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*Выпуск очередного номера нашего журнала приурочен к открытию V Российского национального конгресса с международным участием «Трансплантация и донорство органов». Это значимое событие современной научной, медицинской и общественной жизни России вновь пройдет в очном режиме при участии представителей органов государственной власти, руководителей здравоохранения, общественных организаций, российских и зарубежных лидеров клинической медицины и биомедицинской науки.*

*Пандемия, вызванная коронавирусной инфекцией, внесла свои коррективы не только в нашу медицинскую деятельность, но и в привычный ритм жизни как в нашей стране, так и за рубежом. Главные силы трансплантологического сообщества были направлены на исследования, посвященные лечению и предупреждению тяжелых осложнений у реципиентов, перенесших трансплантацию солидных органов, и больных тяжелыми хроническими заболеваниями с терминальной стадией поражения жизненно важных органов в условиях пандемии COVID-19. Мы получили неоценимый опыт, и в рамках конгресса пройдет конференция, посвященная вопросам трансплантологической помощи пациентам с новой коронавирусной инфекцией.*

*Среди основных тем конгресса – трансплантация органов педиатрическим пациентам: обсуждение организационных и правовых аспектов; обмен опытом и уникальными клиническими наблюдениями в области трансплanta-*

# 5TH NATIONAL CONGRESS OF TRANSPLANT SURGEONS FEATURING INTERNATIONAL PARTICIPANTS

*The next issue of our journal is timed to coincide with the opening of the 5th National Congress of Transplant Surgeons themed "Organ Transplantation and Donation". The congress will be featuring international participants and guests. This event, which is significant in the modern scientific, medical and social life of Russia, will again be held physically. Expected to actively participate at the event are government authorities, heads in the healthcare industry, public organizations, Russian and foreign leaders in clinical medicine and biomedical science.*

*Disruptions caused by the COVID-19 pandemic have fundamentally changed many things not only in our medical activities, but also in our everyday life both in our country and overseas. The transplantation community has channeled its main efforts towards research on treatment and prevention of severe complications in solid organ recipients and patients with end-stage chronic diseases affecting vital organs amidst the COVID-19 pandemic. We have gained invaluable experience and knowledge so far, and the Congress will be featuring a conference devoted to provision of transplant care to COVID-19 patients.*

*Among the main topics of the Congress are "Pediatric Organ Transplantation: Discussion of Organizational and Legal Aspects", "Exchange of Experience and Unique Clinical Cases in Pediatric Transplantation of Heart, Kidney and Liver", and "Development of Artificial Organs".*

*ции сердца, почек, печени детям; разработка искусственных органов.*

*В этом году мы отмечаем 90-летие Валерия Ивановича Шумакова, заложившего основы отечественной трансплантологии и более 33 лет бессменно возглавлявшего НИИ трансплантологии и искусственных органов. Ему принадлежит ведущая роль в становлении клинической трансплантологии в нашей стране, в разработке, создании и внедрении в клиническую практику искусственных органов (клапанов сердца, кардиостимуляторов, искусственного сердца и др.). Именно Валерий Иванович явился инициатором организации и проведения первых всероссийских форумов трансплантологов, ставших традиционной площадкой для обмена опытом и научными достижениями в области трансплантологии и создания искусственных органов, консолидации врачей и ученых на пути развития медицинской науки и внедрения ее достижений в практику.*

*В память о нашем учителе ежегодно проводятся всероссийские конференции «Шумаковские чтения», посвященные наиболее актуальным проблемам трансплантологии. В рамках V Всероссийского конгресса трансплантологов состоятся XII Шумаковские чтения «Педиатрическая трансплантация: возможности сегодня и завтра».*

*С уважением,*
*академик РАН С.В. Готье*

*This year we are celebrating the 90th anniversary of the birth of Valery Shumakov, the founding father of transplantology in Russia, who laid the foundations for organ transplants. For over 33 years, he headed the Shumakov National Medical Research Center of Transplantology and Artificial Organs. Professor Shumakov played a leading role in the development of clinical transplantology in our country. He was a central figure in the development and creation of artificial organs (heart valves, pacemakers, artificial heart and others) and their introduction into clinical practice. Valery Shumakov was the person that initiated the organization and conduct of the first National Forum of Transplantologists, which subsequently became the traditional platform for exchange of experience and scientific achievements in the field of transplantology and creation of artificial organs, for consolidation of doctors and scientists on the path to medical science development and for implementation of relevant achievements in practice.*

*National conference "Shumakov Readings" is held annually in memory of our great teacher. It is dedicated to the most pressing problems in transplantology. The 12th Shumakov Readings "Pediatric Transplantation: Opportunities Today and Tomorrow" will be held as part of the 5th National Congress of Transplant Surgeons.*

*Sincerely,*
*Sergey V. Gautier*
*Member, Russian Academy of Sciences*

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# ORGAN DONATION AND TRANSPLANTATION IN THE RUSSIAN FEDERATION IN 2020

## 13th Report from the Registry of the Russian Transplant Society

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[2] Sechenov University, Moscow, Russian Federation

**Objective:** to monitor the current trends and developments in organ donation and transplantation in the Russian Federation based on the 2020 data. **Materials and methods.** Heads of organ transplant centers were surveyed through questionnaires. Data control was done using the information accounting system of the Russian Ministry of Health. Between separate federal subjects of the Russian Federation and between transplantation centers, comparative analysis of data obtained over years was performed. **Results.** Based on data retrieved from the 2020 Registry, 44 kidney, 29 liver and 16 heart transplantation programs were functioning in the Russian Federation in 2020. The kidney transplant waitlist in 2020 included about 11.5% of the total 60,000 patients receiving dialysis. Organ donation activity in 2020 was 3.9 per million population, with a 74.6% multi-organ procurement rate and an average of 2.9 organs being procured from one effective donor. In 2020, there were 7.7 kidney transplants per million population, 3.8 liver transplants per million population and 1.7 heart transplants per million population. Same year, the number of transplant surgeries performed in the Russian Federation fell by 19.2% to 13.4 per million population against the background of the outbreak caused by the new coronavirus disease COVID-19. The city of Moscow and the Moscow region in 2020 accounted for 13 out of the 14 functioning organ transplantation centers, performing 66.3% of all kidney transplants and 72.4% of all extrarenal transplants in the country. The number of organ recipients in the Russian Federation have exceeded 130 per million population. **Conclusion.** In 2020, despite the new coronavirus disease COVID-19 pandemic and accompanying restrictive measures, transplant centers continued to perform organ transplants, run a waiting list and monitor organ recipients. However, the number of effective donors (–22.9%) and organ transplants (–19.2%) decreased, tentatively to the 2017 levels. In 2021, transplant centers with support from health authorities will have to restore the volume of transplant care with consideration to the real needs of the population and the donor resource. The COVID-19 factor, including vaccination of the population, as well as financial support to transplantation programs, will be decisive in shaping the trend of transplantation care and organ donation in the federal subjects of the Russian Federation in the coming 1–2 years.

*Keywords: organ donation, kidney, liver, heart, lung, pancreas transplantation, transplant center, waiting list, registry, COVID-19.*

## INTRODUCTION

Current trends and developments in organ donation and transplantation in Russia are monitored via the National Registry under the auspices of the organ transplant commission of the Russian Ministry of Health and the Russian Transplant Society. Previous reports have been published in 2009–2019 [1–11].

Information contained in the Registry is sent to the following international registries: International Registry of 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 National Registry has served as a tool for ensuring quality control and data integrity in the information system used for recording human donor organs and tissues, and information about donors and recipients. The information system operates under executive order No. 355n of the Russian Ministry of Health, dated June 8, 2016.

Annual reports of the Registry contain not only statistical data for the reporting period, but also systematic analysis of the data with an assessment of the current situation in transplantology, challenges, trends, and prospects for further development in this healthcare sector.

Since 2019, the Registry has also been used for monitoring the implementation of departmental target pro-

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gram "*Organ Donation and Transplantation in the Russian Federation*", approved via executive order No. 365 of the Russian Ministry of Health on June 4, 2019.

The data for the Registry are collected via questionnaires administered to all transplantation centers in the Russian Federation. Comparative analysis of all data gathered over years from Russian regions, transplant centers and from international registries is performed.

The working group would like to thank all permanent and new participants in the Registry who have provided data, as well as the Russian Ministry of Health and the Central Research Institute for Healthcare Organization and Informatization.

## TRANSPLANT CENTERS AND WAITING LISTS

In the Russian Federation, there are transplant centers in 32 federal subjects with a total population of 99.3 million people, Fig. 1.

In 2020, despite the outbreak caused by the new coronavirus infection COVID-19 and related restrictive measures, most of the 60 functioning transplant centers continued to provide transplant care to the population. Eleven medical institutions that were hosting transplant centers, suspended transplant care for varying periods of time due to reassignment, Table 1.

Kidney transplantation was performed in 44 centers, liver transplantation in 29, heart transplantation in 16, pancreas transplantation in 3, lung transplantation in 3, and small intestine transplantation in 1.

Of the 60 transplant centers, 19 are federal institutions, including 11 institutions under the Russian Ministry of Health, 2 institutions under the Russian Ministry of Science and Higher Education, 4 institutions under the Federal Biomedical Agency, 2 institutions under the Russian Ministry of Defense, and 41 are institutions run by federal subjects of the Russian Federation.

In 2020, there were 6,929 potential recipients on the kidney transplant waiting list in the Russian Federation, i.e. 11.5% of the total number of patients hemodialysis and peritoneal dialysis (approximately 60,000). Of these, 1,433 were waitlisted in 2020 for the first time. The kidney transplant waitlist mortality in the Russian Federation in 2020 was 1.8% (125 patients).

There were 2,237 potential recipients on the liver transplant waiting list in 2020; 780 of them were included in the list for the first time in 2020. Liver transplant waitlist mortality in the Russian Federation in 2020 was 5.5% (124 patients).

There were 708 potential recipients waitlisted for heart transplantation in 2020; 303 of them were included in the waiting list for the first time in 2020. Heart transplant waitlist mortality in Russia was 7.5% (53 patients).

Between 2012 and 2020, as the number of organ transplants increased in the Russian Federation, the number of waitlisted patients for kidney transplantation almost doubled, the liver transplant waiting list increased 4.6 times, while heart transplant list increased 1.8 times. Meanwhile, the average waiting time for organ transplantation remained virtually unchanged. Waitlist mortality in 2012–2019 tends to decrease; taking into account the COVID-19 pandemic, waitlist mortality was higher in 2020 than in 2019.

Table 2 presents the number of potential recipients on waiting lists at transplant centers.

In 2020, 1,960 organ transplants (13.4 per 1 million population) were performed in Russia of which 258 were pediatric organ transplants. See Tables 3 and 4.

Because of the growing incidence of COVID-19 in a number of regions in Russia, medical organizations with functioning transplantation centers were reassigned to provide medical care to patients with suspected or diagnosed COVID-19. In the spring of 2020, known restrictions were imposed on movement of citizens, there were restrictions on routine consultations and hospitalizations of citizens.

