culprit plaques showed that the main and control groups of patients also had different mean calcium arc and maximum calcium arc. The authors conducted additional analysis of the main group of patients, dividing the latter into tertiles according to hemodialysis duration. Analysis of the culprit plaques demonstrated that the patient subgroups differed in mean calcium arc and maximum calcium arc, as well as number of thin intimal calcium, which was defined as an arc of calcium >30° within intima <0.5 mm thick. In the analysis of non-culprit plaques, the patient subgroups differed only in the mean calcium arcs [27].

Sugiyama et al. studied the morphological characteristics of native plaques in patients that were divided into 3 groups according to the values of eGFR calculated by a formula adapted for a Japanese population: the non-CKD group (eGFR ≥60 mL/min/1.73 m^2), CKD group (15 ≤ GFR < 60 mL/min/1.73 m^2) and ESRD group (GFR <15 mL/min/1.73 m^2 and/or hemodialysis). To count some parameters, the CKD and non-CKD groups were combined into a non-ESRD group. The CKD group had a larger lipid arc, longer lipid length and higher prevalence of lipid-rich plaque than the non-CKD group. The ESRD group had a thinner fibrous cap, higher prevalence of plaque rupture, and larger calcification arc than the non-ESRD group. Age, diabetes, and hemodialysis, but not GFR, were independently associated with the presence of calcified plaques [31].

Minami et al. investigated 140 non-culprit plaques in 84 patients with coronary artery disease who were treated with a statin and had two optical coherence tomography imaging: at first admission and 6 months after. Response of thin-cap area (fibrous cap thickness <200 μm) to statin therapy was the criterion. CKD was defined as eGFR <60 mL/min per 1.73 m^2 calculated using the MDRD equation. Compared with the initial OCT, a follow-up OCT revealed that there was a decrease in thin-cap area, average lipid arc, maximum lipid arc, lipid length, lipid index, size of stenosis by area and size of macrophage accumulations. Fibrous cap thickness at the thinnest part increased. Patients with larger thin-cap area at baseline OCT had a more significant reduction in thin-cap area at a follow-up OCT. Compared with those who initially took statins, patients who had not previously taken statins had a more significant reduction in thin-cap area at a follow-up OCT. CKD was a predictor for unfavorable response to statin therapy, while ACS at first hospitalization was a predictor for favorable vascular response to statin therapy [32].

ESRD patients are more likely to be detected with multivessel coronary artery disease and plaques with increased media thickness, activation of macrophages and marked calcification [33]. Most of these factors can be detected via OCT imaging, but such patients have a particularly high risk of developing contrast-induced nephropathy. In order to solve this problem, crystalloid or colloidal solutions (or their mixture) are used in place of an X-ray contrast agents, which avoids deterioration of kidney function. Karimi Galougahi et al. published a clinical case where a patient with advanced CKD (creatinine = 4.5 mg/dL [397.8 μmol/L], eGFR = 13 mL/min/1.73 m^2) not requiring haemodialysis had his LCA and diagonal artery successfully imaged with OCT. An optically clear medium was created by a mixture of saline and colloid infusate. Post-OCT renal function remained stable [34]. Unfortunately, the above study contains limited information on the features of atherosclerotic coronary lesions in this patient and the solution used. Azzalini et al. published a detailed description of a clinical case where a stage IV SD and CKD patient (GFR 16 ml/min/1.73 m^2) had his LCA and diagonal artery successfully imaged with OCT. An optically clear medium was created with a Dextran 40 colloidal solution at an infusion rate of 4.0 mL/min, the total amount of solution introduced being 14.0 mL. Kidney function remained stable during the patient's hospitalization [35]. Koga et al. published a clinical case where a patient receiving hemodialysis was visualized *in vivo* with calcified thrombus by OCT, IVUS and angioscopy. The authors did not indicate which solution was used to create the optically clear medium [36]. Ozaki et al. enrolled 22 pati-

ents with 25 coronary stented lesions in their study. Each patient was subjected to OCT using a contrast medium and OCT using a mixture of dextran 40 and lactated Ringer's solution. It was demonstrated that the number of segments available for analysis did not differ between OCT using contrast medium and OCT using a mixture of dextran 40 and lactated Ringer's solution (97.9% vs. 96.5%, respectively) [37].

CONCLUSION

In patients with impaired renal function, OCT permits more accurate assessment of the morphology of plaques, inflammation severity and "vulnerability" of plaques in general. OCT allows to clearly visualize calcification and evaluate its severity with high accuracy. Only OCT can enable one to see plaque erosion and distinguish it from plaque rupture. This may be important for determining further patient management tactics, such as revascularization feasibility. If revascularization is the choice, the decision then shifts to optimal choice of a stent or scaffold. There are major limitations on the use of OCT in CKD patients due to the risk of developing contrast-induced nephropathy. Therefore, further improvement on the technique for replacing blood with an optically clear medium is needed. One of the crucial tasks at present is to carry out large-scale multicenter studies to clarify the possibility of detecting vulnerable atherosclerotic plaques via routine use of OCT and identifying the possibility of reducing ACS risk. Also critical is to identify the impact on patient survival without adverse events as one of the underlying goals of any intervention.

The authors declare no conflict of interest.

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ACID ION MODIFICATION IN A DIALYSIS FLUID

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Apart from its main electrolytes – sodium, potassium, calcium and magnesium – a dialysis fluid (DF) contains a buffer for correction of acidosis. A small amount of acid is added to the DF to prevent calcium and magnesium precipitation. Acetic acid has traditionally been used for this purpose. Several studies have shown that acetate ion, even in small concentrations, can cause a number of adverse events, such as low blood pressure, production of proinflammatory cytokines, etc. This literature review aims at considering alternative acidic components of DF, such as citric, hydrochloric, and succinic acids, as well as their advantages, possibilities and features of their use in wide clinical practice.

Keywords: dialysis fluid, acetate, citrate, hydrochloride, succinate.

INTRODUCTION

In hemodialysis, the patient's blood contacts the dialysis fluid (DF or dialysate) through the membrane; transmembrane metabolism is the primary physical mechanism behind the method. DF contains the main electrolytes, which are balanced in concentrations with human plasma – sodium, potassium, calcium and magnesium (in the form of chlorides), as well as a buffer designed to correct acidosis. Since kidney failure is characterized by deficit of bicarbonate, the main buffer system, there should be direct replacement of this deficit during treatment. Accordingly, dialysate contains bicarbonate in a concentration exceeding the physiological level (about 32 mmol/L). Bicarbonate liquid needs to be stabilized to prevent precipitation of hardness salts (calcium and magnesium carbonates) and bring the pH to physiological values. Before hemodialysis came onboard, this problem was addressed by saturating DF with carbon dioxide, passing carbon dioxide through it (Fig. 1).

Due to the technical complexity of this approach with the introduction of long-term hemodialysis, bicarbonate was replaced by acetate as the buffer system in the dialysate. The use of stable acetate-based dialysate prepared from a single-component concentrate has greatly simplified the technical implementation of hemodialysis. How-

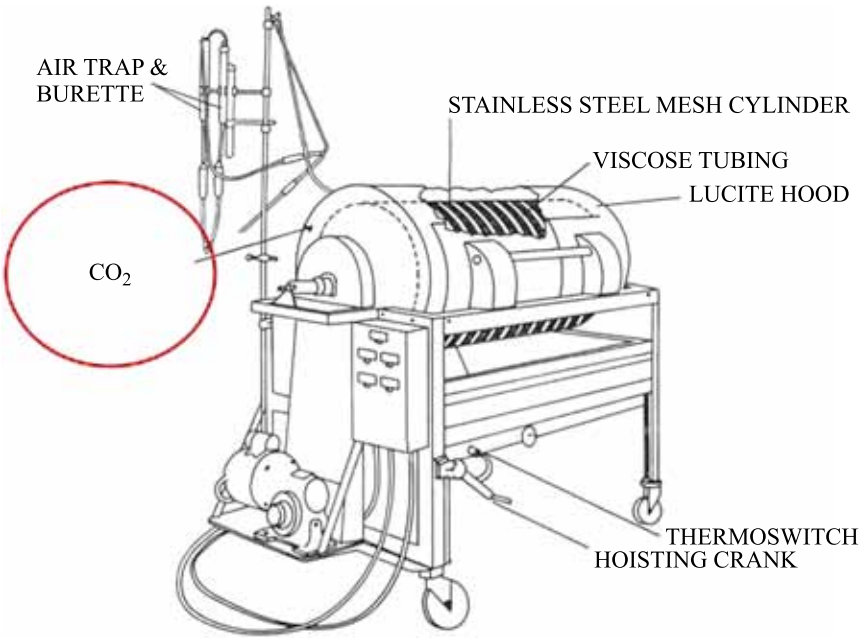

Fig. 1. Stabilization of bicarbonate dialysis fluid with carbon dioxide in one of the first hemodialysis systems (Kolff–Brigham artificial kidney, 1948

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ever, from a clinical point of view, acetate has proven to be unfavorable. In the absence of bicarbonate in DF, its concentration was reduced in the blood plasma after commencement of dialysis, and the absence of sufficient carbon dioxide tension in the solution caused hypoxemia. In addition, high acetatemia, progressing during hemodialysis, was complicated by numerous episodes of intradialytic hypotension associated with peripheral vasodilation [1] and by reduced myocardial contractility. As a result, bicarbonate was reintroduced as the main buffer in dialysis fluids in hemodialysis in the 1980s. The problem of dialysate stabilization was solved by dividing the dialysis concentrate into two components, one of which contained sodium bicarbonate, and the second was added with acid. When mixed in a proportional system, part of bicarbonate ions reacts with hydrogen ions to achieve sufficient carbon dioxide tension and stabilize DF acidity at a level that prevents calcium and magnesium salt precipitation.

NECESSARY CLARIFICATION

Thus, from a chemical point of view, carbonic acid is the only acid that must be present in a dialysate. However, this substance has an extremely low melting point and is characterized by very high volatility at atmospheric pressure. In order to obtain carbonic acid in a solution, you either saturate the liquid with carbon dioxide gas (Fig. 2, a), or obtain carbonic acid in a chemical reaction of bicarbonate with protons, as is the case in modern hemodialysis machines (Fig. 2, b).

The need to deliver hydrogen ions to the DF is why an acid is added to it. The negative ion in the acid composition is only an additive that you have to bear with. Depending on its chemical composition, this ion can play both a positive and a negative role in the body.

ACETATE ION

Compared to acetate-containing bicarbonate dialysate, the content of acetate ion in modern bicarbonate dialysate is an order of lower magnitude, = 3–6 mmol/L, depending on the DF acidification method. In the patient's body, acetate ion is metabolized in the liver and muscles to form an equivalent amount of bicarbonate. Some acetate ions are conjugated with coenzyme A, which requires conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). cAMP triggers nitric oxide (NO) production and vasodilation. In addition, acetate ion stimulates production of potent pro-inflammatory cytokine interleukin-1. There are no clear indications of the clinical consequences of the influence of trace amounts of acetate. Nevertheless, it is assumed that for certain categories of patients, such effects is irrelevant as confirmed by some studies on acetate-free methods [2, 3].

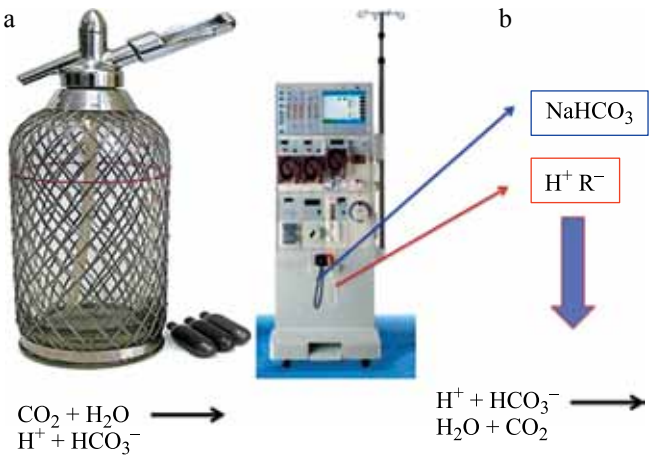

Fig. 2. Carbonic acid production in a solution

CHLORON ION

Since all the electrolytes in a dialysate solution are in the form of chlorides, total concentration of chlorine ions is very high, about 108 mmol/L. It is therefore logical to use hydrochloric acid for DF acidification. A 3 mmol/L increase in concentration of chlorine ions has no effect on the patient's body. At the same time, such DF does not contain any extraneous anions, including acetate ion. A large observational study conducted in France, which included data from over 15,000 patients, showed improved survival in patients aged 70 years and older who have never come in contact with acetate ion-containing DF [4]. A team of authors [2] showed that the use of acetate-free bicarbonate dialysate with slow continuous dialysis in patients with acute kidney injury after cardiac surgery was able to reduce the rate of hemodynamic complications by 3.8 times in comparison with standard acetate-containing bicarbonate dialysate. The use of standard bicarbonate dialysate was associated with 12-fold increased acetate ion concentration in blood plasma from the upper limit of the norm [2].