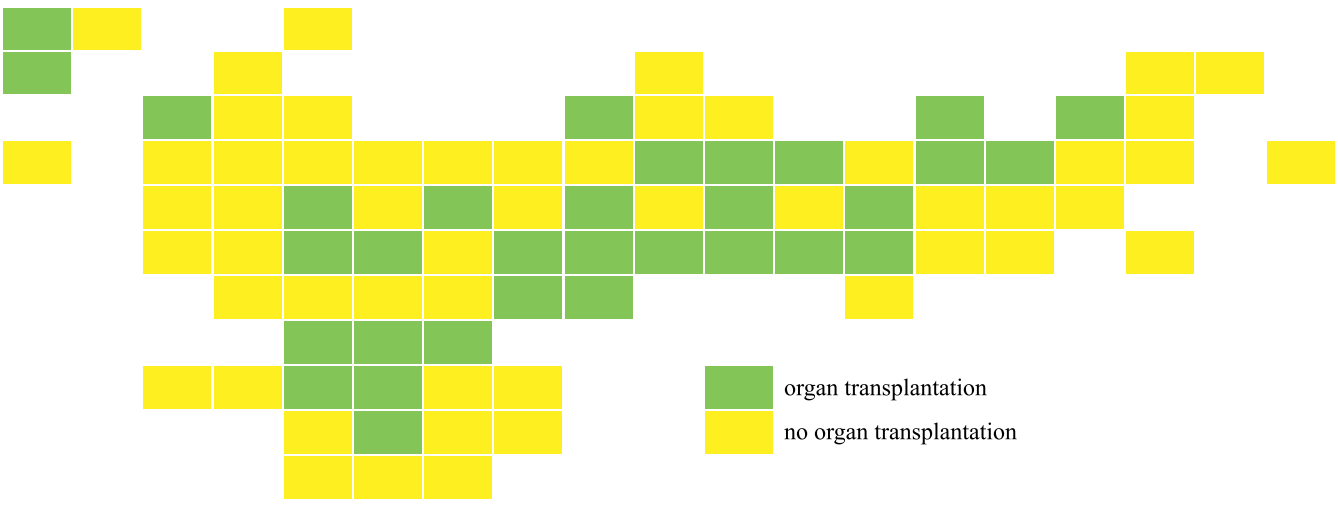


Fig. 1. Geography of organ transplant centers in Russia in 2020

Table 1

**Participation of medical institutions (transplant centers) in provision of medical care to patients with suspected or diagnosed COVID-19**

| No. | Transplant center, region, Federal District | Participation of medical institutions in provision of medical care to patients with suspected or confirmed COVID-19 | Reassignment of the surgical department where organ transplant surgeries are performed, with the suspension of organ transplant surgeries |
|---|---|---|---|
| 1 | 2 | 3 | 4 |
| 1 | Shumakov National Medical Research Center of Transplantology and Artificial Organs, Moscow, Central Federal District | yes | no |
| 2 | Branch of Shumakov National Medical Research Center of Transplantology and Artificial Organs, Volzhsky, Southern Federal District | no | no |
| 3 | Lopatkin Research Institute of Urology and Interventional Radiology – a branch of the National Medical Research Center for Radiology, Moscow, Central Federal District | yes | yes |
| 4 | Russian Children's Clinical Hospital, Moscow, Central Federal District | no | no |
| 5 | Petrovsky National Research Centre of Surgery, Moscow, Central Federal District | no | no |
| 6 | Burnazyan Federal Medical and Biophysical Center, Moscow, Central Federal District | yes | no |
| 7 | Bakulev Scientific Center of Cardiovascular Surgery, Moscow, Central Federal District | no | no |
| 8 | National Medical Research Center for Children's Health, Moscow, Central Federal District | no | no |
| 9 | Botkin City Clinical Hospital, Moscow, Central Federal District | yes | no |
| 10 | Research Institute of Emergency Pediatric Surgery and Traumatology, Moscow, Central Federal District | no | no |
| 11 | Sklifosovsky Research Institute of Emergency Care, Moscow, Central Federal District | yes | no |
| 12 | Morozovskaya Children's City Clinical Hospital, Moscow, Central Federal District | no | no |
| 13 | Vladimirsky Moscow Regional Research Clinical Institute, Moscow Oblast, Central Federal District | yes | yes |
| 14 | Federal Clinical Center for High Medical Technologies under the Federal Medical-Biological Agency (119), Moscow Oblast, Central Federal District | yes | yes |
| 15 | Vishnevsky 3rd Central Military Clinical Hospital, Moscow Oblast, Central Federal District | no | no |
| 16 | St. Joasaphus Belgorod Regional Clinical Hospital, Belgorod, Central Federal District | no | no |
| 17 | Voronezh Regional Clinical Hospital No. 1, Voronezh, Central Federal District | yes | no |
| 18 | Tula Regional Clinical Hospital, Tula, Central Federal District | yes | yes |
| 19 | Ryazan Regional Clinical Hospital, Ryazan, Central Federal District | yes | no |
| 20 | Stavropol Regional Clinical Hospital, Stavropol, North Caucasian Federal District | yes | no |
| 21 | Ochapovsky Regional Clinical Hospital No. 1, Krasnodar, Southern Federal District | yes | no |
| 22 | Regional Clinical Hospital No. 2, Krasnodar, Southern Federal District | yes | no |
| 23 | Volzhsky Regional Urological Center, Volzhsky, Southern Federal District | no | no |
| 24 | Rostov Regional Clinical Hospital, Rostov-on-Don, Southern Federal District | yes | no |
| 25 | Granov Russian Research Center of Radiology and Surgical Technologies, St. Petersburg, Northwestern Federal District | no | no |

End of table 1

| 1 | 2 | 3 | 4 |
|---|---|---|---|
| 26 | Almazov National Medical Research Centre, St. Petersburg, Northwestern Federal District | yes | no |
| 27 | Pavlov First St. Petersburg State Medical University, St. Petersburg, Northwestern Federal District | yes | no |
| 28 | St. Petersburg Dzhanelidze Research Institute of Emergency Medicine, St. Petersburg, Northwestern Federal District | yes | no |
| 29 | Leningrad Regional Clinical Hospital, St. Petersburg, Northwestern Federal District | yes | no |
| 30 | Kirov Military Medical Academy, St. Petersburg, Northwestern Federal District | no | no |
| 31 | City Mariinskaya Hospital, St. Petersburg, Northwestern Federal District | yes | yes |
| 32 | Volosevich First City Clinical Hospital, Arkhangelsk, Northwestern Federal District | yes | no |
| 33 | Republican Hospital No. 1 – National Center of Medicine, Yakutsk, Far Eastern Federal District | yes | yes |
| 34 | Meshalkin National Medical Research Center, Novosibirsk, Siberian Federal District | no | no |
| 35 | State Novosibirsk Regional Clinical Hospital, Novosibirsk, Siberian Federal District | yes | no |
| 36 | Research Institute for Complex Issues of Cardiovascular Diseases, Kemerovo, Siberian Federal District | yes | no |
| 37 | Belyaev Kemerovo Regional Clinical Hospital, Kemerovo, Siberian Federal District | yes | no |
| 38 | Podgorbunsky City Clinical Hospital, Kemerovo, Siberian Federal District | yes | no |
| 39 | Irkutsk Regional Clinical Hospital, Irkutsk, Siberian Federal District | yes | no |
| 40 | Omsk City Clinical Hospital No. 1, Omsk, Siberian Federal District | | |
| 41 | Regional Clinical Hospital, Altai Krai (Barnaul), Siberian Federal District | yes | no |
| 42 | Federal Center for Cardiovascular Surgery, Krasnoyarsk, Siberian Federal District | no | no |
| 43 | Federal Siberian Research and Clinical Center, Krasnoyarsk, Siberian Federal District | yes | yes |
| 44 | Krasnoyarsk Clinical Hospital, Krasnoyarsk, Siberian Federal District | yes | no |
| 45 | Sverdlovsk Regional Clinical Hospital No. 1, Yekaterinburg, Ural Federal District | yes | no |
| 46 | Chelyabinsk Regional Clinical Hospital, Chelyabinsk, Ural Federal District | no | no |
| 47 | Regional Clinical Hospital No. 1, Tyumen, Ural Federal District | yes | no |
| 48 | District Clinical Hospital, Khanty-Mansiysk, Ural Federal District | yes | yes |
| 49 | Samara State Medical University, Samara, Volga Federal District | no | no |
| 50 | Saratov State Medical University, Saratov, Volga Federal District | yes | yes |
| 51 | Regional Clinical Hospital, Saratov, Volga Federal District | yes | yes |
| 52 | Volga District Medical Center, Nizhny Novgorod, Volga Federal District | yes | no |
| 53 | Specialized Cardiac Surgical Clinical Hospital, Nizhny Novgorod, Volga Federal District | no | no |
| 54 | Republican Clinical Hospital, Kazan, Volga Federal District | yes | no |
| 55 | Interregional Clinical Diagnostic Center, Kazan, Volga Federal District | no | no |
| 56 | Republican Clinical Hospital, Ufa, Volga Federal District | yes | no |
| 57 | Republican Cardiology Clinic, Ufa, Volga Federal District | no | no |
| 58 | Perm Regional Clinical Hospital, Perm, Volga Federal District | yes | no |
| 59 | Ulyanovsk Regional Clinical Center for Specialized Types of Medical Care, Ulyanovsk, Volga Federal District | yes | no |
| 60 | City Clinical Hospital for Emergency Medical Care No. 1, Orenburg, Volga Federal District | yes | yes |

Table 2

**Organ transplant waiting lists in regions across Russia in 2020**

| Federal district, region, population in 2020 (million people)* Type of transplantation | Russian Federation | Central Federal District | | | | | Southern Federal District | | | North Caucasian Federal District | Northwestern Federal District | | Siberian Federal District | | | | | | Ural Federal District | | | | Volga Federal District | | | | | | | | Far Eastern Federal District |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Moscow and Moscow Oblast | Belgorod Oblast | Voronezh Oblast | Ryazan Oblast | Tula Oblast | Krasnodar Krai | Volgograd Oblast | Rostov Oblast | Stavropol Krai | St. Petersburg and Leningrad Oblast | Arkhangelsk Oblast without Nenets Autonomous Okrug | Novosibirsk Oblast | Kemerovo Oblast | Irkutsk Oblast | Omsk Oblast | Altai Krai | Krasnoyarsk Krai | Sverdlovsk Oblast | Tyumen Oblast without autonomous okrugs | Khanty-Mansi Autonomous Okrug – Yugra | Chelyabinsk Oblast | Samara Oblast | Saratov Oblast | Nizhny Novgorod Oblast | The Republic of Tatarstan | The Republic of Bashkortostan | Orenburg Oblast | Perm Krai | Ulyanovsk Oblast | Republic of Sakha (Yakutia) |
| | **146.2** | 12.6 | 1.5 | 2.3 | 1.1 | 1.5 | 5.7 | 2.5 | 4.2 | 2.8 | 5.4 | 1.1 | 2.8 | 2.6 | 2.4 | 1.9 | 2.3 | 2.8 | 4.3 | 1.5 | 1.7 | 3.4 | 3.1 | 2.4 | 3.2 | 3.9 | 4.0 | 1.9 | 2.6 | 1.2 | 1.0 |
| | | 7.7 | | | | | | | | | 1.9 | | | | | | | | | | | | | | | | | | | | |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 |
| **KIDNEY** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Number of transplant centers | **46** | 12 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 4 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Number of patients waitlisted for the first time in 2020 | **1433** | 736 | 6 | 7 | 0 | 5 | 41 | 0 | 30 | 9 | 66 | 4 | 40 | 27 | 19 | 15 | 20 | 49 | 25 | 10 | 13 | 26 | 27 | 15 | 35 | 46 | 44 | 2 | 112 | 4 | 0 |
| Total number of waitlisted patients in 2020 | **6929** | 2555 | 48 | 102 | 32 | 15 | 397 | 110 | 122 | 9 | 328 | 70 | 144 | 136 | 70 | 113 | 110 | 189 | 235 | 78 | 153 | 163 | 253 | 123 | 510 | 300 | 241 | 114 | 112 | 35 | 62 |
| Number of waitlisted patients as of 12/31/20 | **5680** | 1894 | 43 | 86 | 24 | 7 | 372 | 91 | 78 | 0 | 267 | 68 | 120 | 94 | 50 | 87 | 94 | 160 | 224 | 63 | 147 | 146 | 203 | 112 | 486 | 251 | 197 | 110 | 110 | 34 | 62 |
| Number of waitlisted patients who died in 2020 | **125** | 22 | 1 | 9 | 0 | 2 | 0 | 0 | 7 | 0 | 0 | 1 | 1 | 3 | 0 | 25 | 0 | 1 | 2 | 4 | 2 | 13 | 3 | 4 | 10 | 9 | 6 | 0 | 0 | 0 | 0 |
| **LIVER** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Number of transplant centers | **31** | 6 | 1 | 0 | 1 | 0 | 2 | 0 | 1 | 1 | 4 | 0 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 |
| Number of patients waitlisted for the first time in 2020 | **780** | 473 | 2 | 0 | 0 | 0 | 5 | 0 | 55 | 4 | 32 | 0 | 66 | 9 | 18 | 4 | 7 | 16 | 12 | 6 | 4 | 11 | 2 | 0 | 23 | 20 | 11 | 0 | 0 | 0 | 0 |
| Total number of waitlisted patients in 2020 | **2237** | 886 | 68 | 0 | 5 | 0 | 51 | 0 | 171 | 4 | 233 | 0 | 101 | 68 | 19 | 4 | 49 | 41 | 109 | 6 | 7 | 38 | 2 | 0 | 202 | 61 | 96 | 0 | 0 | 0 | 16 |

End of table 2

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Number of waitlisted patients as of 12/31/20 | **1554** | 437 | 64 | 0 | 4 | 0 | 41 | 0 | 140 | 0 | 209 | 0 | 59 | 59 | 5 | 4 | 45 | 26 | 92 | 2 | 4 | 31 | 2 | 0 | 190 | 39 | 85 | 0 | 0 | 0 | 16 |
| Number of waitlisted patients who died in 2020 | **124** | 56 | 3 | 0 | 0 | 0 | 2 | 0 | 16 | 0 | 0 | 0 | 8 | 0 | 0 | 0 | 0 | 3 | 15 | 1 | 2 | 5 | 0 | 0 | 6 | 2 | 5 | 0 | 0 | 0 | 0 |
| **HEART** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Number of transplant centers | **18** | 4 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 |
| Number of patients waitlisted for the first time in 2020 | **304** | 157 | 4 | 1 | 0 | 0 | 19 | 0 | 9 | 0 | 34 | 0 | 19 | 16 | 0 | 0 | 7 | 9 | 14 | 0 | 0 | 1 | 0 | 0 | 0 | 8 | 6 | 0 | 0 | 0 | 0 |
| Total number of waitlisted patients in 2020 | **709** | 317 | 14 | 1 | 0 | 0 | 42 | 0 | 25 | 0 | 48 | 0 | 49 | 67 | 0 | 0 | 9 | 35 | 50 | 0 | 0 | 10 | 0 | 0 | 0 | 16 | 26 | 0 | 0 | 0 | 0 |
| Number of waitlisted patients as of 12/31/20 | **404** | 164 | 12 | 0 | 0 | 0 | 22 | 0 | 17 | 0 | 19 | 0 | 25 | 46 | 0 | 0 | 7 | 18 | 34 | 0 | 0 | 8 | 0 | 0 | 0 | 11 | 21 | 0 | 0 | 0 | 0 |
| Number of waitlisted patients who died in 2020 | **53** | 11 | 1 | 0 | 0 | 0 | 1 | 0 | 2 | 0 | 7 | 0 | 9 | 11 | 0 | 0 | 0 | 3 | 5 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| **PANCREAS** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Number of transplant centers | **4** | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Number of patients waitlisted for the first time in 2020 | **14** | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total number of waitlisted patients in 2020 | **114** | 110 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Number of waitlisted patients as of 12/31/20 | **98** | 95 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Number of waitlisted patients who died in 2020 | **0** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **LUNGS** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Number of transplant centers | **3** | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Number of patients waitlisted for the first time in 2020 | **46** | 45 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total number of waitlisted patients in 2020 | **94** | 93 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Number of waitlisted patients as of 12/31/20 | **76** | 76 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Number of waitlisted patients who died in 2020 | **7** | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

* – http://www.gks.ru.

Table 3

**Organ donation and transplantation in Russia in 2020**

| Indicator | Number (abs) | Indicator per million population |
|---|---|---|
| **Organ donation** | | |
| Total number of organ donors | 890 | 6.1 |
| Deceased donor | 564 | 3.9 |
| Living (related) donors | 326 | 2.2 |
| **Organ transplantation** | | |
| Total number of organs transplanted | 1960 | 13.4 |
| *including pediatric transplants* | 256 | |
| Kidney | 1124 | 7.7 |
| as well as cadaveric | 967 | 6.6 |
| from a living donor | 157 | 1.1 |
| *including pediatric transplants* | 119 | |
| Liver | 559 | 3.8 |
| as well as cadaveric | 390 | 2.7 |
| from a living donor | 169 | 1.2 |
| *including pediatric transplants* | 131 | |
| Heart | 251 | 1.7 |
| *including pediatric transplants* | 6 | |
| Pancreas | 16 | 0.1 |
| Lungs | 11 | 0.1 |
| *including pediatric transplants* | 0 | |
| Small intestine | 1 | 0.0 |
| *including pediatric transplants* | 0 | |

* Population of the Russian Federation in 2020: 146.2 million people. (www.gks.ru).

The impact of COVID-19 and related restrictive measures was reflected in the monthly organ transplant statistics in 2020. See Figs. 2 and 3.

In 2020, high-tech medical care for organ transplantation was fully funded. Based on data obtained from the Federal Registry for High-Tech Medical Care, 1842 (94.0%) organ transplant surgeries were performed in 2020 using funds from the compulsory medical insurance system, allocated for provision of high-tech medical care on organ transplant (there were 2119 transplant surgeries (87.3%) in 2019). See Fig. 5.

Since 2010, when funding was included in the Registry as an indicator, the number of organ transplants performed using the funds allocated for provision of high-tech medical care on organ transplant has increased 2.3 times. At the same time, the proportion of organ transplants performed using these funds has increased by 35.8%.

The financial costs per unit of high-tech medical care for transplantation in 2020 were as follows:

– 950,896 rubles for kidney, pancreas, kidney-pancreas, small bowel, lung transplant;
– 1,206,336 rubles for heart and liver transplant;
– 1,723,623 rubles for heart-lung transplant.

(Resolution No. 1610 of the Government of the Russian Federation, dated December 7, 2019).

## ORGAN DONATION

In 2020, donor programs were implemented in 31 (out of 85) federal subjects of the Russian Federation with a population of 98.3 million people. In Perm Krai,

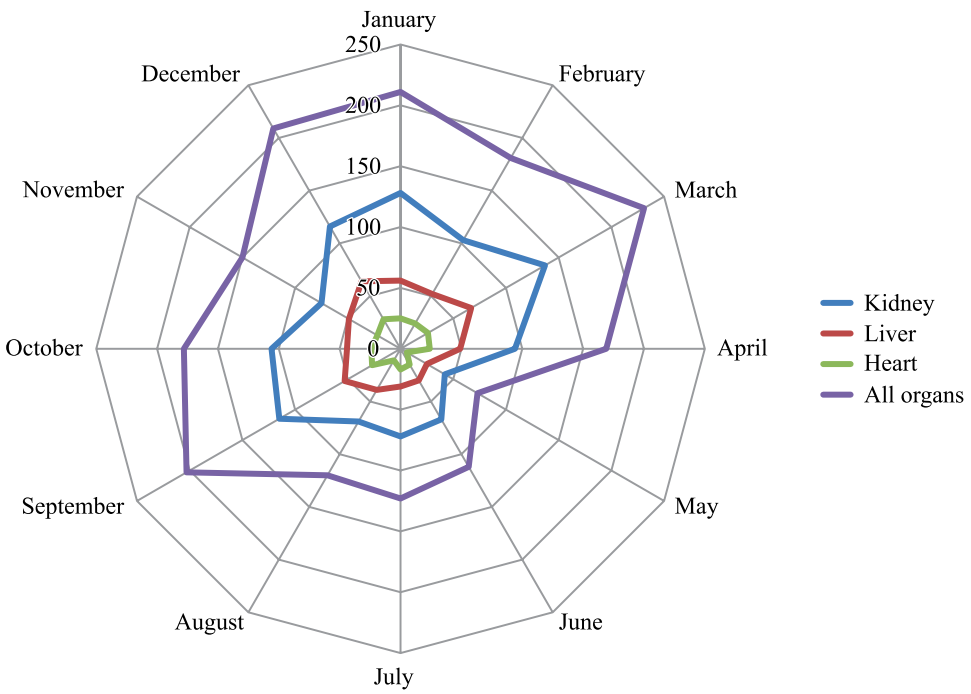


Fig. 2. Number of organ transplants by months in 2020

Table 4

**Transplantation activity in Russia in 2020**

| No. | Transplant center, region, federal district | **Total** | **Kidney (total)** | Kidney (cadaveric) | Kidney (living related) | **Liver (total)** | Liver (cadaveric) | Liver (living related) | **Heart** | **Pancreas** | **Lungs** | **Heart-lungs** | **Small intestine** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| 1 | Branch of Shumakov National Medical Research Center of Transplantology and Artificial Organs, **Moscow, Central Federal District** | **563** | **198** | 134 | 64 | **165** | 57 | 108 | **190** | **2** | **6** | **2** | **0** |
| 2 | Branch of Shumakov National Medical Research Center of Transplantology and Artificial Organs, **Volzhsky, Southern Federal District** | **8** | **8** | 0 | 8 | **0** | 0 | 0 | **0** | **0** | **0** | **0** | **0** |
| 3 | Lopatkin Research Institute of Urology and Interventional Radiology – a branch of the National Medical Research Center for Radiology, **Moscow, Central Federal District** | **28** | **28** | 22 | 6 | **0** | 0 | 0 | **0** | **0** | **0** | **0** | **0** |
| 4 | Russian Children's Clinical Hospital, **Moscow, Central Federal District** | **30** | **30** | 30 | 0 | **0** | 0 | 0 | **0** | **0** | **0** | **0** | **0** |
| 5 | Petrovsky National Research Centre of Surgery, **Moscow, Central Federal District** | **21** | **13** | 6 | 7 | **8** | 1 | 7 | **0** | **0** | **0** | **0** | **0** |
| 6 | Burnazyan Federal Medical and Biophysical Center, **Moscow, Central Federal District** | **59** | **12** | 10 | 2 | **47** | 10 | 37 | **0** | **0** | **0** | **0** | **0** |
| 7 | Bakulev Scientific Center of Cardiovascular Surgery, **Moscow, Central Federal District** | **3** | **0** | 0 | 0 | **0** | 0 | 0 | **3** | **0** | **0** | **0** | **0** |
| 8 | National Medical Research Center for Children's Health, **Moscow, Central Federal District** | **34** | **34** | 8 | 26 | **0** | 0 | 0 | **0** | **0** | **0** | **0** | **0** |
| 9 | Botkin City Clinical Hospital, **Moscow, Central Federal District** | **110** | **75** | 75 | 0 | **35** | 35 | 0 | **0** | **0** | **0** | **0** | **0** |
| 10 | Research Institute of Emergency Pediatric Surgery and Traumatology, **Moscow, Central Federal District** | **1** | **1** | 1 | 0 | **0** | 0 | 0 | **0** | **0** | **0** | **0** | **0** |
| 11 | Sklifosovsky Research Institute of Emergency Care, **Moscow, Central Federal District** | **333** | **198** | 196 | 2 | **118** | 117 | 1 | **1** | **13** | **2** | **0** | **1** |
| 12 | Morozovskaya Children's City Clinical Hospital, **Moscow, Central Federal District** | **0** | **0** | 0 | 0 | **0** | 0 | 0 | **0** | **0** | **0** | **0** | **0** |
| 13 | Vladimirsky Moscow Regional Research Clinical Institute, **Moscow Oblast, Central Federal District** | **54** | **36** | 35 | 1 | **17** | 14 | 3 | **1** | **0** | **0** | **0** | **0** |
| 14 | Federal Clinical Center for High Medical Technologies under the Federal Medical-Biological Agency (119), **Moscow Oblast, Central Federal District** | **8** | **8** | 6 | 2 | **0** | 0 | 0 | **0** | **0** | **0** | **0** | **0** |
| 15 | Vishnevsky 3rd Central Military Clinical Hospital, **Moscow Oblast, Central Federal District** | **0** | **0** | 0 | 0 | **0** | 0 | 0 | **0** | **0** | **0** | **0** | **0** |
| 16 | St. Joasaphus Belgorod Regional Clinical Hospital, **Belgorod, Central Federal District** | **5** | **4** | 4 | 0 | **1** | 1 | 0 | **0** | **0** | **0** | **0** | **0** |