Widespread use of hydrochloric acid concentrate in common hemodialysis is obviously limited by the technical difficulties associated with the aggressiveness of hydrochloric acid. Nevertheless, such concentrates today are available including in the domestic market, and one can expect an increase in their use.

CITRATE ION

If hydrochloric acid in dialysate solution can be considered intact with respect to metabolic processes in the body, then if acetic acid is partially or completely substituted with citric acid in the dialysate, there could be certain positive effects. In an energy-free metabolic process, 1 mmol of citrate produces 3 mmol of bicarbonate. Moreover, citrate metabolism is adequate not only for renal but also for liver failure, as shown by studies on citrate anticoagulation in the treatment of critically ill patients [5–7]. Citrate was found to have a positive effect on exercise tolerance in healthy volunteers [8]. *In vitro*

experiments have shown that citrate causes less activation of complement and granulocytes in comparison with acetate [9–11], and also reduces the severity of oxidative stress [12–14]. Besides, citrate has been widely used as an anticoagulant since 1914. Thus, when using this ion in a DF composition, one can naturally expect decreased need for anticoagulation in the extracorporeal circuit, as well as certain optimization of metabolic processes.

Several studies have shown that heparin dose can be reduced when using a citrate-enriched dialysate. So, Sands et al. showed that one-third reduction in heparin when using dialysate containing citrate does not increase prevalence of thrombosis in the extracorporeal circuit and is not associated with decreased dialysis efficiency [15]. When comparing heparin-free dialysis with periodic flushing of the extracorporeal circuit with dialysis, performed with citrate-enriched dialysate, there was no difference in the incidence of thrombotic complications [16]. The most encouraging results were obtained by Aniort et al. When performing online hemodiafiltration without using anticoagulant, the use of citrate-enriched DF prevented thrombosis in the extracorporeal circuit during 120 treatment sessions [17]. In yet another study [18], citrate was found to have a moderate anticoagulant effect. Besides, the authors noted decreased pre-dialysis levels of C-reactive protein and beta-2 microglobulin, absence of post-dialysis hypocalcemia and moderate concentrations of citrate ion (0.29 mmol/L at a concentration safe for regional citrate anticoagulation = 0.89 mmol/L). Decreased concentrations of C-reactive protein and beta-2 microglobulin was also noted when using citrate-based dialysate for hemodiafiltration [19]. Within 4 months after transferring 29 patients to maintenance hemodialysis with citrate-containing dialysate, Kuragano et al. noted that predialysis bicarbonate levels normalized in patients with initially low serum bicarbonate, the need for erythropoiesis-stimulating agents decreased and albumin levels increased. All positive changes disappeared after patients returned to treatment with standard acetate-containing dialysate [20]. Better acid-base correction with citrate-containing dialysate has been noted in several studies [21–23]. In addition, the authors note a regular decrease in post-dialysis levels of ionized calcium, and, accordingly, a certain increase in parathyroid hormone levels. Such observations are not universal, since in the already mentioned Panichi et al. [18], where dialysate calcium concentration was 1.5 mmol/L, hypocalcemia was not detected. The clinical consequences of the chelating ability of citrate with respect to calcium ions are not yet clear. Some authors suggest that standard concentrations of calcium and magnesium should be reviewed under the use of citrate-containing dialysate [21].

SUCCINATE (AMBER ACID)

In Russia, the acid component of bicarbonate concentrate has been industrially produced for several years. Here, acetic acid is partially, and in recent years, completely replaced with succinic acid. When using this prescription of concentrate in the clinic, there was decreased pre-dialysis sodium levels and systolic blood pressure along with decreased interdialysis hydration and severity of intradialysis hypotension. In addition, there was moderate increase in hemoglobin levels. The authors attribute such effects to the influence of succinate on angiogenesis, which allows mobilizing osmotically inactive sodium ions and making them available for excretion during hemodialysis [24]. Unfortunately, the experience of using succinate-containing dialysate and reports on this topic are very scarce and limited only to national sources.

CONCLUSION

Dialysate solution along with the dialysis membrane form the basis of hemodialysis system, which determines substance transfer patterns during treatment sessions. However, while many studies have been devoted to the study of the properties of dialysis membranes and their effect on dialysis therapy, there is substantially less amount of information on DF. To a certain extent, this is due to widespread introduction of central concentrates delivery systems for hemodialysis. Here, clinicians are limited typically to choosing between only two prescriptions. Nevertheless, excluding acetate ion from dialysate through the use of hydrochloric acid does not require any changes to the existing practice at dialysis centers. Due to the increasing elderly population and consequently, comorbidity of dialysis patients, such conversion seems very urgent. Clarifying the indications for the use of citrate-containing dialysate and determining its optimal composition require further research.

Thus, the process of driving out acetate ion from the commonly used dialysate solution, which began with widespread withdrawal from single-component acetate concentrate, is ongoing. In order to speed up this action and make it more meaningful, research in this direction should be intensified.

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POSSIBLE USE OF SPERMATOGONIAL STEM CELLS IN THE TREATMENT OF MALE INFERTILITY

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Spermatogonial stem cells, which are already present at birth in the testicles, are the progenitors of male gametes. These cells cannot produce mature sperm before puberty due to their dependence on hormonal stimuli. This feature of the reproductive system limits preservation of fertility only to males who can produce an ejaculate. Therefore, the use of cancer treatment which can lead to fertility loss has made sperm cryopreservation a standard practice. Prepubertal cancer boys – who are prescribed chemotherapy that is toxic to their reproductive system – are deprived of this fertility management procedure. This review focuses on the problem of obtaining and preserving spermatogonial stem cells for future transplantation to restore spermatogenesis. Development of these methods is becoming increasingly urgent due to higher survival rates in childhood cancer over the past decades thanks to improvements in diagnosis and effective treatment. Restoring and preserving fertility using spermatogonial stem cells may be the only option for such patients.

Keywords: spermatogonial stem cells, fertility, cell culture.

INTRODUCTION

Male infertility is a pressing issue, whose solution is of medical and psychosocial importance. It can affect the future of a nation to a certain extent. Properly understanding the pathological processes underlying fertility disorders is extremely important since various etiological factors and pathogenetic mechanisms disrupt the quantitative and qualitative parameters of sperm. According to estimates, over 8% of men of reproductive age seek medical help for infertility problems. In about half of subfertile (with reduced fecundity) couples, the male factor is the main cause. In about 12% of subfertile men, severe oligozoospermia or azoospermia is detected. In oligozoospermia, the semen ejaculated during an orgasm contains fewer sperm than normal for fertility. Genetic disorders, inflammatory, endocrine and infectious diseases, alcohol and drug abuse, radiation and use of certain gonadotoxic drugs, heavy metal and carbon dioxide poisoning can all lead to low sperm count. Azoospermia is a medical condition where the semen contains no sperm. There are two types of azoospermia – obstructive and non-obstructive. With obstructive azoospermia, sperm cannot get into the semen due to impaired patency or absence of vas deferens. The ducts located in the epididymis can also be affected. Various infectious and inflammatory diseases, injuries, varicocele, or congenital anomalies of the genitourinary tract sometimes lead to obstruction of ejaculatory ducts. Non-obstructive azoospermia results from endocrine and genetic diseases, after radiation exposure, with some metabolic disorders (diabetes mellitus), and oncological diseases. In the treatment of most cases of azoospermia, especially the non-obstructive forms of genetic origin, assisted reproductive technologies takes priority, with the aim of restoring the qualitative and quantitative parameters of sperm or ensuring sperm maturation *ex vivo*. Therefore, the possibility of obtaining the required amount of mature sperm cells via *in vitro* manipulation gives hope to men with severe spermatogenesis of becoming biological fathers.

Spermatogonial stem cells (SSCs), which are already present at birth, are stem cells of the testicle [1, 2]. These cells cannot produce mature sperm before puberty due to their dependence on hormonal stimuli. This biological characteristic of the reproductive system limits fertility preservation only to males capable of producing an ejaculate, because the standard procedure for fertility preservation is semen cryopreservation, which usually guarantees future achievement of paternity [3–5]. This possibility becomes very important in men whose reproductive system has been exposed to the toxic effects of infertility-causing chemotherapy and radiation for cancer [2, 6].

Over the past decades, childhood cancer survival rates have increased due to improved diagnosis and more effective therapy [4]. However, anti-cancer treatment often has a devastating effect on the reproductive system of prepubertal boys. Preliminary sperm preservation in the boys is impossible since there is no sperm to preserve. Cryopreservation of testicular tissue for future extraction and transplantation of SSCs is an option currently being developed for restoration of spermatogenesis after chemotherapy in prepubertal boys. Successes

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recorded in restoring spermatogenesis in animals after SSC transplantation hold out hope that this direction will succeed [7–11]. Development of autologous transplantation technology for SSCs capable of differentiating into mature spermatozoa will be of great importance for practical medicine [12]. With successful implementation of necessary research on SSC production, storage and transplantation, prepubertal boys with cancer will be able to father children in the future [3]. The following describes the basics of obtaining (isolating), identifying and cultivating SSCs *in vitro* for proliferation. Prospects for using SSCs to preserve fertility in prepubertal boys are assessed.

ISOLATION OF SPERMATOGONIAL STEM CELLS

Cell isolation studies are typically based on their ability to self-renew [13, 14] and on optimization of procedures increasing the purity of SSCs obtained, primarily by preventing contamination with other cell types. Generally, SSCs are isolated by enzymatic digestion, usually involving a combination of enzymes such as collagenase, trypsin, and DNase I [15]. Various methods have been developed to obtain highly pure population of SSCs, such as morphology-based selection associated with differential precipitation, extracellular matrix selection, fluorescence-activated cell sorting (FACS), and magnetic-activated cell sorting (MACS).

Morphology-based selection of SSCs is the simplest and cheapest method, but it has the lowest efficiency [16]. Since this method is based on enzymatic isolation of cells and subsequent precipitation at different times, the resulting samples are contaminated with various types of testicular cells, such as Sertoli cells, Leydig cells, myoid cells, and fibroblasts [16, 17]. These cells may release growth factors, hormones and extracellular matrix elements to some extent and thus interfere with *in vitro* self-renewal and proliferation of SSCs [17]. Extracellular matrix selection uses various extracellular proteins, such as laminin and fibronectin, to stimulate SSCs adhesion. These substrates are capable of binding other extracellular matrix components, and they are widely used in *in vitro* cell culture to facilitate cell attachment and stimulate cell proliferation [18]. Since SSCs have weak adhesion potential, it is necessary to facilitate their attachment by coating with substrates to maintain viability *in vitro* [18]. A high-pure SSC population can be obtained using sorting assays such as FACS and MACS. Although the described methods can basically provide SSC cultures of high purity, they have drawbacks due to the resource-intensive procedures and technical difficulties, which often lead to low cell production and insufficient cell viability [15, 18].

To obtain highly pure cell suspensions, it is advisable to use a combination of different SSC isolation methods and look for new methodological approaches. Analysis of the protocols used has shown that most of them involve differential seeding of the initial cell suspension to eliminate other types of testicular cells. This method separates the cells according to their distinctive attachment features. Cells are seeded directly on tissue culture plates or matrix-coated plates using gelatin, laminin, fibronectin or collagen [16, 17]. A recent study has demonstrated effective removal of contaminating cells and high-precision selection of SSCs using two-step purification. First, the collected SSCs were cultured on somatic and Sertoli cells, and after isolation, the cells were purified by centrifugation in bovine serum albumin density gradient. With this method, most unnecessary cells were removed, and the purity of the SSC culture reached above 91.5% [18].

Given that the main purpose of isolating, culturing, and transplanting SSCs is to preserve and restore fertility, and that most patients who benefit from SSCs transplantation have cancer at the time of testicular biopsy, the risk of re-introducing malignant cells must be eliminated. It is necessary to obtain high-pure SSC cultures by a certain combination of identified positive and negative selection markers in order to exclude malignant contamination (1). Despite this, it has not yet been possible to identify – with enough certainty – a combination of positive and negative markers that would obtain a pure SSC suspension [19]. However, a recent study showed that with a combination of CD90 (positive marker) and CD45 (negative marker), the germ cell suspension appeared to be free of cancer cells [12].

CULTIVATION OF SPERMATOGONIAL STEM CELLS *IN VITRO*

As with stem cells in many tissues, the proportion of SSCs is substantially lower than that of surrounding somatic cells. In mice, SSCs located in the epithelium of the seminiferous tubules account for only about [12, 20]. To get enough SSCs for future autotransplantation, their mass needs to be artificially increased, given that the volume of an adult testis is approximately 60 times larger than the biopsy taken from a prepubertal boy. The table shows the steps for obtaining and transplanting SSCs.

Successful long-term *in vitro* proliferation of SSCs was first demonstrated in mice and rats [21–27]. It was shown that during cultivation, the quantity of rodent SSCs can increase exponentially, and they can maintain their biological potential for productive spermatogenesis and restoration of fertility after transplantation into the testes of infertile recipient mice [9, 10, 24–28]. Preservation of non-human primate SSCs by short-term cultivation was then reported [29], and several groups reported both short-term and long-term cultivation of human SSCs, both in adult males and in prepubertal boys [30–36]. In one study [30], cultivation of human testis cells for 64 days increased SSC numbers by more than 18,000 times.

Table

Key achievements in *in vitro* production of SSCs for use in restoring fertility

Year	Authors	Most significant research	Type
1971	Huckins	Spermatogonia renewal and differentiation model and spermatogonial stem cell detection	Rat
1994	Brinster and Avarbock	First successful transplantation of testicular cells from one mouse to another, emergence of offspring from a donor	Mouse
1998	Nagano et al.	Preserving *in vitro* SSCs for 4 months using a somatic cell feeder layer	Mouse
1999	Schlatt et al.	Xenotransplantation of a testicular cell suspension obtained from one primate into the testes of another	Monkey
2002	Nagano et al.	First report on successful cell colonization in mouse testes after xenotransplantation of human SSCs	Human
2003	Kanatsu-Shinohara et al.	Prolonged *in vitro* SSC propagation using GDNF without cell immortalization in culture	Mouse
2005	Keros et al.	Evidence of successful cryopreservation of testis biopsies without compromising their structural integrity	Human
2005	Kanatsu-Shinohara et al.	Long-term propagation of SSCs in serum-free feeder medium	Mouse
2009	Sadri-Ardekani et al.	Long-term *in vitro* propagation of SSCs obtained from adult testis, while maintaining functionality	Human
2011	Sadri-Ardekani et al.	Long-term propagation of SSCs obtained from prepubertal testes, while maintaining their function	Human
2012	Hermann et al.	Production of functionally active sperm capable of fertilizing oocytes by immature macaques subjected to SSC autotransplantation	Monkey
2014	Langenstroth et al.	Separation of somatic and germ cells to create SSC cultures	Monkey
2018	Sharma et al.	Differentiation of xenograft SSCs of monkeys depending on gender and fertility of recipient mice	Monkey

The culture system developed for SSCs is usually based on the use of a feeder medium supplemented with hormones and growth factors, as well as feeder layer of somatic cells [35]. A key factor for most cell cultures is animal fetal serum, but it has proven to be unfavorable for SSC proliferation [25]. Various serum concentrations in the growth medium were used in SSC cultivation, but none of them was able to enhance SSC proliferation compared to the serum-free medium [25]. Purified proteins and additives are required to compensate for the absence of hormones or growth factors supplied by serum [37]. Some of the identified growth factors required for SSC proliferation are the glial cell-derived neurotrophic factor (GDNF) and fibroblast growth factor 2 [14, 26, 38].

An increase in the number of SSCs in the *in vitro* culture system should ideally resemble the *in vivo* situation [39]. *In vivo*, there is a complex niche environment where SSCs and somatic support cells interact to establish the necessary intracellular signaling. Various factors required for stem cell preservation have been identified. Artificially simulating a niche environment is a very difficult task because there are many factors behind interaction between SSCs and somatic cells, and most of them are not well characterized. The use of a feeder layer consisting of somatic cells (often inactivated mouse embryonic fibroblasts, MEFs) is considered important for successful SSC propagation [8, 40]. Growth of spermatogonia on the feeder layer can lead to formation of three-dimensional aggregates, called clusters, which contain many cell types, including SSCs [41]. In order to increase their number, it is necessary to provide *in vitro* microenvironment that is as close as possible to the SSC niche in the testes [13, 39]. SSC niche consists of various supporting cells, such as Sertoli, peritubular, myoid and Leydig cells [18]. However, enzymatic treatment to dissociate tissue and isolate cells destroys the integrity of such an important microenvironment. Feeder layer has a positive effect on SSC preservation, since the cells contained in it produce growth factors and cytokines, which contribute to the conditioning of the medium [8, 42–44]. Similarly, a feeder layer (MEF) provides a convenient surface for SSC attachment. Like any other type of cell, they directly depend on the topography, roughness, and stiffness of substrates [45, 46]. The use of several techniques that improve SSC cultivation conditions has facilitated long-term increase in the pool of cells obtained from different strains of mice of different age groups. SSCs remained undifferentiated for 6 months without loss of function and ability to restore normal spermatogenesis after transplantation [26]. However, clinical use of SSCs requires development of culture systems without xenogenic and feeder culture systems in order to avoid pathogenic contamination [47].

POTENTIAL USE OF SPERMATOGONIAL STEM CELLS IN THE FUTURE

Since the late 1990s, scientists have been able to restore spermatogenesis in animal models. Since then, great

progress has been made in studying the characteristic features of spermatogonial stem cells and the possibility of preserving them *in vitro* by developing culture systems [42, 47, 48].

For future clinical use in humans, the culture medium should preferably not contain animal serum due to possible zoonotic or xenotoxic effects. The use of somatic cells existing in testicular biopsies helps preserve SSCs and can replace the use of exogenous cells located in the feeder layer. On the other hand, it can be imagined that cultivation in a medium in which serum is absent [49] or some growth factors [40] can affect the function of SSCs and decrease their potential [44]. Intervention in cultural conditions is a "double-edged sword": on one hand, there is increased quantity of SSCs due to addition of certain factors, while on the other hand, their functionality may be impaired by the same additives. Ability to modulate *in vitro* conditions involved in self-renewal control, as opposed to SSC differentiation, can lead to production of functionally active gametes *in vitro* [42]. These products, associated with transplantation methods of animal and human models, will facilitate the study of molecular and cellular biology of differentiation of male germ cells and make it possible to develop new infertility treatment strategies [16, 42].

Prospects for restoring male fertility have great potential in basic and applied science [15, 50]. Development of culturing techniques may offer future hope for preserving fertility in cases with no other way out, for example, in prepubertal boys with cancer. Many clinics already cryopreserve testicular tissue from men with cancer. However, methods should be developed to eliminate the risk of reintroducing malignant cells during SSC transplantation [2].

In addition, various studies have shown that SSCs can differentiate into various types of *in vitro* cells, such as cardiomyocytes and nerve cells. It is important to note that these cells have several advantages over embryonic stem cells, such has absence of ethical concerns about their use and origin. In addition, they have lower incidence of tumorigenesis and graft rejection. Based on the-

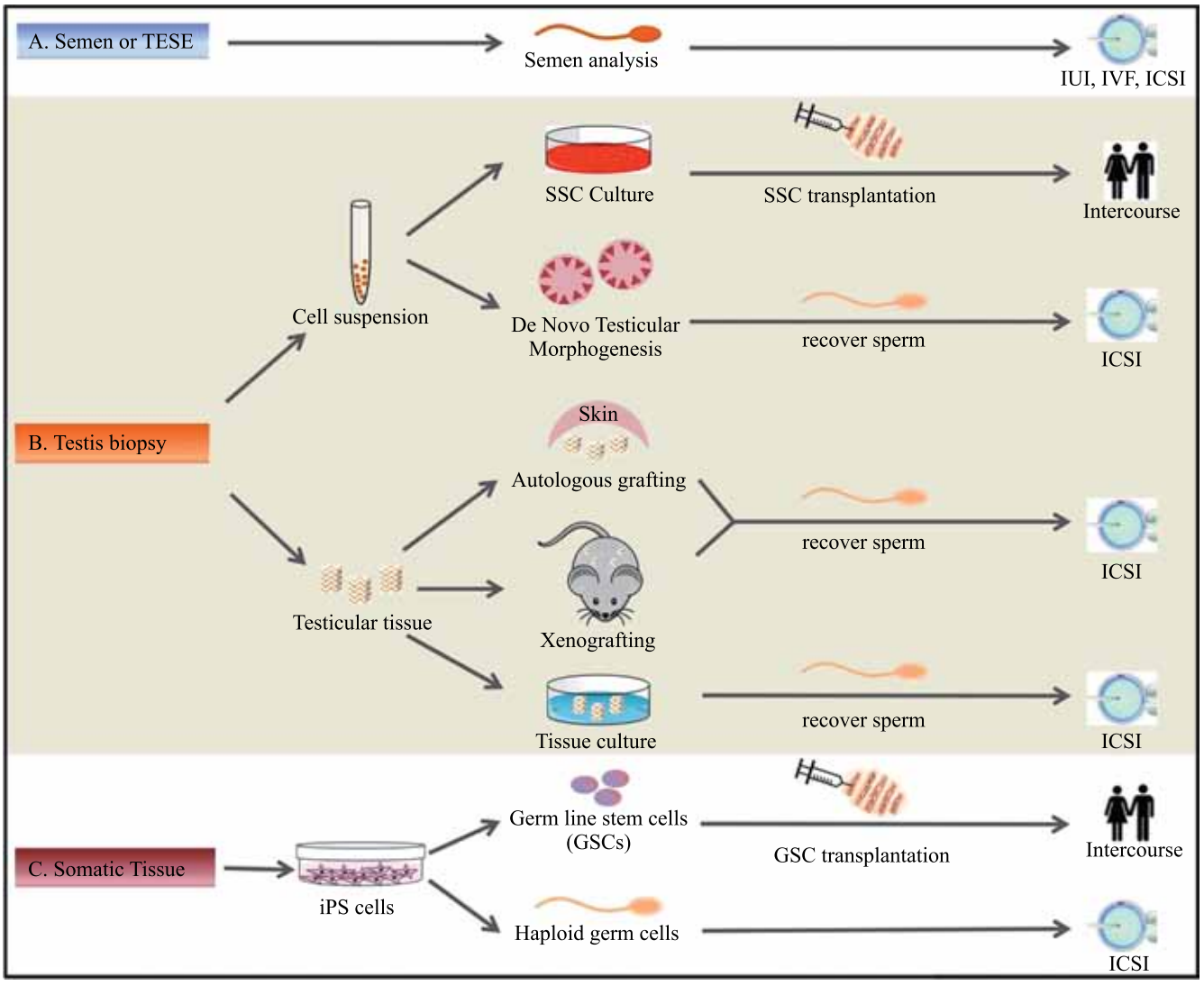

Fig. Standard and experimental treatment options for male infertility (by K. Gassei, K.E. Orwig, 2015)

se assumptions, SSCs may be one of the most promising candidates for clinical applications in cell therapy [23].

As mentioned above, the size of biopsy that can be obtained from prepubertal male testis is relatively small and may contain small amount of SSCs. The amount of SSCs that will be required to regenerate spermatogenesis and achieve fertility in humans is not exactly yet known. However, it is reasonable to assume that the number should be substantially increased in culture prior to transplantation to ensure reliable engraftment and effective spermatogenesis. Each group of researchers reporting on human SSC culture used different cell isolation and cultivation techniques, different feeder media and matrix substrates [51, 52], different sets of growth factors and different methods for evaluating results. Until recently, no method for obtaining human SSC cultures has been independently replicated by another research group. Replication is needed in order to confirm true success and achieve real progress [19]. Besides, while SSC transplantation to regenerate spermatogenesis using functionally active spermatozoa and production of offspring is the gold standard for assessing the quality of rodent SSCs obtained, there is no equivalent analysis of human SSCs. Molecular markers and xenotransplantation between humans and mice may be reasonable surrogate analyzes, but so far there is no agreement within the scientific community that would allow experiments with human SSCs. Perhaps, implementation of *de novo* morphogenesis of testis and/or use of decellularized testes will help create a complete model of human spermatogenesis and conduct the necessary experiments.