Continuation table 4

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 17 | Voronezh Regional Clinical Hospital No. 1, **Voronezh, Central Federal District** | **8** | **7** | 7 | 0 | **0** | 0 | 0 | **1** | **0** | **0** | **0** | **0** |
| 18 | Tula Regional Clinical Hospital, **Tula, Central Federal District** | **6** | **6** | 3 | 3 | **0** | 0 | 0 | **0** | **0** | **0** | **0** | **0** |
| 19 | Ryazan Regional Clinical Hospital, **Ryazan, Central Federal District** | **9** | **8** | 8 | 0 | **1** | 1 | 0 | **0** | **0** | **0** | **0** | **0** |
| 20 | Stavropol Regional Clinical Hospital, **Stavropol, North Caucasian Federal District** | **13** | **9** | 9 | 0 | **4** | 3 | 1 | **0** | **0** | **0** | **0** | **0** |
| 21 | Ochapovsky Regional Clinical Hospital No. 1, **Krasnodar, Southern Federal District** | **33** | **22** | 22 | 0 | **7** | 7 | 0 | **4** | **0** | **0** | **0** | **0** |
| 22 | Regional Clinical Hospital No. 2, **Krasnodar, Southern Federal District** | **1** | **0** | 0 | 0 | **1** | 1 | 0 | **0** | **0** | **0** | **0** | **0** |
| 23 | Volzhsky Regional Urological Center, **Volzhsky, Southern Federal District** | **19** | **19** | 18 | 1 | **0** | 0 | 0 | **0** | **0** | **0** | **0** | **0** |
| 24 | Rostov Regional Clinical Hospital, **Rostov-on-Don, Southern Federal District** | **56** | **37** | 37 | 0 | **15** | 14 | 1 | **3** | **1** | **0** | **0** | **0** |
| 25 | Granov Russian Research Center of Radiology and Surgical Technologies, St. Petersburg, **St. Petersburg, Northwestern Federal District** | **17** | **0** | 0 | 0 | **17** | 17 | 0 | **0** | **0** | **0** | **0** | **0** |
| 26 | Almazov National Medical Research Centre, **St. Petersburg, Northwestern Federal District** | **18** | **0** | 0 | 0 | **0** | 0 | 0 | **18** | **0** | **0** | **0** | **0** |
| 27 | Pavlov First St. Petersburg State Medical University, St. Petersburg, **St. Petersburg, Northwestern Federal District** | **18** | **15** | 9 | 6 | **2** | 2 | 0 | **0** | **0** | **1** | **0** | **0** |
| 28 | St. Petersburg Dzhanelidze Research Institute of Emergency Medicine, **St. Petersburg, Northwestern Federal District** | **22** | **20** | 20 | 0 | **2** | 2 | 0 | **0** | **0** | **0** | **0** | **0** |
| 29 | Leningrad Regional Clinical Hospital, **St. Petersburg, Northwestern Federal District** | **20** | **20** | 20 | 0 | **0** | 0 | 0 | **0** | **0** | **0** | **0** | **0** |
| 30 | Kirov Military Medical Academy, **St. Petersburg, Northwestern Federal District** | **3** | **0** | 0 | 0 | **3** | 3 | 0 | **0** | **0** | **0** | **0** | **0** |
| 31 | City Mariinskaya Hospital, **St. Petersburg, Northwestern Federal District** | **6** | **6** | 6 | 0 | **0** | 0 | 0 | **0** | **0** | **0** | **0** | **0** |
| 32 | Volosevich First City Clinical Hospital, **Arkhangelsk, Northwestern Federal District** | **1** | **1** | 1 | 0 | **0** | 0 | 0 | **0** | **0** | **0** | **0** | 0 |
| 33 | Republican Hospital No. 1 – National Center of Medicine, **Yakutsk, Far Eastern Federal District** | **0** | **0** | 0 | 0 | **0** | 0 | 0 | **0** | **0** | **0** | **0** | **0** |
| 34 | Meshalkin National Medical Research Center, **Novosibirsk, Siberian Federal District** | **5** | **0** | 0 | 0 | **0** | 0 | 0 | **5** | **0** | **0** | **0** | **0** |
| 35 | State Novosibirsk Regional Clinical Hospital, **Novosibirsk, Siberian Federal District** | **57** | **23** | 21 | 2 | **34** | 24 | 10 | **0** | **0** | **0** | **0** | **0** |
| 36 | Research Institute for Complex Issues of Cardiovascular Diseases, **Kemerovo, Siberian Federal District** | **7** | **0** | 0 | 0 | **0** | 0 | 0 | **7** | **0** | **0** | **0** | **0** |
| 37 | Belyaev Kemerovo Regional Clinical Hospital, **Kemerovo, Siberian Federal District** | **39** | **39** | 38 | 1 | **0** | 0 | 0 | **0** | **0** | **0** | **0** | **0** |

End of table 4

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 38 | Podgorbunsky City Clinical Hospital, **Kemerovo, Siberian Federal District** | **9** | **0** | 0 | 0 | **9** | 9 | 0 | **0** | **0** | **0** | **0** | **0** |
| 39 | Irkutsk Regional Clinical Hospital, **Irkutsk, Siberian Federal District** | **34** | **20** | 19 | 1 | **14** | 14 | 0 | **0** | **0** | **0** | **0** | **0** |
| 40 | Omsk City Clinical Hospital No. 1, **Omsk, Siberian Federal District** | **1** | **1** | 1 | 0 | **0** | 0 | 0 | **0** | **0** | **0** | **0** | **0** |
| 41 | Regional Clinical Hospital, **Altai Krai (Barnaul), Siberian Federal District** | **20** | **16** | 16 | 0 | **4** | 4 | 0 | **0** | **0** | **0** | **0** | **0** |
| 42 | Federal Center for Cardiovascular Surgery, **Krasnoyarsk, Siberian Federal District** | **0** | **0** | 0 | 0 | **0** | 0 | 0 | **0** | **0** | **0** | **0** | **0** |
| 43 | Federal Siberian Research and Clinical Center, **Krasnoyarsk, Siberian Federal District** | **14** | **12** | 12 | 0 | **2** | 2 | 0 | **0** | **0** | **0** | **0** | **0** |
| 44 | Krasnoyarsk Clinical Hospital, **Krasnoyarsk, Siberian Federal District** | **29** | **16** | 16 | 0 | **10** | 10 | 0 | **3** | **0** | **0** | **0** | **0** |
| 45 | Sverdlovsk Regional Clinical Hospital No. 1, **Yekaterinburg, Ural Federal District** | **17** | **9** | 9 | 0 | **5** | 5 | 0 | **3** | **0** | **0** | **0** | **0** |
| 46 | Chelyabinsk Regional Clinical Hospital, **Chelyabinsk, Ural Federal District** | **7** | **4** | 4 | 0 | **2** | 2 | 0 | **1** | **0** | **0** | **0** | **0** |
| 47 | Regional Clinical Hospital No. 1, **Tyumen, Ural Federal District** | **14** | **11** | 11 | 0 | **3** | 3 | 0 | **0** | **0** | **0** | **0** | **0** |
| 48 | District Clinical Hospital, **Khanty-Mansiysk, Ural Federal District** | **5** | **4** | 4 | 0 | **1** | 1 | 0 | **0** | **0** | **0** | **0** | **0** |
| 49 | Samara State Medical University, **Samara, Volga Federal District** | **47** | **47** | 47 | 0 | **0** | 0 | 0 | **0** | **0** | **0** | **0** | **0** |
| 50 | Saratov State Medical University, **Saratov, Volga Federal District** | **8** | **8** | 0 | 8 | **0** | 0 | 0 | **0** | **0** | **0** | **0** | **0** |
| 51 | Regional Clinical Hospital, **Saratov, Volga Federal District** | **0** | **0** | 0 | 0 | **0** | 0 | 0 | **0** | **0** | **0** | **0** | **0** |
| 52 | Volga District Medical Center, **Nizhny Novgorod, Volga Federal District** | **20** | **14** | 8 | 6 | **6** | 5 | 1 | **0** | **0** | **0** | **0** | **0** |
| 53 | Specialized Cardiac Surgical Clinical Hospital, **Nizhny Novgorod, Volga Federal District** | **1** | **0** | 0 | 0 | **0** | 0 | 0 | **1** | **0** | **0** | **0** | **0** |
| 54 | Republican Clinical Hospital, **Kazan, Volga Federal District** | **60** | **40** | 34 | 6 | **20** | 20 | 0 | **0** | **0** | **0** | **0** | **0** |
| 55 | Interregional Clinical Diagnostic Center, **Kazan, Volga Federal District** | **4** | **0** | 0 | 0 | **0** | 0 | 0 | **4** | **0** | **0** | **0** | **0** |
| 56 | Republican Clinical Hospital, **Ufa, Volga Federal District** | **44** | **38** | 38 | 0 | **6** | 6 | 0 | **0** | **0** | **0** | **0** | **0** |
| 57 | Republican Cardiology Clinic, **Ufa, Volga Federal District** | **4** | **0** | 0 | 0 | **0** | 0 | 0 | **4** | **0** | **0** | **0** | **0** |
| 58 | Perm Regional Clinical Hospital, **Perm, Volga Federal District** | **2** | **2** | 0 | 2 | **0** | 0 | 0 | **0** | **0** | **0** | **0** | **0** |
| 59 | Ulyanovsk Regional Clinical Center for Specialized Types of Medical Care, **Ulyanovsk, Volga Federal District** | **1** | **1** | 0 | 1 | **0** | 0 | 0 | **0** | **0** | **0** | **0** | **0** |
| 60 | City Clinical Hospital for Emergency Medical Care No. 1, **Orenburg, Volga Federal District** | **4** | **4** | 2 | 2 | **0** | 0 | 0 | **0** | 0 | 0 | 0 | 0 |
| **Total** | | **1960** | **1124** | **967** | **157** | **559** | **390** | **169** | **249** | **16** | **9** | **2** | **1** |

Ulyanovsk Oblast and Saratov Oblast, only living-related donor kidney transplants were performed.

There was a total of 564 effective deceased donors (3.9 per million population) in 2020. See Table 5.

In 2020, some of the hospitals involved in donor support for transplantation centers reassigned their intensive care units to provide medical care to patients with severe COVID-19.

The past year continued the trend of increase in the proportion of effective organ donors over 60 years of age; it exceeded 20% in 2020. Male donors were 73.0%, female donors were 27%. The age structure of effective organ donors is shown in Fig. 5.

Donor activity per population of the regions implementing donor programs (95.9 million) amounted to 5.9 per million population. See Tables 6 and 7. The steadily high European level indicator of donor activity was retained in Moscow (20.9), while the indicators in Kemerovo Oblast (10.0), Samara Oblast (7.5), Irkutsk Oblast (6.7) and Leningrad Oblast (6.1) were also stea-