Generalizing the numerous male infertility studies described in this review to a certain extent, they can be shown in the form of a small diagram (Fig.) and comments, which are presented below [25].

A. Spermatozoa obtained from ejaculated sperm or by testicular sperm extraction (TESE) from the testes of infertile men can be used to facilitate pregnancy via intrauterine insemination (IUI), *in vitro* fertilization (IVF) or IVF with intracytoplasmic sperm injection (ICSI).

B. Where it is not possible to obtain spermatozoa by biopsy, testicular tissue containing SSCs can be obtained. Testicular tissue can be digested with enzymes to produce a cell suspension from which significant amount of SSCs can be obtained by culturing, which, in turn, can be transplanted into the patient's testes. This method can regenerate spermatogenesis, and possibly natural fertility. Heterogeneous suspensions of testicular cells also have the potential for *de novo* morphogenesis of testis with seminiferous tubules and polarized epithelium surrounded by basal membrane with germ cells inside and interstitial cells outside the tubules. Sperm generated in "rebuilt" testes can be used to fertilize eggs by ICSI. Intact testis tissues from prepubertal animals can be auto- or xenotransplanted under the skin or in the scrotum and produce mature sperm that can be used to fertilize eggs by ICSI. Sperm can also be generated when immature testicular tissue is preserved in culture and used to fertilize eggs via ICSI.

C. Patient-specific induced pluripotent stem cells (iPSCs) can be derived from the patient's somatic tissues (e.g., skin or blood) and differentiated into germline stem cells (GSCs) for further introduction into the patient's testes. This technique may have the potential for regeneration of spermatogenesis and natural fertility. It is also possible to differentiate iPSCs cells into spermatozoa, which can be used to fertilize eggs by ICSI.

Apparently, help from social efforts can play a significant role in initiating and supporting scientific research on prevention and treatment of male infertility. The society is beginning to realize the benefits of fertility preservation in pediatric and adolescent patients suffering from cancer and undergoing gonadotoxic chemotherapy, as well as those with severe sexual development problems [53]. Certain efforts are being made to create multidisciplinary community groups that would assist in developing studies on fertility preservation for pediatric patients and assessing relevant ethical issues and necessary material costs. In particular, the Oncofertility Consortium has been established to provide guidance for health care providers aiming to develop programs at institutions lacking pediatric fertility preservation services [54].

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CURRENT STATE OF THE PROBLEM AND RESULTS OF *EX VIVO* PERFUSION OF DONOR HEARTS

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Patients with drug refractory end-stage heart failure fall into the severe category of cardiological patients. Numerous studies have shown the superior efficacy of heart transplantation over other treatments for end-stage chronic heart failure. However, despite decades of achievements in transplantology, shortage of donor organs remains a pressing and unresolved issue. The only way to reduce shortage of donor organs is to use donors with advanced criteria, which requires the use of latest technologies in organ resuscitation and conditioning.

Keywords: heart failure, heart transplantation, ex vivo perfusion.

Over the past 15 years, heart failure has remained the leading worldwide cause of death. The disease affects 1 to 2% of the total population with the risk of development in people over 55 is 33 and 28% for men and women, respectively [1]. With the increasing life expectancy, such risk factors as arterial hypertension and coronary heart disease continue, and the predicted prevalence of heart failure will increase by 20% by 2030, thus remaining the primary cause of death [2].

Despite more than half a century of research in the treatment of chronic heart failure, the development of various devices for assisted circulation, stem cell therapy, etc., there is still no treatment comparable in effectiveness to a human donor heart transplant [3]. Heart transplant is the "gold standard" for treating patients with end-stage chronic heart failure. Unfortunately, an acute shortage of donor organs has been and remains the vulnerable spot of this treatment. Thus, due to donor organs shortage, the number of heart transplants performed in the United Kingdom and many Western countries has fallen sharply in recent decades, while the number of patients on the waiting list continues to grow [4].

In the UK, of the approximately 750,000 patients requiring heart transplant, only 0.02% receive it. Due to this discrepancy between the need and possibility, almost 10% of patients on the waiting list die annually [3]. According to the report by the Canadian Institute of Medical Information, in Canada for the past 10 years, the annual mortality rate of patients awaiting heart transplant has been 16% [5].

The first successful clinical cadaver heart transplant was performed with an organ donated after death from circulatory arrest in 1967 by Christian Barnard and his team at Groote Schuur Hospital [6]. In that time, before the criteria were established for brain death, a heart transplant could be performed only if the donor and recipient were in close proximity to each other. When the term "brain death" was introduced at the legislative level, it allowed the remote sampling of donor organs. At the same time, for years, the use of the hearts of donors who died from circulatory arrest has been discontinued.

However, in some time, the idea of using such organs for transplant returned to life. To meet the needs in donor organs, surgeons were forced to expand the criteria for donor organ collection, in particular through the use of organs received from donors who died from circulatory arrest or had an asystole episode. In the literature, such donor organs are called "organs from expanded criteria donors (ECD)", "organ donors after irreversible cardiac arrest" or "asystolic donors".

The strategy aimed at reducing the need for donor organs through the use of transplants from expanded criteria donors has proved safe and has been formed in protocols in accordance with national and international standards in Australia, Belgium, the Netherlands, Spain, the UK and the USA. According to G. Citerio et al., *ex vivo* restoration of a marginal donor organ would increase the donor pool by 15–30% [7, 8].

The use of such a donor pool became possible due to significant progress in the field of resuscitation and conditioning of donor organs, in particular due to the development of organ perfusion systems that are able to solve a number of such difficult tasks as assessing the functional status of the transplant, time of ischemia, and logistics of donor to recipient delivery.

The main issue of using hearts after donor death from circulatory arrest is the time of thermal ischemia, as well as the need to maintain myocardial viability during delivery. Despite the fact that pharmacological cold cardioplegia is the standard for preserving donor organs, after

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four hours the transplant function can be compromised by a long ischemic period, especially in patients of the older age group [9].

This technique of organ preservation is the greatest risk factor for primary allograft dysfunction and death [10, 11]. An increase in the time of cold ischemia from 3 to 6 hours doubles the risk of death one year after transplant, compared to 50% decrease in predicted one-year mortality, if the period of ischemia is less than one hour [12]. These data were also confirmed by US scientists, proving that reducing ischemic time by one hour increases survival by 2.2 years [13]. J. Kobashigawa et al. found that ischemia exceeding 4 hours significantly increases the risk of primary transplant dysfunction which is associated with 8% mortality after 30 days and increased mortality in 5 and 15 years after transplant [14].

The use of expanded criteria for the donor organs collection, though providing increase in the availability of heart transplants, can be accompanied by a number of complications [15]. Therefore, it became apparent that expanding the criteria for organ harvesting needs alternative, more physiological conditioning techniques. *Ex vivo* warm perfusion of the heart is an alternative technique of preserving the transplant, which allows improving the function of the donor organ and expanding the donor pool, neglecting the time required to deliver the organ from a donor to the recipient [16].

TransMedics (Massachusetts) system (TMS) is the first commercially available device to transport a donor heart in a normothermic perfusion state. Perfusate is a patented pouring solution with the addition of insulin, antibiotic, methylprednisolone, sodium bicarbonate, multivitamins and fresh donated blood [3].

A number of studies have proven the advantage of exothermic normothermic perfusion *ex vivo* over hypothermic preservation of donor hearts. It is important to note that thermal ischemia tolerance of the donor hearts donated after circulatory arrest is higher than that of hearts from donors with brain death [17]. TMS can be successfully used to assess the functional capabilities of "expanded criteria" organs, heart donors with low EF, previous cardiac arrest, long-term (>4 h) predicted ischemia and unknown coronary bed status due to the absence of coronary angiography before the implantation stage, thus avoiding the potential risk of dangerous complications and death for recipients [18, 19].

Heart EXPAND Trial results showed that 75 of 93 donor hearts perfused with TransMedics system were successfully transplanted, resulting in 81% utilization rate. The average OCS perfusion time was 6.35 h. 30-day and 6-month survival rates were 94.7 and 88%, respectively [20].

The use of TMS allows can solve another very important problem that reduces the donor pool: the problem of logistics of organ delivery to the recipient. According to various estimates, about 60% of potential allografts are considered unsuitable for transplant for various reasons, including the impossibility of the earliest possible organ delivery to the recipient [21]. In the United States, only 30–35% of donor hearts are used for transplant due to storage restrictions using standard pharmacological cold protection.

TMS extends the time for the donor organs outside the body to at least 8 hours, expands the potential geography of donor bases and allows angiography of the donor organ inside the system, which is especially important for donors of the older age group. For instance, in 2015 in Australia, a donor heart was successfully transplanted after 10.5 hours of TMS perfusion. In the UK, supposedly, this would provide for an international exchange of organs with Europe and the eastern United States. Such an expansion of the donor pool is one of TMS main potential advantages [22].

In 2018, Rymbay Kaliyev et al. reported the successful 16-hour perfusion of the donor heart followed by successful transplant to the recipient. The TransMedics donor organ support system made it possible to deliver the organ over a distance of 500 km by rail due to poor weather conditions and the inability to use air transport [23].

The use of TMS by the transplant team allows eliminating the urgency associated with the desire to shorten the ischemia period and avoiding the dangerous high-speed team traffic earlier associated with serious injuries and deaths among team members [3].

Every year, the number of cases using *ex vivo* perfusion systems is rising. In Diana García S. MD. et al., thirty hearts are reported to be saved with TMS from February 2013 to January 2014, 26 of which (86.7%) were transplanted. All these transplant procedures were classified as high-risk due to long delivery time: over 2.5 h with an estimated ischemia time of over 4 h, EF less than 50%, left ventricular hypertrophy, cardiac arrest donors, alcohol/drug abused donors, donors with coronary heart disease or increased pulmonary vascular resistance. According to 2015 data, the system for the donor organs transportation was used in 246 orthotopic heart transplants around the world [3].

M.A. Quader et al. did not find any differences in the results of heart transplants with good left ventricular function between the TransMedics system and the standard pharmacological cold protection in cases when the total period of ischemia was up to 2 h. Nevertheless, allografts with longer ischemia times showed worse left ventricular function and elevated troponin levels. Assessing the functional status of an organ *ex vivo* in combination with the decreased time of cold ischemia minimizes the risk of primary allograft dysfunction and potentially increases the donor pool [24].

According to J.M. Tikkanen et al., long-term survival, improved quality of life and graft function are comparable among recipients with the heart transplant, survived pharmacocholastic ischemia and *ex vivo* thermal perfusion [25]. Vipin Mehta et al. reported 100% 30-day survival rate of recipients who received hearts after *ex vivo* reperfusion and 86% 90-day survival rate. This result is comparable with S. Messer et al.; according to their data, the 30-day and 90-day survival rates were 100% and 93%, respectively [26]. According to Joshua L. Chan, MD et al., there was no significant difference in two-year survival between groups of patients who underwent cardiac transplantation after *ex vivo* perfusion and pharmacological cold ischemia. The two-year survival rate of the recipients was 72.2 and 81.6%, respectively (p 0.38) [27].

However, the wide use of TMS is limited by the high cost of the system. For the UK, the National Institutes of Health reports the cost of a one-time TMS perfusion kit of about £30,000 [28]. It should be noted that this estimate includes only the cost of the device and does not consider additional expenditures. It should be borne in mind that approximately 10–20% of the funds will be spent on hearts subsequently recognized as unsuitable for transplant. However, according to Vipin Mehta et al., the heart transplant from donors after blood circulation stop and using *ex vivo* perfusion can lead to a 23% increase in heart transplant activity and should be accepted by more institutions around the world [29].

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SERGEI BRUKHONENKO – THE FOUNDER OF CARDIOPULMONARY BYPASS (PHILOSOPHICAL, METHODOLOGICAL AND SOCIOCULTURAL CONTEXT)

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This paper is dedicated to Sergei Brukhonenko (1890–1960), a physiologist and inventor of the world's first heart-lung machine. His immense contributions to the development of modern science and technology are investigated. Brukhonenko's invention is seen as modern biotechnology that has attracted an all-round strong public interest. A philosophical analysis of the differences between the concepts "*technique*" and "*technology*" is presented. The paper discusses the sociocultural impact of biotechnology on the society and culture.

Keywords: Sergei Brukhonenko, cardiopulmonary bypass, technology, scientific priority, truant doctor.

1. INTRODUCTION

The invention of the cardiopulmonary bypass (CB) technology is one of the most outstanding high-tech achievements of the 20th century biomedicine. The word "high-tech" here requires clarification. For example, such a scientific discovery as anesthesia and its introduction into surgical practice of (mid-19th century) did not require preclinical studies at all. In contrast to anesthesia, the CB method (in the 1950s) was introduced into clinical practice after decades of development of physiological knowledge and, simultaneously, improvement of methods for open perfusion of isolated organs (e.g. heart). Only then could the problem of perfusion of the whole organism be solved. That is, the "small" and "large" circles of blood circulation known to science since the time of M. Servetus and W. Harvey had to be reproduced in the form of a single technological circuit if the "artificial heart plus artificial lungs".

The priority for creating the first CB automatic device belongs to the Russian doctor, physiologist, inventor S.S. Bryukhonenko (1890–1960) [1, 2, 3, etc.]. S.S. Bryukhonenko called his CB device the "auto-jector". Generally speaking, the historical fate of the terms that the authors themselves used to designate their great scientific discoveries and technical inventions is always fascinating when viewed in the context of the history of science. For instance, Isaac Newton called his law of universal gravitation the "law of inverse proportionality", and V.K. Zvorykin named his television set an "iconoscope". The name S.S. Bryukhonenko gave to his CB device holds a worthy place in this set of the most important discoveries and inventions in science and technology. Already in the first decade the CB method was used in clinics (late 1950s – early 1960s), several technical modifications of the "auto-jector" existed in Russia, and in other countries, over 70 [4].

We use the term "technology" to reveal a deeper, more general cultural meaning of the contribution to science S.S. Bryukhonenko made, focusing on philosophical issues, sometimes just slightly mirrored in his works. The concept of technology is used in modern philosophy of science and technology both in narrow and broad senses [5]. In a narrow sense, this is clear to every literate person of today (e.g. the technology of metallurgical production known since the time of the first civilizations). The concept of technology in the broad sense is most often associated with such achievements of modern civilization as information technology or biotechnology. In this sense, the concept of technology implies, first of all, an innovative component, that is, a permanent solution in line with this scientific and technical progress solving more and more scientific tasks, the implementation of novel technical projects; secondly, a fundamentally new model of the relationship between science (new forms of organization of scientific research in particular) and social practices; thirdly, the "embeddedness" in the accelerating processes of scientific and technological development proper of the value component (moral, philosophical, religious, etc. reflection) [6].

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CB as a method of experimental and clinical surgery is also a technology, if only because the creation of this method made possible the accelerated development of resuscitation and transplantation with philosophical problems "embedded" in these clinical practices.

S.S. Bryukhonenko's pioneering in creating the CB technique has been noted in reports at scientific congresses, publications in scientific journals, Soviet and foreign, from the mid 1920-s. Many Soviet authors have emphasized the significance of the patents S.S. Bryukhonenko got on his CB device: in Germany and the UK (1929), in France (1930) and the USSR (1934). Despite that, it was sorry to hear the words of the clinical heart transplant pioneer K. Barnard during his visit to Moscow leading surgical clinics in 1960[1], he did not see any open heart surgery and was "so disappointed with the state of surgery in the USSR that he would not recommend it to anyone as the place for training and experience in this area" [7].

Nevertheless, if truth be told, it should be mentioned that the first operation in the USSR with the use of the Soviet-made CB device was made by A.A. Vishnevsky November 27, 1957 [2]. In general, the fact of a certain lag in the application of the technique in the Soviet medical practice is undeniable. However, let us note another point: the first stage of introducing the CB technique into clinical practice can be called a "dramatic medicine" (G. Glazer). Here we find a concretization of the problem of the demarcation of two concepts, engineering and technology. The ethical dilemmas that the hero of the novel by N.M. Amosov (1913–2002) "Thoughts and Heart" is only the beginning. Very soon, with the development of clinical transplant, the further progress of surgery in this area will depend on the legitimation of a new criterion for death (brain death), on the creation of a new ethical and legal basis for justification, reasoning and regulation of organ donation practices.

2. S.S. BRYUKHONENKO'S AUTO-JECTOR: "LIVING HEAD" SENSATION EXPERIMENT[2]

The years S.S. Bryukhonenko formed himself as a creative person, coincided with the Russian Revolution and the following turbulent decades of the Soviet history. His father was a railway engineer, and from his youth he showed a fondness of invention. Entering the gymnasium in Saratov, he finished his education in Moscow. In the 1910–1914, as a student at the medical faculty of the Imperial Moscow University, he was fascinated by bacteriology. In 1914, having graduated from the university and signed the "Faculty promise" (a kind of Hippocratic oath, a set of ethical and professional rules for a doctor in pre-revolutionary Russia), he went to the WWI front as a regimental doctor. Having returned home from the front, already to the Soviet Russia, he again found himself "at the front" of the fight against typhus and cholera.

At that time, Sergey Bryukhonenko met Professor F.A. Andreev, the author of 1913 work "On Experience in Restoring the Activity of the Heart, Respiration and Functions of the Central Nervous System". F. Andreev was a prominent Russian clinician and an outstanding scientist whose innovative systematic ideas largely predetermined the development of such high-tech areas of biomedicine of the 20th century as resuscitation and transfusiology. P.M. Bogopolsky et al. note: "Since 1907, F.A. Andreev has researched into various issues, among which… the concept of "imaginary" death in the period of living existence of isolated organs and the whole organism; the restoration of heart function in conditions of adequate coronary perfusion; injection of Ringer-Locke solution with adrenaline or defibrinated blood into the carotid artery … simultaneously with intra-arterial infusion, suction of blood from the superior vena cava … use of hirudin and peptone to reduce blood coagulation … study the possibility of restoring the central nervous system and the whole body with CB" [3]. S.S. Bryukhonenko worked as an assistant to Professor Andreev from 1919 to 1924.

A young doctor started the creation of CB technique with the *methods for an experimental study of the fundamental problems of pathophysiology*, looking for new therapeutic agents for the treatment of typhus. A kind of a "natural experiment" was the cholera locus broke out among typhoid patients. Bryukhonenko drew attention to the fact that bacteriologically proven cholera which began on the 6th–7th day of the disease with typhus seemed to cut it off. It also turned out that cholera was accompanied by a decrease in blood coagulability, while typhus was associated with its increase [2]. Having discovered this clinical phenomenon, Bryukhonenko began to inject anticoagulants and some other pharmacological substances into the veins of such patients. As a result, in 95% of cases an artificially caused crisis arose, and in 5% of cases it was possible to interrupt the disease course. F. Andreev suggested that his assistant find an explanation for the revealed phenomenon, exploring separately the role of the nervous and humoral factors of the thermoregulation mechanism. Bryukhonenko set an ambitious goal of studying the mechanics of thermore-

[1] Barnard visited the USSR during the 27th All-Union Congress of Surgeons (May 23 to May 28, 1960).

[2] In this brief sketch of S.S. Bryukhonenko's life, we used not only published works [2, etc.], but also personal memoirs of professor-biologist L.F. Kurilo, who worked in 1957–1958 a laboratory assistant in the Physiological Laboratory at the Scientific Research Institute of Experimental Surgical Instruments and Instruments (NIIEHAiI) of the Ministry of Health of the USSR led by S.S. Bryukhonenko. For this we are sincerely grateful.

gulation on the biological model of the *"isolated head" of a hemathermal animal*.

S.S. Bryukhonenko recalled: "Between 1920 and 1923. *I invented and constructed an apparatus for cardiopulmonary bypass*, an auto-jector, and developed the method of isolating a dog's head …" (italics added – A.Ya., O.N., O.V.) [2]. Some publications express an opinion that the first CPB pump was created by S.S. Bryukhonenko in collaboration with S.I. Chechulin. In this regard, A.G. Lapchinsky (who was at that time S. S. Bryukhonenko's employee) in his report to the Moscow Society of Surgeons in 1961 (in connection with the anniversary of the death of S.S. Bryukhonenko) remarked a "mistake that somehow crept in" [1]. S.P. Glossy emphasizes that S.I. Chechulin took part in the improvement of the "auto-jector", though this improvement concerned only minor details [8].

The attempts to ensure the vital activity of the "isolated head" of a hemathermal animal (using perfusion of blood vessels) have been repeatedly undertaken by scientists before, A.A. Kulyabko, in particular; however, all of them were unsuccessful. Why? Here it is appropriate to cite I.P. Pavlov in that "science moves in jumps, depending on the successes made by the technique" [9]. No one was able to achieve the result of "keeping alive" such an organ as the head of a hemathermal animal, as no one had created a scientific task adequate for the scientific method.

S.S. Bryukhonenko and S.I. Chechulin carried out together the physiological experiments with a biological model of the "isolated head". In their work published in 1928, "Experiments on Isolating a Dog's Head (with a Demonstration of the Instrument)" [10] they published the "Background". In the beginning of the review, the idea expressed by J.J. S. Le Gallois is cited: through the transfusion of blood, to revitalize the brain (after decapitation). In general, the review provided an analysis of more than a dozen works from 1834 to 1924 by French and English authors[3] (including Charles Edouard Brown-Séquard). The authors emphasize: "One cannot fail to see attempts to create conditions for truly artificial blood circulation". However, the analysis of the difficulties in solving many physiological and technical issues drew them to conclusion that "none of the researchers could do this in full" [10]. A reason was that for most physiologists, the method of separating the head from the trunk of experimental animals seemed "barbaric"[4]: "The decapitation of the animal was carried out primitively, with the help of a specially arranged guillotine … Therefore, we chose a *purely surgical path*, and the head was removed gradually and in a certain sequence" (italics added – A.Ya., O.N., O.V.) [10].

Let us dwell on the latter words of Russian scientists, recalling that the very creation by Andreas Vesalius of the modern scientific anatomy is genetically related to the new cultural meaning of the relationship to the human body. The rigorous scientific observation, the scientific objectivity of the research activities of the anatomists here turned into a more careful, more respectful, more humane attitude to human organs and tissues.

The scale and prominence of the scientific and technical invention made by S.S. Bryukhonenko goes beyond the frame of the technical progress as such. Proving the effectiveness of the auto-jector, a new discipline of clinical and experimental surgery has emerged, i.e. perfusion. An optimistic thought was expressed in one of the recent reviews of the history and achievements of perfusion (2015) on the theoretically possible *ideal perfusion* of the future (italics added – A.Ya., O.N., O.V.) [11]. But in fact, while we are dealing with the symbiosis of a machine and a person, it's just a prosthetics of such an attribute of life as blood circulation. And then the techno-pessimistic question arises, "Is it possible to create a "perfect graft" of blood circulation in general?" CB *as a technology* most eloquently illustrates the trend of a modern individuum to transform "at the level" of being, existence itself, as a result of which a person in the modern world acquires the quality of an "artifact" [12].

Since 1925, S.S. Bryukhonenko and S.I. Chechulin have repeatedly demonstrated to the public their experiment of "revitalizing the dog's head" at the All-Russian congresses of pathologists and physiologists (and at the 4th congress in 1930, in the presence of invited foreign scientists). The demonstrations continued at the 6th Congress (after the death of S.I. Chechulin in 1938). The fact should be emphasized that representatives of the highest authorities of the Soviet state were usually present at such demonstrations. After a successful show at Moscow State University in 1928, to expand the research of S.S. Brukhonenko, a sim of 30 thousand rubles was allocated and an experimental therapy laboratory was organized. Finally, after a series of scientific forums where the demonstration of "revitalizing the dog's head" was repeated over and over again, the 15th International Congress of Physiologists held in 1935 in Moscow under the chairmanship of Academician I.P. Pavlov, was a climax (the congress was generously funded by the state as it was given great political importance).

After the triumphal demonstration of the "revitalizing the dog's head" experiment at the 15th International Congress of Physiologists, the Scientific Council of People's

[3] S.S. Brukhonenko was fluent in English, German and French; in the last years of his life he studied Spanish [2].

[4] Historians of science emphasize that Brown-Séquard in his experiments separated the dog's head from the body with a saber strike [2].

Commissars of the USSR opened the Scientific Research Institute of Experimental Physiology and Therapy (NIIEFT) with S.S. Bryukhonenko as its director. At the same time, he was awarded the degree of Doctor of Medical Sciences (without defending a thesis). The staff of the new institute reached 150. The team developed and manufactured auto-jectors, which *aroused lively interest* after demonstrating experiments at scientific congresses and congresses" (italics added – A.Ya., O.N., O.V.) [2].