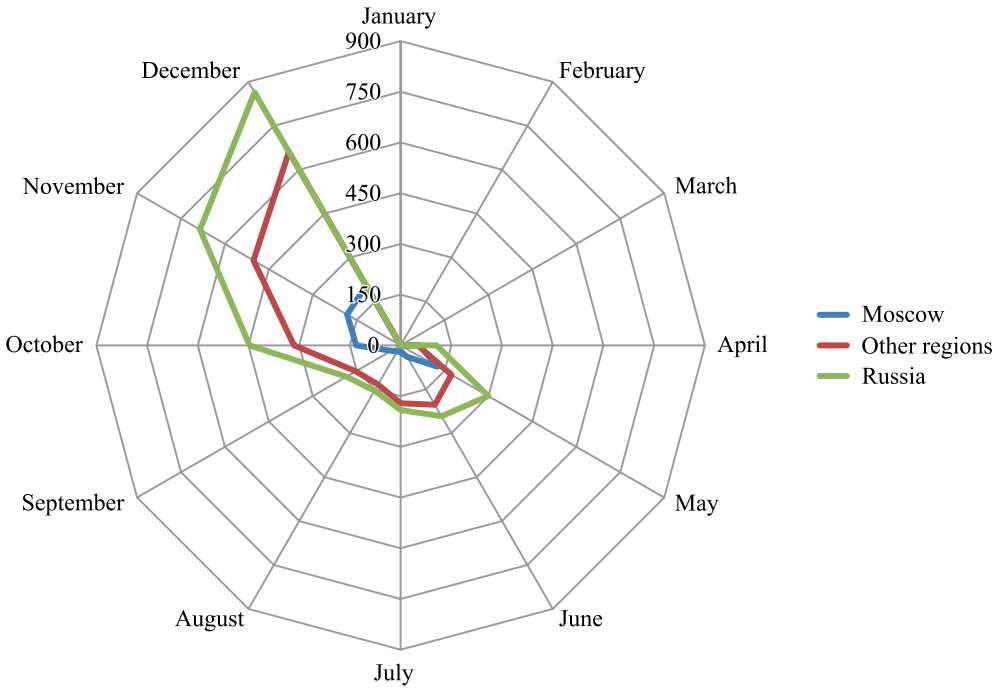


Fig. 3. COVID-19 incidence by months in 2020

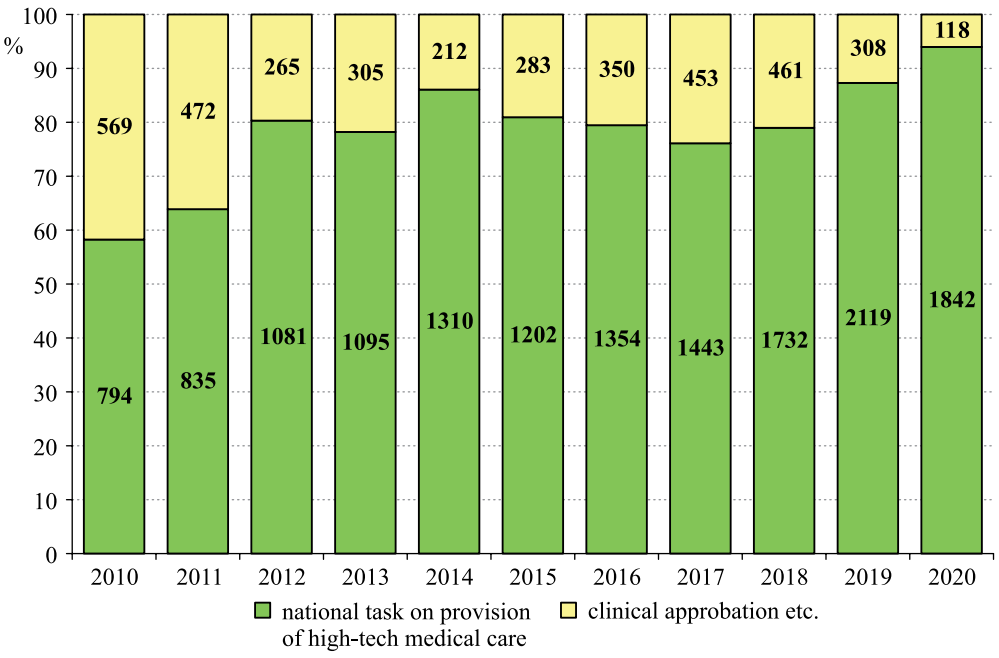


Fig. 4. Funding for Organ Transplants in Russia in 2010–2020

dily high. Moscow and Moscow Oblast accounted for 50.3% (284) of effective donors in 2020.

In 2020, the practice of brain death pronouncement continued to expand in Russia. There were 547 effective brain-dead donors – 97.0% (94.5% in 2019) of the total pool of effective donors. See Fig. 6.

There are no organ donor programs in the country that do not use a protocol to diagnose human death based on diagnosis of brain death. In 24 federal subjects of Russia, organ donor programs worked only with brain-dead donors. Kemerovo Oblast, which lags behind other regions in this indicator, has seen a consistent increase in the proportion of brain-dead donors: 36.7% in 2018, 47.5% in 2019 and 66.7% in 2020.

In 2020, a total of 421 multi-organ procurements were performed, accounting for 74.6%. For comparison, it was 71.8% in 2019. There were 16 organ donor programs involving a high proportion of multi-organ procurements (over 70%). In 4 of the programs, multiple organs were procured from all (100%) the patients.

Moscow and Moscow Oblast accounted for 239 (56.8%) multi-organ donors in the country in 2020.

The average number of organs procured from one donor remained the same with that of 2019 and 2018 – 2.9 procurements. The highest number of organ procurements were, as before, performed at federal subjects where extrarenal organs were transplanted and (or) at federal subjects where there was interregional coordination: Tula Oblast (3.7), Moscow (3.3), Ryazan Oblast (3.2), Sverdlovsk Oblast (3.2), and Nizhny Novgorod Oblast (3.2). The lowest number of procurements was recorded in Omsk Oblast (1.5), in Volgograd Oblast (2.0), and in Irkutsk Oblast (2.0).

In 2020, the rate of procurement and use of donor kidneys was 86.7%. In 17 regions, this indicator was in the optimal 90%–100% range, in 7 regions it was between

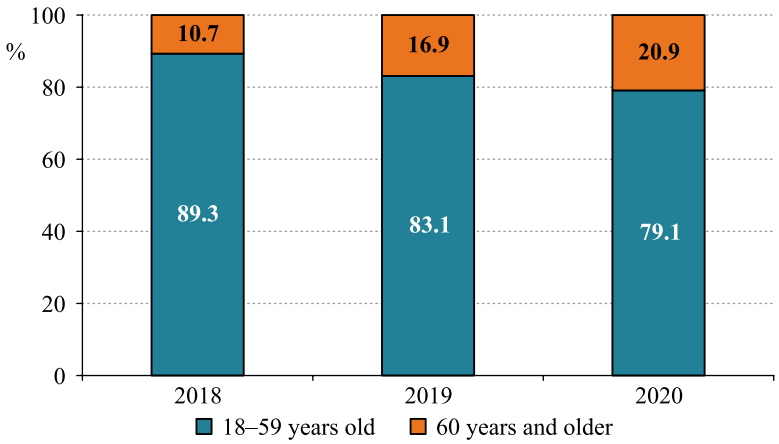


Fig. 5. Age structure of effective organ donors in 2018–2020

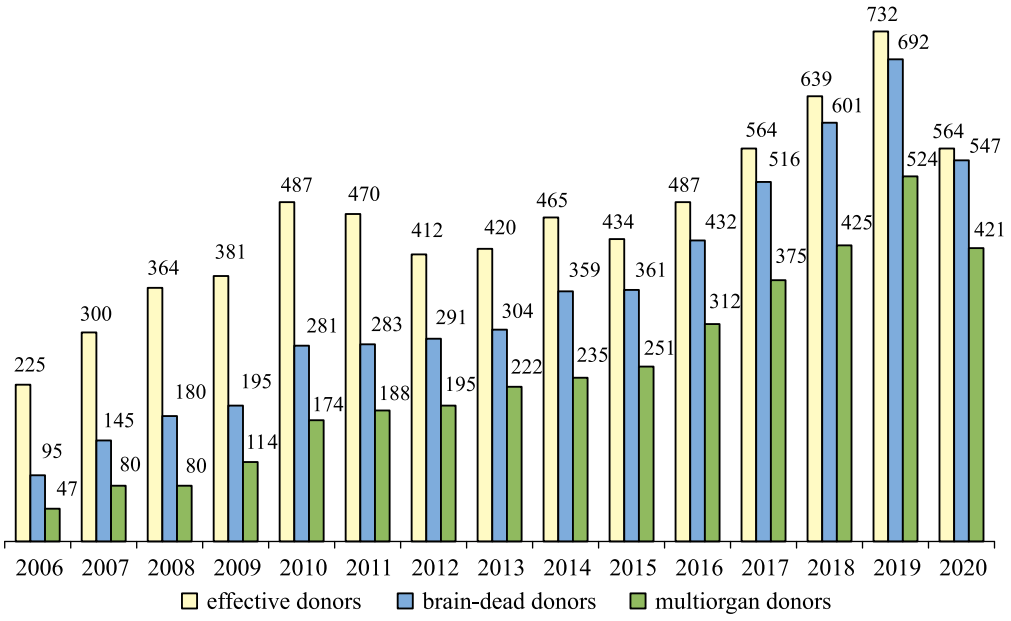


Fig. 6. Structure of effective organ donors in the Russian Federation in 2006–2020

Table 5

**Indicators associated with donor activity in Russian regions in 2020**

| No. | Region | Organ Donation Coordinating Center, region | Population (million) | Number of donor bases | Effective donors (abs., per million population) | | including those diagnosed with brain death (abs., %) | | including multi-organ donors (abs., %) | | Total organs procured | including procured kidneys | Number of organs/number of donors ratio | Percentage of kidneys procured |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| 1 | Moscow | Moscow Coordinating Center for Organ Donation, Moscow (Botkin City Clinical Hospital) | 12.6 | 17 | 263 | 20.9 | 260 | 98.9 | 219 | 83.3 | 857 | 457 | 3.3 | 86.9 |
| 2 | Moscow Oblast | Vladimirsky Moscow Regional Research Clinical Institute, Moscow | 7.5 | 33 | 21 | 2.8 | 21 | 100.0 | 20 | 95.2 | 54 | 42 | 2.6 | 100.0 |
| 3 | Belgorod Oblast | St. Joasaphus Belgorod Regional Clinical Hospital, Belgorod | 1.5 | 1 | 2 | 1.3 | 2 | 100.0 | 1 | 50.0 | 5 | 4 | 2.5 | 100.0 |
| 4 | Voronezh Oblast | Voronezh Regional Clinical Hospital No. 1, Voronezh | 2.3 | 10 | 4 | 1.7 | 4 | 100.0 | 1 | 25.0 | 9 | 7 | 2.3 | 87.5 |
| 5 | Tula Oblast | Tula Regional Clinical Hospital, Tula | 1.5 | 1 | 3 | 2.0 | 3 | 100.0 | 3 | 100.0 | 11 | 6 | 3.7 | 100.0 |
| 6 | Ryazan Oblast | Ryazan Regional Clinical Hospital, Ryazan | 1.1 | 1 | 6 | 5.5 | 6 | 100.0 | 5 | 83.3 | 19 | 12 | 3.2 | 100.0 |
| 7 | Krasnodar Krai | Ochapovsky Regional Clinical Hospital No. 1, Krasnodar | 5.6 | 2 | 13 | 2.3 | 13 | 100.0 | 9 | 69.2 | 35 | 22 | 2.7 | 84.6 |
| 8 | Volgograd Oblast | Volzhsky Regional Urological Center, Volzhsky | 2.5 | 11 | 10 | 4.0 | 10 | 100.0 | 0 | 0.0 | 20 | 20 | 2.0 | 100.0 |
| 9 | Rostov Oblast | Rostov Regional Clinical Hospital, Rostov-on-Don | 4.2 | 1 | 18 | 4.3 | 18 | 100.0 | 14 | 77.8 | 53 | 35 | 2.9 | 97.2 |
| 10 | Stavropol Krai | Stavropol Regional Clinical Hospital, Stavropol | 2.8 | 1 | 13 | 4.6 | 13 | 100.0 | 11 | 84.6 | 37 | 26 | 2.8 | 100.0 |
| 11 | St. Petersburg | Center for Organ and Tissue Donation, St. Petersburg (St. Petersburg Dzhanelidze Research Institute of Emergency Medicine) | 5.4 | 14 | 25 | 4.6 | 25 | 100.0 | 22 | 88.0 | 69 | 33 | 2.8 | 66.0 |
| 12 | Leningrad Oblast | Leningrad Regional Clinical Hospital, St. Petersburg | 1.8 | 1 | 11 | 6.1 | 11 | 100.0 | 7 | 63.6 | 29 | 22 | 2.6 | 100.0 |
| 13 | Arkhangelsk Oblast | Volosevich First City Clinical Hospital, Arkhangelsk | 1.1 | 1 | 1 | 0.9 | 1 | 100.0 | 1 | 100.0 | 3 | 2 | 3.0 | 100.0 |
| 14 | Novosibirsk Oblast | State Novosibirsk Regional Clinical Hospital, Novosibirsk | 2.8 | 10 | 15 | 5.4 | 15 | 100.0 | 11 | 73.3 | 32 | 15 | 2.1 | 50.0 |
| 15 | Kemerovo Oblast | Belyaev Kemerovo Regional Clinical Hospital, Kemerovo | 2.7 | 15 | 27 | 10.0 | 18 | 66.7 | 17 | 63.0 | 70 | 44 | 2.6 | 81.5 |
| 16 | Irkutsk Oblast | Irkutsk Regional Clinical Hospital, Irkutsk | 2.4 | 1 | 16 | 6.7 | 15 | 93.8 | 13 | 81.3 | 32 | 19 | 2.0 | 59.4 |
| 17 | Omsk Oblast | Omsk City Clinical Hospital No. 1, Omsk | 1.9 | 2 | 2 | 1.1 | 2 | 100.0 | 1 | 50.0 | 3 | 2 | 1.5 | 50.0 |
| 18 | Altai Krai | Regional Clinical Hospital, Altai Krai (Barnaul) | 2.3 | 1 | 9 | 3.9 | 9 | 100.0 | 9 | 100.0 | 27 | 18 | 3.0 | 100.0 |

End of table 5

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 19 | Krasnoyarsk Krai | Krasnoyarsk Clinical Hospital, Krasnoyarsk | 2.9 | 12 | 10 | 3.4 | 10 | 100.0 | 9 | 90.0 | 29 | 17 | 2.9 | 85.0 |
| 20 | Sverdlovsk Oblast | Sverdlovsk Regional Clinical Hospital No. 1, Yekaterinburg | 4.3 | 8 | 6 | 1.4 | 6 | 100.0 | 5 | 83.3 | 19 | 11 | 3.2 | 91.7 |
| 21 | Chelyabinsk Oblast | Chelyabinsk Regional Clinical Hospital, Chelyabinsk | 3.5 | 1 | 3 | 0.9 | 3 | 100.0 | 2 | 66.7 | 9 | 6 | 3.0 | 100.0 |
| 22 | Tyumen Oblast | Regional Clinical Hospital No. 1, Tyumen | 1.5 | 1 | 5 | 3.3 | 5 | 100.0 | 3 | 60.0 | 14 | 10 | 2.8 | 100.0 |
| 23 | Khanty-Mansi Autonomous Okrug – Yugra | District Clinical Hospital, Khanty-Mansiysk | 1.7 | 8 | 3 | 1.8 | 3 | 100.0 | 2 | 66.7 | 8 | 6 | 2.7 | 100.0 |
| 24 | Samara Oblast | Samara State Medical University, Samara | 3.2 | 5 | 24 | 7.5 | 20 | 83.3 | 4 | 16.7 | 52 | 48 | 2.2 | 100.0 |
| 25 | Saratov Oblast | Regional Clinical Hospital, Saratov | 2.4 | 1 | 0 | 0.0 | 0 | – | 0 | – | 0 | 0 | – | – |
| 26 | Nizhny Novgorod Oblast | Volga District Medical Center, Nizhny Novgorod | 3.2 | 9 | 5 | 1.6 | 5 | 100.0 | 4 | 80.0 | 16 | 8 | 3.2 | 80.0 |
| 27 | The Republic of Tatarstan | Republican Clinical Hospital, Kazan | 3.9 | 2 | 21 | 5.4 | 21 | 100.0 | 19 | 90.5 | 57 | 34 | 2.7 | 81.0 |
| 28 | The Republic of Bashkortostan | Republican Clinical Hospital, Ufa | 4.1 | 12 | 18 | 4.4 | 18 | 100.0 | 6 | 33.3 | 46 | 36 | 2.6 | 100.0 |
| 29 | Orenburg Oblast | City Clinical Hospital for Emergency Medical Care No. 1, Orenburg | 2.0 | 2 | 1 | 0.5 | 1 | 100.0 | 1 | 100.0 | 3 | 2 | 3.0 | 100.0 |
| 30 | The Republic of Sakha (Yakutia) | Republican Hospital No. 1 – National Center of Medicine, Yakutsk | 1.0 | 1 | 0 | 0.0 | 0 | – | 0 | – | 0 | 0 | – | – |
| 31 | Departmental program of the Federal Medical-Biological Agency of Russia | Burnazyan Federal Medical and Biophysical Center, Moscow, Central Federal District | – | 28 | 1 | – | 1 | 100.0 | 0 | 0.0 | 1 | 0 | 1.0 | 0.0 |
| 32 | Departmental program of the Federal Medical-Biological Agency of Russia | Federal Siberian Research and Clinical Center, Krasnoyarsk | – | 5 | 8 | – | 8 | 100.0 | 2 | 25.0 | 17 | 14 | 2.1 | 87.5 |
| | | **Total** | **146.2** | **218** | **564** | **3.9** | **547** | **97.0** | **421** | **74.6** | **1636** | **978** | **2.9** | **86.7** |

80% and 90%, and in 4 programs it was less than 80% (66.0% in St. Petersburg, 50.0% in Novosibirsk Region, 59.4% in Irkutsk Region and 50.0% in Omsk Oblast).

Thus, with reduced number of effective donors against the background of the COVID-19 pandemic, the indicators of donor work efficiency, namely the proportion of brain-dead donors, the proportion of multi-organ donors, the average number of organs transplanted from one effective donor, did not worsen. No cases of donor-to-recipient transfer of COVID-19 infection were recorded.

In 2020, the number of organ donations from living related donors was 326 – 36.6% of the total number of procurements (890).

## KIDNEY TRANSPLANTATION

In 2020, a total of 1,124 kidney transplantations were performed (7.7 per million population). See Fig. 7.

There were 967 deceased-donor kidney transplants (6.6 per million population) in 2020. There were 157 living-related donor kidney transplants (1.1 per million population) in 2020.

Table 8 and Fig. 8 show the kidney transplant centers with the highest number of kidney transplants in 2020.

The rating primarily demonstrates the leadership and sustainability of the transplantation programs of leading transplant centers in Moscow, which in turn is a consequence of the effective work by the Moscow Coordination Center for Organ Donation. The positive dynamics of transplantation programs in Samara Oblast and Rostov Oblast should also be noted. The negative impact of COVID-19 was largely reflected in the indicators for Kemerovo Oblast, St. Petersburg, and Moscow Oblast. The total transplant activity in the presented 10 leading kidney transplant centers is 750.

Table 6

**Rating of regions by donor activity in 2020**

| Subject of the Russian Federation (Oblast) | Population in 2020, million | Number of effective donors per million population | | Rating | | Change in rating |
|---|---|---|---|---|---|---|
| | | 2020 | 2019 | 2020 | 2019 | |
| Moscow | 12.6 | 20.9 | 22.0 | 1 | 1 | No |
| Kemerovo Oblast | 2.7 | 10.0 | 14.8 | 2 | 2 | No |
| Samara Oblast | 3.2 | 7.5 | 7.8 | 3 | 7 | +4 |
| Irkutsk Oblast | 2.4 | 6.7 | 6.7 | 4 | 8 | +4 |
| Leningrad Oblast | 1.8 | 6.1 | 3.9 | 5 | 19 | +14 |
| Ryazan Oblast | 1.1 | 5.5 | 11.8 | 6 | 3 | –3 |
| Novosibirsk Oblast | 2.8 | 5.4 | 8.2 | 7 | 6 | –1 |
| The Republic of Tatarstan | 3.9 | 5.4 | 3.8 | 8 | 20 | +12 |
| Voronezh Oblast | 2.3 | 5.4 | 3.5 | 9 | 22 | +13 |
| St. Petersburg | 5.4 | 4.6 | 9.8 | 10 | 4 | –6 |
| Stavropol Krai | 2.8 | 4.6 | 1.1 | 11 | 29 | +18 |
| The Republic of Bashkortostan | 4.1 | 4.4 | 5.9 | 12 | 9 | –3 |
| Rostov Oblast | 4.2 | 4.3 | 5.0 | 13 | 13 | No |
| Volgograd Oblast | 2.5 | 4.0 | 4.0 | 14 | 18 | +4 |
| Krasnoyarsk Krai* | 2.9 | 3.4 | 4.5 | 15 | 15 | No |
| Tyumen Oblast | 1.5 | 3.3 | 8.7 | 16 | 5 | –11 |
| Moscow Oblast | 7.5 | 2.8 | 5.5 | 17 | 12 | –5 |
| Krasnodar Krai | 5.6 | 2.3 | 4.1 | 18 | 17 | –1 |
| Tula Oblast | 1.5 | 2.0 | 1.3 | 19 | 27 | +8 |
| Khanty-Mansi Autonomous Okrug – Yugra | 1.7 | 1.8 | 2.9 | 20 | 25 | +5 |
| The Republic of Sakha (Yakutia) | 1 | 1.7 | 3.0 | 21 | 24 | +3 |
| Nizhny Novgorod Oblast | 3.2 | 1.6 | 3.8 | 22 | 21 | –1 |
| Altai Krai | 2.3 | 1.6 | 3.5 | 23 | 23 | No |
| Sverdlovsk Oblast | 4.3 | 1.4 | 5.6 | 24 | 10 | –14 |
| Belgorod Oblast | 1.5 | 1.3 | 2.7 | 25 | 26 | +1 |
| Omsk Oblast | 1.9 | 1.1 | 1.1 | 26 | 30 | +4 |
| Arkhangelsk Oblast | 1.1 | 0.9 | 4.5 | 27 | 14 | –13 |
| Chelyabinsk Oblast | 3.5 | 0.9 | 1.1 | 28 | 28 | No |
| Orenburg Oblast | 2 | 0.5 | 5.5 | 29 | 11 | –18 |
| Saratov Oblast | 2.4 | 0.0 | 4.2 | 30 | 16 | –14 |
| Russia (85 federal subjects of the Russian Federation) | 146.2 | 3.9 | 5.0 | – | – | |

* Excluding the donor program FSRCC under FMBA, Krasnoyarsk.