As one can see, the experimental physiological and clinical CB method S.S. Bryukhonenko created had, as an indirect consequence, the *widest public response*. A.A. Kulyabko, in an article published in 1928 in Pravda newspaper, stressed that S.S. Bryukhonenko's invention "made an enchanting impression". Of particular note is the international public interest: for example, Bernard Shaw in his usual ironic tone said he was ready to give Dr. Bryukhonenko his head for his experiments [2]. In 30 years, an experiment was made in 1953 by V.P. Demikhov (and his younger colleague and ally V.M. Goryainov) on a puppy dog head and front paws transplant onto the neck of an adult dog. Another decade passed, and the first clinical heart transplant by K. Barnard at the end of 1967 caused a public outcry, which was comparable in strength to the society reaction to the launch of the Soviet Sputnik in 1957 and the first manned flight into space in 1961.

All the above examples are cases of the *development of modern technologies*. As mentioned earlier, the concept of technology in the broad sense, bears something of a "virus"[5] of progressive innovations. In the mass consciousness of society, certain high expectations are associated with such technologies. Modern technology is such a striking *present* in our imagination of human life that is always promising an even *more amazing future*. A person's perception of modern technology can be described with "charm". Thus, modern technologies, being initially the stage of scientific and technological progress, include two other components, social (global "public response") and psychological ("charming and enchanting effect").

As mentioned earlier, the concept of technology in the broad sense implies an analysis of the *ideological, philosophical issues* that are "embedded" in them. S.S. Bryukhonenko and S.I. Chechulin's physiological experiments confirm this methodological position. They wrote in 1928, "The phenomena accompanying agony and death deserve special attention, that is why we are going to dwell on them … more in detail" [10]. On the following pages of their work, we find anticipation of the key problems of resuscitation (which emerged as an independent scientific discipline in the second half of the 20th century) problems equally belonging to both scientific and philosophical sphere. First of all, Bryukhonenko and Chechulin clearly formulate the basic category of resuscitation and intensive care as a "clinical death", but also use other terms, "temporary loss of function and reactivity", "apparent death", "imaginary death", "delayed death", as opposed to "final", real, actual death "[10].

The concepts of clinical and biological death were later introduced by V.A. Negovsky whose work gained fame abroad, which proves the priority of the Soviet science in the area. However, the mismatch of terms denoting the subject hides philosophical and methodological issues that require special analysis. Note that the term "clinical death" is *Scheintod* in German, that is, "apparent death". And the Russian literature expressed the point regarding the appropriateness of abandoning the terms "clinical" and "biological" death as contradicting the laws of logic [13].

Having justified the possibility of restoration of signs of life in an "isolated head" in his experiments with the help of an auto-jector (after 8 to 20-minute death), S.S. Bryukhonenko and S.I. Chechulin formulated the main question, "Is it possible to identify the phenomena of life and death that we observed on an isolated head with what we see on the whole organism?" [10] and further, "No one will doubt that we have a number of functions in which the nerve endings, conductors, sections of the central nervous system, some sensory organs and muscle groups take part. But is it possible to go further and call it all by that tempting term, namely, life? …We suppose this question is easier to solve than the question, What is life? Indeed, physiology has not yet been able to give a clear and precise definition of what can be called alive and what is dead"[6].

The above citations anticipate philosophical and methodological issues related to the legitimation by the modern society of a new criterion for the death of a person as "brain death". Let us look at the definition in the current legal act of the Ministry of Health of the Russian Federation: "The death of a human brain occurs with the complete and *irreversible termination* of all functions of the brain … The moment of death of the human brain is the *moment of human death*"[7] (italics added – A.Ya., O.N., O.V.).

Many philosophical debates surrounding the problem of brain death are focused precisely on the point

[5] It is no accident that in modern information technologies the concept of a "virus" has become their inherent characteristic.

[6] Spacing by Brukhonenko and Chechulin.

[7] The procedure for establishing the diagnosis of human brain death: Appendix No. 1 to the order of the Ministry of Health of the Russian Federation of 12.25.2014, No. 908n.

of straight identification of the both parts of the given definition. Here, S.S. Bryukhonenko and S.I. Chechulin in 1928 perceptively saw a certain "gap" (in both ontological and epistemological meanings) between the physiological facts of the *manifestation of life* in an "isolated head" and *life as such*. Here they were closer to their senior contemporary V.I. Vernadsky's opinion who wrote that the question of the essence of life is still being solved mainly at the level of religion or philosophy, that "science should approach this problem itself. Now, it's not the case". [14]. Therefore, following strict scientific criteria, he preferred to use the words "living substance" instead of "life". Obviously, in the philosophical dimension, the categories of life and death lie "on the same scale". It is well known that the legitimation of the new criterion of death ("brain death") is inextricably linked with the prospects for the development of transplant practice. This was most consistently reflected in the Japan Transplantation Law of 1997, "A patient who is diagnosed with brain death is legally dead *only if* being alive he approved the previous transplantation statement of brain death" (italics added – A.Ya., O.N., O.V.) [15].

Here is another philosophical reflection of Brukhonenko and Chechulin, "It is possible to imagine a state where excitability and functions are not detected but can be detected if there are suitable conditions" [10]. Here, they seemed to foresee the current philosophical doubts of some authors on whether the patient with the diagnosis of "brain death" is really dead.

3. S.S. BRYUKHONENKO, A DISCOVERER OF NATURAL GIFTS

Early 1920s in Russia were marked by radical breakdown of all social institutions, including science as one. S.S. Bryukhonenko recalled the details of the birth of his auto-jector, "The initial stage of the work was inventive in nature … one of the models … was a chaotic pile of metal and glass parts mounted on one tripod. Screws were cut from ordinary nails with coins soldered on them. How the details used medical syringes, electric bells, chemical glassware, etc." [2]. S.S. Bryukhonenko, having no special engineering education, was a mathematician, engineer, and designer; he, for example, in drawings of individual parts of the auto-projector, *minimized (to a square centimeter) the area of contact between the dead flesh of the machine and blood*.

As is known, for the first time in clinical practice, the CB apparatus was successfully used in 1953 in the USA by J.H. Gibbon (1903–1973). An essential detail is that in the history of the invention of the first American CB devices, an active role was played by IBM, the company which in the second half of the 20th century became a leader in computer manufacturing. However, the priority of S.S. Bryukhonenko in the development of clinical cardiac surgery cannot be denied. *In the article "Artificial blood circulation of a whole organism (dog) with a heart turned off" published in the USSR in 1928 and in France in 1929 (written in late 1926 – early 1927)*, Bryukhonenko proposed the possibility of operations on a stopped heart. In ten years, he conducts a decisive experiment (*experimentum crucis*): "I now recall this first and decisive experiment. In a dog under anesthesia, the chest cavity was opened, the cardiopulmonary bypass was connected to the body and turned on. I check the impeccability of his work and stop the work of the heart of the dog by simply pressing it with his hand. Usually in such cases agony and death quickly ensue. With natural excitement, I watch if these formidable symptoms appear. Not. A minute passes, then tens of minutes. The dog remains alive. On the same day, I phoned my colleague in previous work, surgeon Professor N.N. Terebinsky. After telling him about this experiment, I asked if he would be interested in the prospects that open up before surgeons due to the possibility of using this discovery for intracardiac operations with temporary heart switch-off" [2, p. 62][8].

Reading one of his most important works, "An apparatus for cardiopulmonary bypass (of the hemathermal)" published in 1928, any doctor understands that one of the main tasks that the author solved when modeling circular arterial and venous blood flow was to ensure automatic regulation within physiological norms of the pressure level in the bloodstream. However, the solutions to dozens of specific technical problems (for instance, avoiding danger of "causing contact soldering or oxidation" [16]) simply cannot be comprehended by the vast majority of specialists in the field of biomedicine. We would give an analogy: only professional musicians are able to appreciate many chapters of the book "Bach" by the world-famous philosopher, musician and doctor Albert Schweitzer (e.g. "The musical language of cantatas"). We believe that, having put the names of Schweitzer and Bryukhonenko in one row, we are dealing with the phenomenon of a creative personality of a renaissance scale, in both cases.

A remarkable confirmation of what has been said can be found in yet another fact of S.S. Bryukhonenko life. A few decades before the appearance of a personal computer, the scientist anticipated 3D computer graphics. He invented a device for stereoscopic three-dimensional objects imaging. He gave a scientific and physiological explanation to his invention: binocular vision peculiar to humans (when each eye sees an object, speaking the language of geometry, from a different angle) just provides

[8] On the contribution to the development of cardiac surgery N.N. Terebinsky see Klinicheskaya i eksperimental'naya khirurgiya. Zhurnal imeni akademika B.V. Petrovskogo. 2015. No. 3.

us with a three-dimensional perception of the world of material things. S.S. Bryukhonenko wrote: "If humanity still had a plane for its drawings, now we are giving it the opportunity to draw in three dimensions. I have been painting stereoscopically for five years and I believe that spatial images, which I could not always reproduce before, can now be drawn within five minutes. The result was learning spatial thinking" [2]. His invention was, in fact, the forerunner of modern 3D computer models used by modern medical students: "medical students could study with the anatomical atlas of stereo images, engineers could depict future devices in the form that the finished device should have, instead of complex layouts, architects could make simple drawings, etc." [2, p. 73].

4. S.S. BRYUKHONENKO AS A TRUANT PHYSICIAN

The words *truant physician* was first used by the famous English surgeon Lord Berkeley Moyniham (1865–1936). The well-known Russian professor in resuscitation science A.P. Zilber drew our attention to this fact. Zilber, who has been studying this cultural phenomenon for many years gives it the following definition, "Medical truentism is the fruitful desire of doctors to use creative work outside medicine" [13]. Many pages of his monograph "Ethics and Law in Critical Medicine" are devoted to such truants as N. Copernicus, N.I. Pirogov, A.A. Bogdanov and others. A. Schweitzer of course, also belongs to the most outstanding truants [17].

Let us dwell on the constant passion S.S. Bryukhonenko felt to creativity in the humanitarian sphere. Sergei Sergeyevich had an absolute ear for music. As a student, he worked part time as a tapeur in silent cinemas. Later, as his friend, the famous pianist Heinrich Neygauz recalled, Sergey Sergeyevich masterfully performed "God Save the Tsar" on the piano with one hand and the "International" with the other [19]. Under the influence of Bryukhonenko's experiments with a "living head", two science fiction novels were written, the well-known "Head of Professor Douel" by A. Belyaev and "Generator of Miracles" by Yu. Dolgushin. The prototype of the protagonist of the latter is S.S. Bryukhonenko. Yu. Dolgushin recalled, "My acquaintance with the famous physiologist and inventor, Professor Sergey Sergeyevich Bryukhonenko, had a huge impact on my work. When I first came to him, he revived dead dogs. Then I saw an "artificial heart" created by him, an apparatus that miraculously replaced a real heart for an animal while it returned to life. It was a preparation for experiments on humans. And it was already real science fiction… And *then Sergey Sergeyevich edited part of the chapters of the "Generator of Miracles"* (italics added – A.Ya., O.N., O.V.) [2]. The very title of the work of Dolgushin confirms our earlier distinction between the concepts of "technology" and "engineering" (the latter, as it were, charged with a "charming virus"). We cannot to avoid mentioning that S.S. Bryukhonenko knew M.A. Bulgakov[9] evidenced in the Diary (1933) of the writer's wife Elena Sergeevna, "We went to Yakimanka to the Institute of Blood Transfusion[10]. Bryukhonenko (Sergey Sergeevich) was very sorry that he could not show the revival of the cut off head of the dog – there is no suitable specimen. He showed some of his achievements. But most importantly, M.A. persistently suggested *to write a play – along with him* – based on some of his scientific experiments" (italics added – A.Ya., O.N., O.V.) [18].

S.S. Bryukhonenko biographers write in their "Afterword" in the book about him, "In the 1920s, he flew on an airplane of one of the first designs, with his legs still hanging in the air, and later he went down under the water in a diver's suit. He was an excellent swimmer and sought to develop new swimming techniques. Having learned to skate, he immediately went on to figure skating … On the eve of one of the most complex, fourth operations, he enthusiastically talked about the solution to a difficult mathematical problem he found … Sergey Sergeyevich was always far from fighting for titles and positions … He generously scattered ideas and did not regret it, because he had never run dry" [2].