Table 7

**Deceased organ donors (effective donors) in 2006–2020**

| No. | Oblast | 2006 | 2007 | | 2008 | | 2009 | | 2010 | | 2011 | | 2012 | | 2013 | | 2014 | | 2015 | | 2016 | | 2017 | | 2018 | | 2019 | | 2020 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Number of effective donors | Number of effective donors | Change over the year (abs.) | Number of effective donors | Change over the year (abs.) | Number of effective donors | Change over the year (abs.) | Number of effective donors | Change over the year (abs.) | Number of effective donors | Change over the year (abs.) | Number of effective donors | Change over the year (abs.) | Number of effective donors | Change over the year (abs.) | Number of effective donors | Change over the year (abs.) | Number of effective donors | Change over the year (abs.) | Number of effective donors | Change over the year (abs.) | Number of effective donors | Change over the year (abs.) | Number of effective donors | Change over the year (abs.) | Number of effective donors | Change over the year (abs.) | Number of effective donors | Change over the year (abs.) |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 |
| 1 | Moscow | 87 | 126 | +39 | 135 | +9 | 136 | +1 | 151 | +15 | 135 | –16 | 111 | –24 | 125 | +14 | 151 | +26 | 142 | –9 | 183 | +41 | 195 | +12 | 218 | +23 | 277 | +59 | 263 | –14 |
| 2 | Moscow Oblast | 24 | 45 | +21 | 59 | +14 | 52 | –7 | 71 | +19 | 82 | +11 | 61 | –21 | 56 | –5 | 51 | –5 | 44 | –7 | 39 | –5 | 75 | +36 | 68 | –7 | 41 | –27 | 21 | –20 |
| 3 | Belgorod Oblast | | 2 | +2 | 3 | +1 | 2 | –1 | 5 | +3 | 6 | +1 | 3 | –3 | 1 | –2 | 2 | +1 | 5 | +3 | 4 | –1 | 4 | 0 | 4 | 0 | 4 | 0 | 2 | –2 |
| 4 | Voronezh Oblast | 6 | 2 | –4 | 8 | +6 | 2 | –6 | 0 | –2 | 1 | +1 | 6 | +5 | 6 | 0 | 5 | –1 | 7 | +2 | 4 | –3 | 1 | –3 | 8 | +7 | 8 | 0 | 4 | –4 |
| 5 | Tula Oblast | | | | | | | | | | | | | | | | | | | | | | | | | | 2 | +2 | 3 | +1 |
| 6 | Ryazan Oblast | | | | | | | | | | | | | | | | | | | | | | | | 2 | +2 | 13 | +11 | 6 | –7 |
| 7 | Krasnodar Krai | | | | | | 3 | +3 | 39 | +36 | 52 | +13 | 42 | –10 | 41 | –1 | 23 | –18 | 25 | +2 | 24 | –1 | 19 | –5 | 20 | +1 | 23 | +3 | 13 | –10 |
| 8 | Volgograd Oblast | 5 | 0 | –5 | 11 | +11 | 15 | +4 | 16 | +1 | 17 | +1 | 19 | +2 | 15 | –2 | 18 | +3 | 8 | –10 | 8 | 0 | 9 | +1 | 9 | 0 | 10 | +1 | 10 | 0 |
| 9 | Rostov Oblast | | | | | | | | | | | | | | | | | | 1 | +1 | 7 | +6 | 13 | +6 | 19 | +6 | 21 | +2 | 18 | –3 |
| 10 | Stavropol Krai | | | | | | | | | | | | | | | | | | | | | | | | 2 | +2 | 3 | +1 | 13 | +10 |
| 11 | St. Petersburg | 30 | 45 | +15 | 47 | +2 | 47 | 0 | 41 | –6 | 34 | –7 | 22 | –12 | 13 | –9 | 23 | +10 | 31 | +8 | 29 | –2 | 31 | +2 | 34 | +3 | 53 | +19 | 25 | –28 |
| 12 | Leningrad Oblast | 12 | 8 | –4 | 11 | +3 | 11 | 0 | 13 | +2 | 10 | –3 | 10 | 0 | 10 | 0 | 9 | –1 | 7 | –2 | 12 | +5 | 11 | –1 | 15 | +4 | 7 | –8 | 11 | +4 |
| 13 | Arkhangelsk Oblast | | | | | | | | | | | | | | | | | | | | | | | | 5 | +5 | 5 | 0 | 1 | –4 |
| 14 | Novosibirsk Oblast | 17 | 11 | –6 | 18 | +7 | 29 | +11 | 35 | +6 | 25 | –10 | 20 | –4 | 17 | –3 | 11 | –6 | 14 | +3 | 9 | –5 | 14 | +5 | 17 | +3 | 23 | +6 | 15 | –8 |
| 15 | Kemerovo Oblast | 16 | 13 | –3 | 18 | +5 | 18 | 0 | 22 | +4 | 12 | –10 | 26 | +14 | 26 | 0 | 31 | +5 | 28 | –3 | 34 | +6 | 22 | –12 | 30 | +8 | 40 | +10 | 27 | –13 |
| 16 | Irkutsk Oblast | | | | 4 | +4 | 6 | +2 | 10 | +4 | 9 | –1 | 8 | –1 | 6 | –2 | 9 | +3 | 4 | –5 | 3 | –1 | 2 | –1 | 7 | +5 | 16 | +9 | 16 | 0 |
| 17 | Omsk Oblast | 10 | 15 | +5 | 13 | –2 | 19 | +6 | 19 | 0 | 14 | –5 | 11 | –3 | 14 | +3 | 16 | +2 | 11 | –5 | 4 | –7 | 4 | 0 | 3 | –1 | 2 | –1 | 2 | 0 |
| 18 | Altai Krai | | | | | | | | | | | | | | 3 | +3 | 5 | +2 | 4 | –1 | 4 | 0 | 8 | +4 | 8 | 0 | 8 | 0 | 9 | +1 |

End of table 7

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 19 | Krasnoyarsk Krai | | | | | | | | | | | | | | | | 3 | +3 | 6 | +3 | 18 | +12 | 27 | +9 | 16 | note | 13 | –3 | 10 | –3 |
| 20 | Sverdlovsk Oblast | 14 | 13 | –1 | 12 | –1 | 13 | +1 | 14 | +1 | 15 | +1 | 14 | –1 | 18 | +4 | 23 | +5 | 18 | –5 | 15 | –3 | 22 | +7 | 24 | +2 | 24 | 0 | 6 | –18 |
| 21 | Chelyabinsk Oblast | | | | | | | | 6 | +6 | 2 | –4 | 7 | +5 | 6 | –1 | 10 | +4 | 9 | –1 | 11 | +2 | 8 | –3 | 4 | –4 | 4 | 0 | 3 | –1 |
| 22 | Tyumen Oblast | | | | | | | | | | | | | | | | | | | | | | 4 | +4 | 13 | +9 | 13 | 0 | 5 | –8 |
| 23 | Khanty-Mansi Autonomous Okrug – Yugra | | | | | | | | | | | | | | | | | | | | | | 3 | +3 | 4 | +1 | 5 | +1 | 3 | –2 |
| 24 | Samara Oblast | 4 | 17 | +13 | 24 | +7 | 18 | –6 | 20 | +2 | 21 | +1 | 19 | –2 | 21 | +2 | 20 | –1 | 18 | –2 | 26 | +8 | 28 | +2 | 23 | –5 | 25 | +2 | 24 | –1 |
| 25 | Saratov Oblast | | | | | | | | | | | | | | 4 | +4 | 7 | +3 | 7 | 0 | 7 | 0 | 7 | 0 | 8 | +1 | 10 | +2 | 0 | –10 |
| 26 | Nizhny Novgorod Oblast | | | | | | 7 | +7 | 11 | +4 | 12 | +1 | 10 | –2 | 8 | –2 | 12 | +4 | 10 | –2 | 11 | +1 | 10 | –1 | 12 | +2 | 12 | 0 | 5 | –7 |
| 27 | The Republic of Tatarstan | | 3 | +3 | 1 | –2 | 3 | +2 | 12 | +9 | 16 | +4 | 9 | +7 | 6 | –3 | 6 | 0 | 4 | –2 | 1 | –3 | 3 | +2 | 4 | +1 | 15 | +11 | 21 | +6 |
| 28 | The Republic of Bashkortostan | | | | | | | | 2 | +2 | 7 | +5 | 14 | +7 | 18 | +4 | 19 | +1 | 14 | +5 | 20 | +6 | 22 | +2 | 20 | –2 | 24 | +4 | 18 | –6 |
| 29 | Orenburg Oblast | | | | | | | | | | | | | | | | | | 3 | +3 | 8 | +5 | 9 | +1 | 8 | –1 | 11 | +3 | 1 | –10 |
| 30 | The Republic of Sakha (Yakutia) | | | | | | | | | | | | | | | | | | | | 2 | +2 | 4 | +2 | 4 | 0 | 3 | –1 | 0 | –3 |
| 31 | Federal Medical-Biological Agency, Moscow | | | | | | | | | | | | | | 6 | +6 | 11 | +5 | 14 | +3 | 16 | +2 | 9 | –7 | 5 | –4 | 1 | –4 | 1 | 0 |
| 32 | Federal Medical-Biological Agency, Krasnoyarsk | | | | | | | | | | | | | | | | | | | | | | | | 24 | note | 16 | –8 | 8 | –8 |
| | TOTAL in the Russian Federation | 225 | 300 | +75 | 364 | +64 | 381 | +17 | 487 | +106 | 470 | –17 | 412 | –58 | 420 | +8 | 465 | +45 | 434 | –31 | 499 | +53 | 565 | +78 | 639 | +74 | 732 | +93 | 564 | **–168** |

*Note.* Donor activity of FSRCC under FMBA, Krasnoyarsk, is presented as a separate program.

In 2020, only 3 kidney transplant centers performed more than 50 surgeries per year. These were the Shumakov National Medical Research Center of Transplantology and Artificial Organs (Shumakov Center) (206 kidney transplants), Sklifosovsky Research Institute of Emergency Care (Sklifosovsky Institute) (198) and Botkin City Clinical Hospital (75). Eight centers performed from 30 to 49 operations during the year, another 10 centers performed from 15 to 29, the remaining 23 centers or 52.3% performed less than 15 kidney transplants.

In 2020, 20 centers out of 44 performed related-donor kidney transplants. A total of 157 transplants were performed, 72 of which were done at the Shumakov Center. The average frequency of living-donor kidney transplants in 2020 was 13.4% of the total number of kidney transplants performed (12.4% in 2019).

In 2020, 8 centers carried out pediatric kidney transplants. A total of 119 kidney transplants were done (101 in 2019), of which 114 (94.2%) were in Moscow – Shumakov Center (48), National Medical Research Center for Children's Health (34), and the Russian Children's Clinical Hospital (30). See Fig. 9.

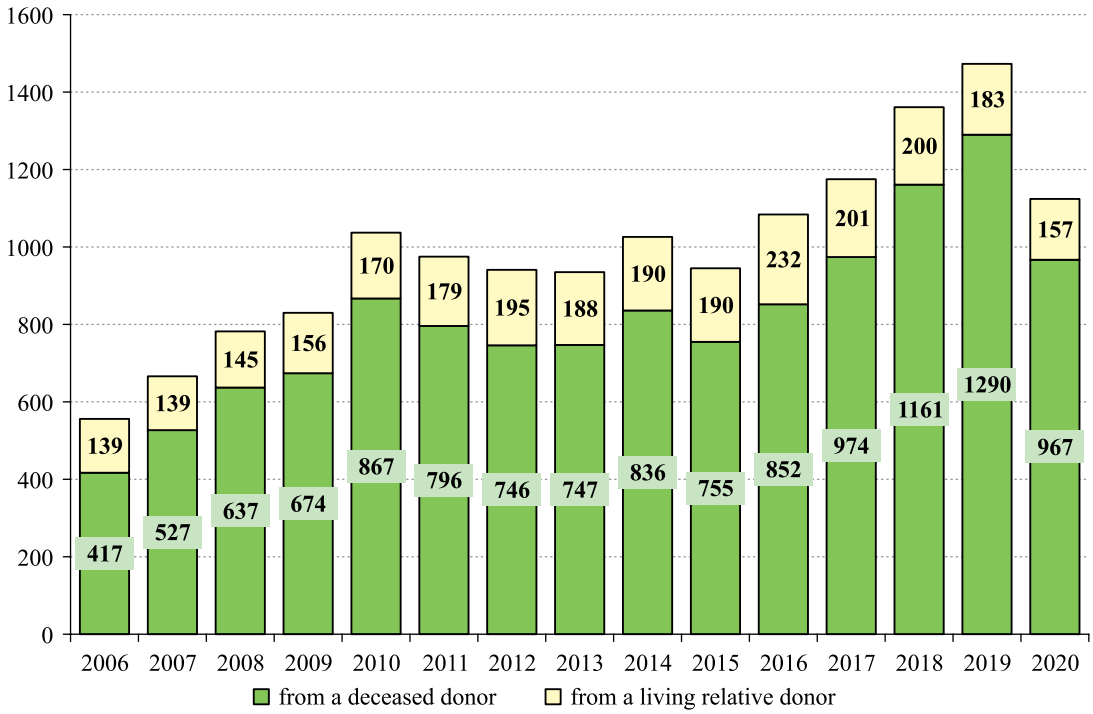


Fig. 7. Kidney transplantation in Russia in 2006–2020

Table 8

**Leading medical institutions in terms of number of kidney transplants performed**

| Rank | Centers – leaders in terms of number of kidney transplants performed | Number of kidney transplants performed in 2020 |
|---|---|---|
| 1 | Shumakov National Medical Research Center of Transplantology and Artificial Organs, Moscow | 206 |
| 2 | Sklifosovsky Research Institute of Emergency Care, Moscow | 198 |
| 3 | Botkin City Clinical Hospital, Moscow | 75 |
| 4 | Samara State Medical University, Samara | 47 |
| 5 | Republican Clinical Hospital, Kazan | 40 |
| 6 | Belyaev Kemerovo Regional Clinical Hospital, Kemerovo | 39 |
| 7 | Republican Clinical Hospital, Ufa | 38 |
| 8 | Rostov Regional Clinical Hospital, Rostov-on-Don | 37 |
| 9 | Vladimirsky Moscow Regional Research Clinical Institute, Moscow Oblast | 36 |
| 10 | National Medical Research Center for Children's Health, Moscow, Central Federal District | 34 |
| | **TOTAL** | **750** |
| | 66.7% of the total number of kidney transplants performed in the Russian Federation (1124) | |

## EXTRARENAL ORGAN TRANSPLANTATION

In 2020, there were 251 heart transplants (1.7 per million population) performed of which 6 were pediatric transplant surgeries. Heart transplants were performed at 16 centers.