5. CONCLUSION

Unfortunately, the priority of the successful application of CB technology in clinical surgery does not belong to Russia, but to Western countries. The main reason was that, in methodological terms, the CB technique (like all modern technologies) has a fundamentally interdisciplinary nature, and the general level of technological progress in the West at that time (1920–50s) was higher. P.M. Bogopolsky et al. critically evaluate the stage of creative searches S.S. Bryukhonenko in the 1930s–40s, when he predominantly dealt with the problem of "reviving the dead", stubbornly believing that the period of clinical death of approximately five minutes using the CB method could, in principle, be extended. It must be recognized that in the light of all subsequent experimental and clinical experience on resuscitation, the position of these authors is scientifically sound. For our part, we would add the following. Scientific worldview of S.S. Bryukhonenko was formed in the first decades of the 20th century, when scientific megaprojects, for

[9] This fact was brought to our attention by I.A. Ivanyushkin, Cand. Philos.

[10] In 1931–1935, Brukhonenko was the head of the experimental therapy laboratory at the Central Institute of Hematology and Blood Transfusion.

example, eugenics, were popular (in Russia, the works of N.K. Koltsov, Yu.A. Filipchenko and others are devoted to it). The idea that predominated, since 1920s, in the scientific work of S.S. Bryukhonenko, of "revitalization after death" was consonant with the very "spirit of revolution" in the then Russian society.

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THE ROLE OF ALTRUISM AND EMPATHY IN ANTICIPATING THE ATTITUDE TOWARD ORGAN DONATION AMONG NURSES IN INTENSIVE CARE UNITS OF QAZVIN: A CROSS-SECTIONAL STUDY

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Nurses, especially nurses in ICUs play an important role in organ donation; their performance in relation to their role is more affected by their attitude in this regard, identification of effective factors on the positive attitude of nurses towards organ donation is essential. **Objective:** this study was conducted aimed to determine the empathy and altruism with the nurses' attitudes in intensive care unit towards organ donation and brain death. **Methods.** In this cross-sectional study, which was conducted in 2019, 222 nurses from intensive care unit were selected by stratified random sampling. For collecting data the questionnaires of altruism, empathy and attitude toward organ donation were completed by nurses. Data was analyzed using pearson correlation, multiple linear regression. **Results.** There was a significant correlation between altruism ($p < 0.001$, $r = 0.24$) and its components (anonymous prosocial behaviors ($r = 0.33$, $p < 0.001$), emotional prosocial behaviors ($r = 0.14$, $p = 0.03$), dire prosocial behaviors ($r = 0.14$, $p = 0.03$) and compliant prosocial behaviors ($r = 0.21$ and $p = 0.001$)) with attitudes and also between empathy ($r = 0.04$, $r = 0.13$), perspective taking component ($p = 0.02$, $r = 0.152$) and imaginary empathy ($r = 0.14$, $p = 0.03$) with nurses' attitudes. The components of anonymous prosocial behaviors in altruism ($p < 0.0001$), gender ($p = 0.007$) and having organ donation card ($p = 0.012$) are positive predictors of nurses' attitudes towards organ donation. **Conclusion.** The results showed that altruism and having organ donation card were two of the most important factors in the tendency of nurses to organ donation. Therefore, the implementation of educational programs regarding changing nurse's attitude in order to increase the sense of altruism and empathy about organ donation is necessary. By strengthening the sense of altruism and empathy in the family of brain death patients, they can be helped to decide on donate their patient's organ with more certainty.

Keywords: Altruism, Empathy, Attitude, Organ Donation, Nurses.

INTRODUCTION

The issue of the organ transplantation has long been propounded in social and scientific circles. It is considered as the best treatment for the terminal and irreversible organ failures and it saves lives in patients with such failures and improves their quality of life [1]. Transplantation is a therapeutic method that is used when all other therapeutic methods fail. It is one of the complex methods that require an expert team from the very beginning of supplying the organ [2]. One of the potential and considerable sources of supplying organs for transplantation is the brain death patients [3]. Many years ago, after the adoption of the law on organ donation, the concept of the organ transplantation was established in Iran and many efforts have been made to promote this humanitarian action with ethical considerations. However, the rate of organ donation is still very low compared to the number of brain deaths [4]. According to the report of the Iranian Association of Organ Donation, every ten minutes one person is added to the waiting list and every two hours a patient who needs the organ transplantation loses his or her life. It is while a person dies with a brain death every 70 minutes. Due to a shortage of donated organs, the number of organ transplantations is much lower in Iran than Europe and the United States so that thousands of people die every year due to lack of access to the required

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organs [5]. In our country, the rate of organ donation per one million people is 2.3 transplantations while Spain with 35 transplantations per million populations has the best situation in this regard, and in other European and American countries, this number ranges from 10 to 25 transplantations per one million people [6].

The difference between the number of people waiting for organ transplantation and the number of people donating their organs is annually increasing worldwide, and this continuing condition makes it necessary to understand how psychological factors can increase people's willingness to donate their organs [7]. According to studies, factors such as prior knowledge of the deceased's wishes (such as signing a donor card), the presence of relatives at the time of the donor's injury, clear and accurate information about the cause and diagnosis of brain death and the benefits of organ donation, the understanding of tests confirming brain death, encouraging organ donation, willingness to help others, ensuring high quality care and respect for the donor's body, good and proper relationship with families and meeting their needs (including mental and social needs), and altruism and empathy are the main reasons for the agreement of the families of brain dead patients with organ donation [8].

Altruism which is one of the prosocial behaviors is often recognized as one of the fundamental principles of organ transplantation [9]. According to the research, four types of prosocial behaviors have been introduced so far, including altruistic prosocial behaviors that are defined as voluntary helping motivated primarily by concern for the needs and welfare of others and often induced by sympathy responding and internalized norms and principles consistent with helping others [10]. Buttson believes that altruism is to understand the others' suffering and to experience the sympathetic concern for others [11]. compliant prosocial behaviors that are defined as helping others in response to their verbal or non-verbal requests [12]; emotional prosocial behaviors that are defined as helping others under emotionally evocative circumstances and are divided into two subsets of emotional prosocial behaviors and dire prosocial behaviors. Highly emotionally evocative situations lead to overarousal and personal distress for some people, while the response may be sympathy for others [13]; and public prosocial behaviors that are divided into the two subsets of anonymous behavior as helping without knowledge of who is helped and public prosocial behavior as a tendency to perform prosocial acts in front of others [10]. In various studies, including Milaniak (2018), Khani (2017), Newton (2011), and Hill (2016), it has been recognized that altruism is one of the main reasons for the willingness to donate organs [7, 14, 15, 16].

Empathy is also considered as an important predictor of various helping behaviors [17]. In studies conducted on empathy, the main focus has been on its definition, cognition, and sometimes on emotion. According to Davis, empathy is not defined as a single dimensional structure (cognitive or emotional), but it consists of a set of constructs structures. Davis considers four components for empathy, including perspective taking as the ability to consider others' perspectives, fantasy as the tendency to imaginary replace oneself into feelings and actions of fictitious characters in books and movies, empathic concern as other-oriented feelings of sympathy and concern for others in distress, and personal distress as self-oriented feelings of personal distress, discomfort, and anxiety in interpersonal conditions [18]. In general, empathy involves sympathy and a willingness to comfort others' suffering [11]. Therefore, empathy can also be linked to the willingness of people to donate an organ [17]. However, the role of empathy in decision making on the organ donation has rarely been considered and only a few studies have been conducted in this field [7]. Cohen et al. (2012) concluded in their study that there was a positive and significant relationship between empathy and the willingness to donate an organ among the students [17]. In the study by Milaniak (2018), a significant correlation was recognized between altruism, empathy and decision making about organ donation among nursing and paramedical students [7], while no significant relationship was found between the consent for the organ donation and the level of empathy in the study conducted by Wilczek et al. (2014) [19].

The organ donation process begins by determining which patients can be suitable for the donation of an organ. For organ donation, brain death should be confirmed by the brain death confirmation association, and the shorter the time interval between the admission of the patient and the confirmation of brain death, the more the number of organs, especially the lungs, which can be transplanted [20]. According to the mortality pattern, the need for living donors can be reduced in developing countries, such as Iran and other Mediterranean countries, by improving the quality of health care, identifying effectively brain death, and obtaining the consent using appropriate strategies [21].

As the primary caregiver of the patient, a nurse in intensive care units may be the first person who diagnoses the patient's lack of response as a sign of brain death [22]. Nurses, especially nurses in the emergency and intensive care units, play an important role in the organ donation process, ranging from identifying and evaluating potential donors to supporting their families to participate in organ donation. Providing information accurately, transparently, sensitively and in a professional manner information to the families of these patients, the nurses make them ready to understand why their dear ones are in such critical conditions and help them to accept easily their deaths, and thus consider the option of donating organs [23]. The performance of nurses in relation to their role in the organ donation process is more affected by their attitudes toward the organ donation, and those who have a more positive attitude to it have better performance in this regard [24].

Different studies conducted in Iran and other countries to assess the knowledge and attitude and performance of nurses in relation to the brain death and organ donation process indicate that nurses have poor knowledge, attitude, and performance toward the organ donation process [6, 24, 25]. Given that we live today in a society where thousands of patients in need of organs are waiting for transplantation with a painful life while we face every day with many brain dead patients whose organs can improve the life quality of patients who need the organs, as well as considering the role of nurses in the organ donation process, it is very important to recognize the factors affecting the attitude of nurses toward organ donation. Therefore, to determine the relationship between empathy and altruism and the attitudes of nurses in intensive care units toward organ donation and brain death, the present study has been conducted to understand which steps should be taken to strengthen the status of this important current priority in the medical community.

METHOD

The present research has been a cross-sectional descriptive-analytic study. The statistical population of this study included all nurses who were working in ICU, dialysis and emergency units of educational and health centers in Qazvin at the time of the research. Using the equation $n = (Z^2 \times SD^2)/d^2$, the sample size was calculated to be 208 people considering the confidence level of 95%, the d = 0.8, and the maximum standard deviation (SD) of nurses' empathy score of 5.88, obtained in the study by Milaniak et al. (2018) [7]. Taking the probability of sample loss into account, the sample size was considered to be 229 people to enhance the validity of the results of the study.

The sampling was performed using a stratified random method so that the units of each hospital were considered as one stratum and the samples were taken using a simple random method from each stratum which included various intensive care units based on the ratio of nurses in those units to the total population by drawing lots from the list of nurses in the random number table. The exclusion criteria in the present study were having the work experience less than one year in intensive care units, lack of interest in participating in research, having a history of donating an organ or transplantation and or the need for an organ donation in family and relatives, the withdrawal from participating in the study, the completion of years of service, and the change of service place before the completion of the research.

THE TOOLS USED TO COLLECT DATA IN THIS STUDY INCLUDED:

– Demographic and occupational information questionnaire with 8 questions to collect data such as age, gender, marital status, religion, type of employment, degree of education, organizational position, and work experience of the participant in intensive care units.
– Carlo's prosocial tendencies measure (PTM-R) with 25 questions, including 5 subscales of anonymous prosocial behaviors (7 items), altruistic and public prosocial behaviors (8 items), emotional prosocial behaviors (3 items), dire prosocial behaviors (4 items), and compliant prosocial behaviors (3 items). The questionnaire was scored by a 5-point Likert scale ranging from "doesn't describe me at all" to" describes me fully" with points 1, 2, 3, 4, and 5 respectively. The 5th subscale was scored in reverse order. In this test, score 5 indicates the highest degree of altruistic behavior and the highest score is equal to 125. The validity and reliability of this tool have been analyzed separately for each subscale by Carlo et al. (2003) and it has been recognized that they are desirable [26]. Additionally, its validity and reliability have been studied in Iran by Kajbaf et al. (2010) and using a test-retest method, the reliability coefficient of this tool has been calculated by the Cronbach's alpha of 0.86 to be 0.863 [27].

Davis Empathy Questionnaire:

– Empathy questionnaire with 28 questions, which has been developed by Davis in 1983, to measure empathy. It measures the four subscales of personal distress, empathic concern, perspective taking, and fantasy. Each subscale in this test includes 7 sentences that are scored based on a 5-point Likert scale (ranging from 0 = strongly disagree to 4 = strongly agree) and the questions 3, 4, 7, 12, 13, 14, 15, 18, and 19 are scored in reverse. The minimum and maximum score for each subscale can be 0 and 28, respectively. A higher score indicates more empathy. In the study by Davis, the test-retest reliability of the tool has been equal to 0.61–0.79 for men and 0.62–0.81 for women, while the internal reliability has been equal to 0.71–0.77. and its validity is reported to be desirable [28]. In Iran, the study conducted by Khodabakhsh et al. (2012) has calculated the test-retest reliability of the tool to be 0.71 [29]. In the study conducted by Alah Gholilo et al., the Cronbach's alpha coefficient and the reliability coefficient of this tool has been reported as 0.77 and 0.76, respectively [30].
– Attitudes toward organ donation questionnaire with 13 questions, which has been developed by Chakradhar et al. The scores given to each item in this tool range from 1 = strongly disagree to 5 = strongly agree and the total score may vary from 13 to 65. Obtaining a higher score indicates a more positive attitude toward organ donation. The validity of this questionnaire has been determined by the content validity in a study by Purbahram et al. (2017). Its reliability has also been calculated by Cronbach's alpha coefficient in the same study to be 76.3%, which is considered as acceptable reliability [31].

The present study has been conducted as a student thesis. After obtaining a license from the Ethics Committee of the Qazvin University of Medical Sciences (IR.QUMS.REC.1397.179), obtaining a letter of introduction from the university's research deputy, providing the necessary explanations regarding the purpose of the research, and obtaining the written consent of the participants, the data collection began. To comply with ethical standards, the questionnaires were distributed as anonymous and the confidentiality of data obtained from the participants was ensured. After collecting the questionnaires, data analysis was performed using SPSS 23 and descriptive statistics (frequency, percentage, central tendency indexes, and dispersion).

RESULTS

The total number of samples studied in this research was reduced from 229 to 222 because of the incompleteness of some of the questionnaires and their removal from the study. The majority of samples were female (84.7%) and married (75.2%) with the mean age of 32.30 ± 6.21 and the average amount of work experience in intensive care units of 6.18 ± 4.52. 92.8% of the studied samples were nurses with a bachelor degree and 5.9% of them had higher degrees. 105 cases of nurses worked in ICU, 89 in the emergency unit, and 28 in the dialysis unit. 49 cases of the studied samples had signed a donor card (Table 1).

Table 1

Demographic characteristics of nurses in ICU, emergency and dialysis units of Educational and health Centers affiliated to Qazvin University of Medical Sciences

	Variable	Number	Percentage
Unit	ICU	105	47.3
	Emergency	89	40.2
	Dialysis	28	12.7
Gender	Male	34	15.3
	Female	188	84.7
Marital status	Single	54	24.4
	Married	167	75.6
Employment status	Permanent	92	41.6
	Temporary-to-permanent	21	9.5
	Passing a training course	53	24.0
	Contractual	55	24.9
Organizational position	Supervisor	7	3.3
	Nurse	203	96.7
Education level	Bachelor	206	93.6
	Master	13	5.9
	PhD	1	0.5

According to the results, the mean score of 89 ± 12.17 was obtained for altruism, 59.35 ± 8.91 for the empathy, and 48.25 ± 8.50 for the attitude. The mean score of attitude was higher in women than men (p = 0.006) and in married people than the single ones (p = 0.02). The mean score of attitude was also higher in supervisors than nurses (p = 0.41). Additionally, it was higher in nurses working in ICU and dialysis units than those in the emergency units (p = 0.23). A significant relationship was not found between the attitude and education level (p = 0.84) and employment status (p = 0.81).

The results of the present study have shown that there was a positive correlation between altruism ($p < 0.001$ and $r = 0.24$) and its components, including anonymous prosocial behaviors ($p < 0.001$ and $r = 0.33$), emotional prosocial behaviors ($p = 0.03$ and $r = 0.14$), dire prosocial behaviors ($p = 0.03$ and $r = 0.14$), and compliant prosocial behaviors ($p = 0.001$ and $r = 0.21$), and the attitudes of nurses toward organ donation. However, there was a negative correlation between public prosocial behaviors and the attitudes of nurses ($p = 0.87$ and $r = -0.03$). Concerning the variable of empathy, there was a positive correlation between the total score of empathy ($p = 0.04$ and $r = 0.13$), perspective taking ($p = 0.02$ and $r = 0.152$), and fantasy ($p = 0.03$ and $r = 0.10$) and the attitudes of nurses. There was no positive correlation between personal distress ($p = 0.13$, $r = 0.10$) and empathic concern ($p = 0.99$ and $r = 0.00$) (Table 2). The two variables of altruism and empathy have also had a positive correlation ($p < 0.001$ and $r = 0.420$).

The results of multiple linear regression analysis, which was done to investigate the predictive role of research variables (quantitative and qualitative) in relation to the organ donation, have shown that gender, marital

Table 2

Correlation between altruism and empathy with the attitudes of nurses in ICU, emergency and dialysis units of Educational and Health Centers affiliated to Qazvin University of Medical Sciences

	The total score of attitude		The total score of attitude		
	Correlation coefficient	P-value		Correlation coefficient	P-value
Total score of altruism	0.24	<0.001	Total score of empathy	0.133	0.048
Anonymous prosocial behaviors	0.338	<0.001	Personal distress	0.102	0.131
Altruistic and public prosocial behaviors	–0.010	0.878	Empathic concern	0.000	0.997
Emotional prosocial behaviors	0.144	0.031	Perspective takes	0.152	0.023
Dire prosocial behaviors	0.143	0.033	Fantasy	0.140	0.037
Compliant prosocial behaviors	0.219	0.001			

Table 3

Predictors of the attitudes of nurses in ICU, emergency, and dialysis units toward organ donation in Educational and Health Centers affiliated to Qazvin University of Medical Sciences

Variable	B	Std. Error	Beta	P-value	95% confidence interval	
					Minimum	Maximum
Constant value	19,922	5,339		0.000	9/396	30/448
Gender	4,172	1.540	0.175	0.007	1,136	7,208
Marital status	0,999	1,323	0.51	0.451	–1.609	3,608
Signing a donor card	3,223	1.279	0.159	0.012	0,702	5.744
Anonymous prosocial behaviors	0,582	0,148	0,349	0,000	0,290	0,874
Altruistic and public prosocial behaviors	–0,64	0,291	–0,18	0,828	–0,637	0,510
Emotional prosocial behaviors	–0,431	0,263	–0,148	0,103	–0,951	0,88
Dire prosocial behaviors	0,321	0,288	0,083	0,266	–0,246	0,889
Personal distress	–0,020	0,159	–0,009	0,900	–0,333	0,293
Empathic concern	0,141	0,228	0,048	0,536	–0,308	0,591
Perspective takes	0,239	0,240	0,074	0,321	–0,234	0,711

status, signing a donor card, and altruism have been positive predictors of nurses' attitude toward organ donation and empathy has been the negative predictor (Table 3).

Linear regression was performed to assess the predictors of the attitudes toward organ donation. Variables that had $p > 0.2$ in the single-variable model were entered into multiple linear regressions by "inter" method, that according to the results, the components of the anonymous prosocial behaviors ($p < 0.0001$) in altruism and gender variables ($p = 0.007$) and having organ donation card ($p = 0.012$) were significant predictors of nurses' attitude towards organ donation and in total, these variables predicted 20% change in attitude (Table 3).

DISCUSSION

The results obtained from the present study that has aimed to investigate the relationship between empathy and altruism with the attitude of nurses toward organ donation confirm the effect of altruism on the attitude of nurses toward organ donation. There was a direct and significant relationship between the total score of altruism and such components as anonymous prosocial behaviors, emotional prosocial behaviors, dire prosocial behaviors, and compliant prosocial behaviors with the attitudes of nurses toward organ donation. This is in agreement with the results obtained from the study by Khani et al. (2016), which showed a significant direct relationship between altruism and the attitude toward organ donation as well as a significant indirect relationship between the altruism caused by practicing religious beliefs and the attitude toward the organ donation [14]. Hill et al. (2016) demonstrated that there was a moderate positive correlation between the altruism and the attitude toward the organ donation and among the altruism and big five personality dimensions, only altruism was an important predictor of decision making on organ donation [16]. In the study by Khani et al. (2014) that investigated the role of social capitals and altruism in the prediction of medical doctors' attitudes toward organ donation, a positive and significant correlation was recognized between the attitude toward the organ donation and the altruism [32]. In the study conducted by Newton et al. (2011), the altruistic motivation to help others was introduced as the most commonly identified motivator for becoming an organ donor [15]. It can be said that the feeling of altruism has a significant effect on the attitude of nurses toward organ donation and improves the performance of nurses in identifying brain death patients, caring for them, and increasing the consent of their families for the organ donation.

The results have also shown that there was a direct and significant correlation between the total score of empathy, perspective taking, and fantasy with the attitude of nurses. However, there was no correlation between distress and empathic concern. In study of Cohen et al. (2012), empathic concern and self-interest (personal distress) were considered as a positive predictor of tendency to organ donation, while paying attention to the interests of others (empathic attention) was not the positive predictor of tendency to organ donation. In the present study, there was a significant relationship between imaginary empathy and perspective taking with attitude toward organ donation, but none of the subscales of empathy was not a positive predictors of nurses' attitude toward donation [17]. The research conducted by Wilczek et al. (2014) demonstrated that there was no significant correlation between empathy and the willingness to donate organs of brain death patients. It is in contrast to the results of the present study. Their study showed that the highest percentage of empathy was associated with the female participants [19], which is in agreement with this study.

The results of the study by Milaniak et al. (2018) showed a significant correlation between altruism and empathy with the causes of posthumous organ donation. In their study, the highest percentage of willing to sign a donor card was associated with the group who had a moderate level of altruism, and the highest percentage of

those who had signed previously a donor card was seen in the group with a high level of altruism [7]. These are consistent with the findings of the present study. In terms of empathy, only perspective taking was considered as a significant predictor and there was a poor relationship between the perspective taking and the dependent variable. There was a significant difference between the level of personal distress and disagreement with the organ donation. There was a positive and significant correlation between the fantasy and the agreement with the organ donation after the death of relatives, which was in agreement with the present study, but in the present study, none of the empathy subscales were positive predicator of the attitude toward organ donation [7]. There was no correlation between altruism and empathy that this finding was not in agreement with our study [7]. Empathy affected the agreement with donating organs of relatives after brain death and those with a higher level of empathy tended to develop a more positive attitude toward organ donation. According to the findings of the study it can be concluded that nurses with a high level of empathy can improve the status of organ donation in the society by developing more positive attitudes toward organ donation in relatives of brain death patients. In addition, the present study has shown that the score of attitude has been higher in those who have signed a donor card and signing a donor card has a positive and significant correlation with a positive attitude toward organ donation. In the study by Purbahram et al. (2017), the score of attitude in nurses who signed a donor card was higher than those who didn't sign a donor card. However, this difference was not significant [31]. The study conducted by Rodrigue (2004) also demonstrated that those who signed a donor card had more positive attitudes toward organ donation than those who didn't sign a donor card [34]. In the present study the gender, marital status and the component of anonymous prosocial behaviors have been a positive predictor of attitude toward organ donation while the effect of age, marital status, other component of altruism and also empathy and its components has not been significant. It is not in agreement with the findings of the study by Hill (2016), in which the age was the positive predictor of the attitude toward the organ donation and the gender was the negative predictor of it [16], as well as the results of study by Rodrigue (2004), in which the gender and marital status had no relationship with the attitudes and beliefs toward the organ donation [34].

Considering the necessity of research in the field of organ donation and the increasing need of the society for it, it is suggested that different methods to be used to increase the feelings of altruism and empathy to strengthen the positive attitude toward the organ donation among nurses in intensive care units.

CONCLUSION

The results of our research show that empathy and altruism can lead to positive attitudes of nurses toward the organ donation and improve their performance in the organ donation process and ultimately, promote the status of organ donation in the society. More research is needed to understand how empathy and altruism affect the organ donation process. The results showed that altruism and having organ donation card were the most important factors in the tendency of nurses to organ donation. Therefore, the implementation of educational programs regarding changing nurse's attitude in order to increase the sense of altruism and empathy about organ donation is necessary. By strengthening the sense of altruism and empathy in the family of brain death patients, they can be helped to decide on donate their patient's organ with more certainty.

LIMITATIONS

The present study has been of the kind of correlational research and as a limitation, it cannot be said that the predictor variables have been the main cause of the independent variable. It is suggested that the effects of other psychological and social characteristics on the attitudes of nurses toward the organ donation to be investigated in the next research.

The present research is correlational and as a limitation, it can not be said that the predictor variables are the main cause of the independent variable. Secondly, a questionnaire was used to collect the data. Therefore, considering that the questionnaires had an aspect of self-evaluation, there might be a bias (orientation) in the responses. Therefore, it is suggested that interview and observation be used in subsequent studies. It is also suggested that in the subsequent studies, other tools for measuring altruism and empathy and the effects of other psychological and social characteristics on the nurses' attitude towards organ donation should be investigated.

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