The Shumakov Center (Moscow) accounted for 75.7% (190 heart transplant surgeries) of the total number of heart transplants in Russia. The heart transplant program at this center continues to drive the level of availability of this type of transplant care in the country.

While 7 transplant centers performed 10 or more heart transplants in 2019, only 2 did so in 2020 – Shumakov Center (190) and Almazov National Medical Research Centre (18). Meshalkin National Medical Research Center in Novosibirsk, Sklifosovsky Institute in Moscow, Ochapovsky Regional Clinical Hospital No. 1 in Kras-

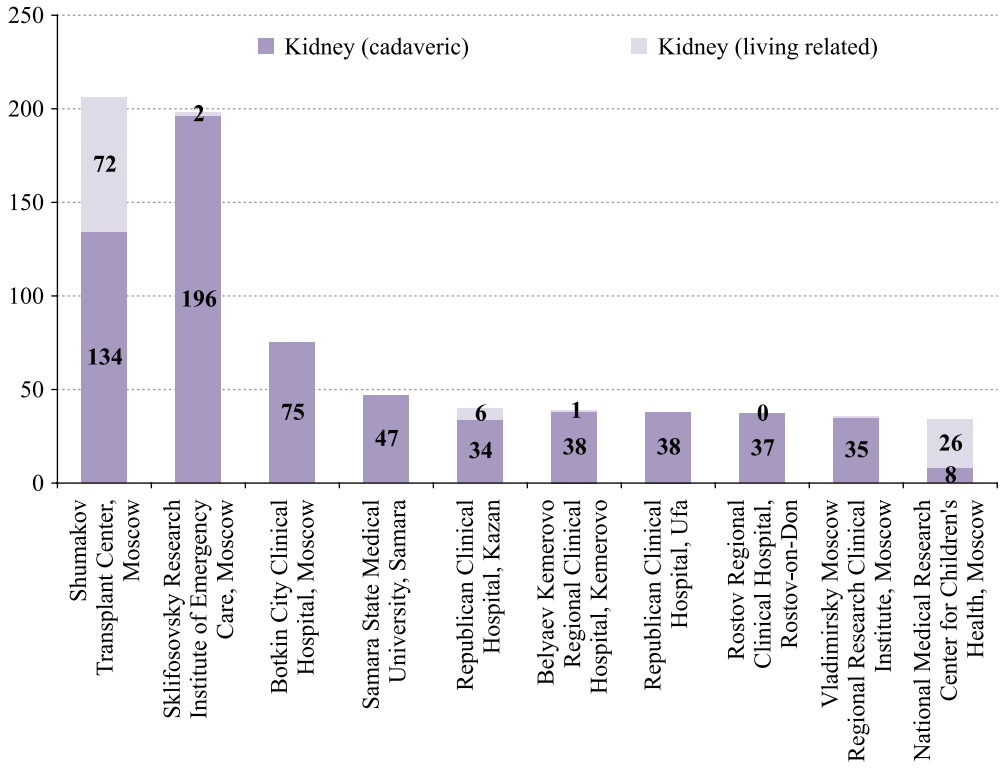


Fig. 8. Leading medical institutions in terms of number of kidney transplants performed

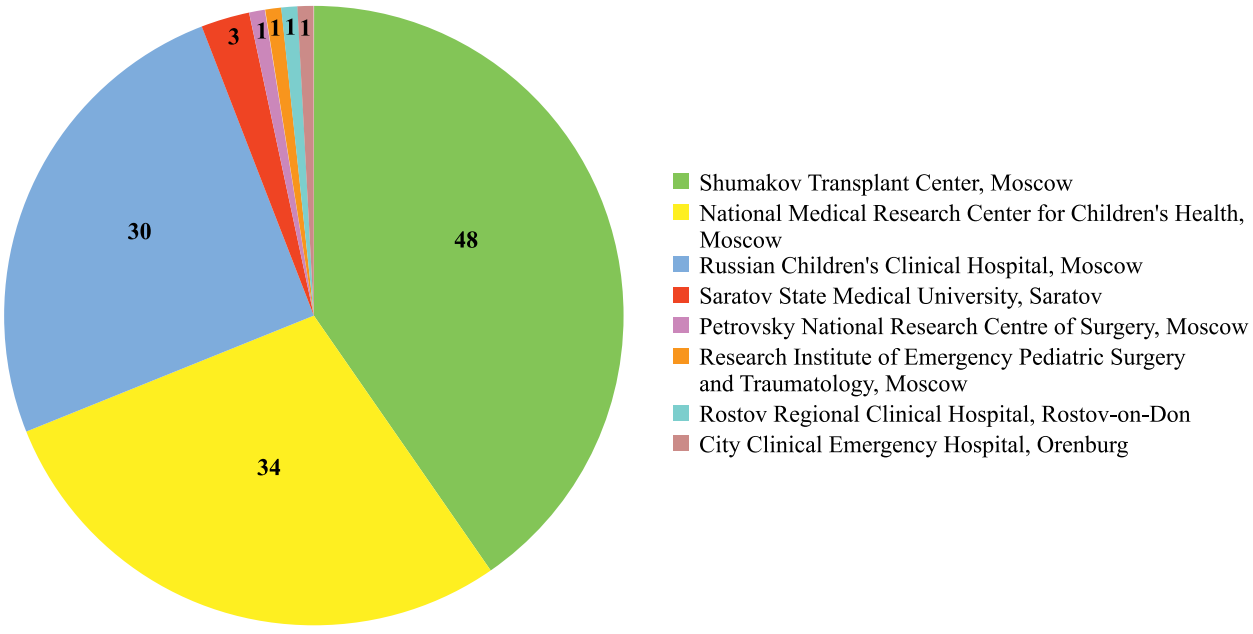


Fig. 9. Pediatric kidney transplantation in Russia in 2019

nodar, Sverdlovsk Regional Clinical Hospital No. 1 in Yekaterinburg, and the Research Institute for Complex Problems of Cardiovascular Diseases in Kemerovo were unable to maintain transplantation activity during the COVID-19 pandemic.

Lung transplantations in 2020 were performed at 3 transplantation centers. A total 9 lung transplants were conducted; Shumakov Center (6 lung transplants), Sklifosovsky Institute (2), and Pavlov First St. Petersburg State Medical University in St. Petersburg (1). The Shumakov Center also performed 2 heart-lung transplants.

Table 9 and Fig. 10 show the thoracic organ transplant centers that performed the highest number of heart and lung transplants in 2020.

Table 9

**Leading medical institutions in terms of number of thoracic organ transplantations performed**

| Rank | Centers – leaders in terms of number of heart transplants performed | Number of kidney transplants performed in 2020 |
|---|---|---|
| 1 | Shumakov National Medical Research Center of Transplantology and Artificial Organs, Moscow | 190 |
| 2 | Almazov National Medical Research Centre, St. Petersburg | 18 |
| 3 | Research Institute for Complex Issues of Cardiovascular Diseases, Kemerovo | 7 |
| 4 | Meshalkin National Medical Research Center, Novosibirsk | 5 |
| 5 | Ochapovsky Regional Clinical Hospital No. 1, Krasnodar | 4 |
| 6 | Interregional Clinical Diagnostic Center, Kazan | 4 |
| 7 | Republican Cardiology Clinic, Ufa | 4 |
| 8 | Bakulev Scientific Center of Cardiovascular Surgery, Moscow, Central Federal District | 3 |
| 9 | Rostov Regional Clinical Hospital, Rostov-on-Don | 3 |
| 10 | Krasnoyarsk Clinical Hospital, Krasnoyarsk | 3 |
| | Sverdlovsk Regional Clinical Hospital No. 1, Yekaterinburg | 3 |
| | **TOTAL** | **244** |
| | 98.0% of the total number of heart transplants performed in the Russian Federation (249) | |

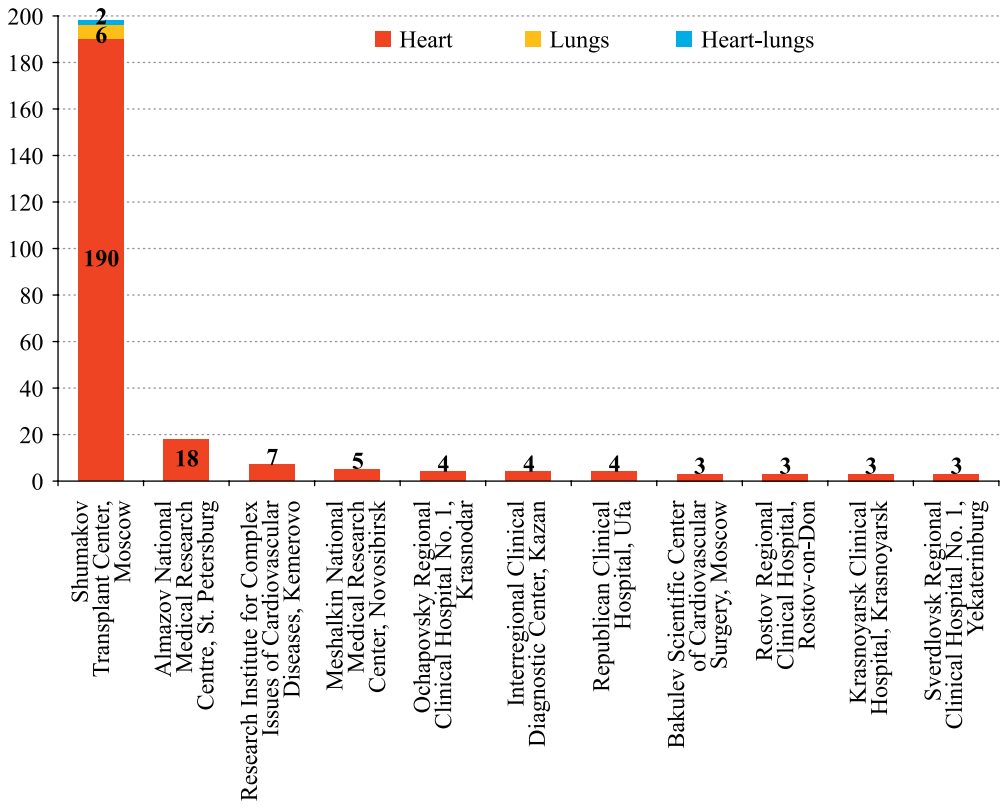


Fig. 10. Leading medical institutions in terms of number of thoracic organ transplantations performed

In 2020, a total of 559 liver transplants (3.8 per million population) were performed. Liver transplants were performed at 29 centers.

A new liver transplant program was launched in 2020 – 3 deceased-donor liver transplants were performed at Tyumen Regional Clinical Hospital No. 1. The liver transplantation program at the Republican Clinical Hospital in Kazan has become more active, the number of transplants in 2020 compared to 2019 increased by 53.8% to 20. In 2020, 6 transplantation centers performed 20 or more liver transplants each: Shumakov Center (165), Sklifosovsky Institute (118), Burnazyan Federal Medical and Biophysical Center (47), Botkin City Clinical Hospital (35); State Novosibirsk Regional Clinical Hospital (34), Republican Clinical Hospital, Kazan (20).

Moscow-based transplant centers (6) accounted for 69.8% (390 transplants) of liver transplantation in 2020 and 64.4% (376 transplants) in 2019.

Table 10 and Fig. 11 show the liver transplant centers with the highest number of liver transplants in 2020.

Table 10

**Leading medical institution in terms of number of liver transplantations performed**

| Rank | Centers – leaders in terms of number of liver transplants performed | Number of kidney transplants performed in 2020 |
|---|---|---|
| 1 | Shumakov National Medical Research Center of Transplantology and Artificial Organs, Moscow | 165 |
| 2 | Sklifosovsky Research Institute of Emergency Care, Moscow | 118 |
| 3 | Burnazyan Federal Medical and Biophysical Center, Moscow | 47 |
| 4 | Botkin City Clinical Hospital, Moscow | 35 |
| 5 | State Novosibirsk Regional Clinical Hospital, Novosibirsk | 34 |
| 6 | Republican Clinical Hospital, Kazan | 20 |
| 7 | Vladimirsky Moscow Regional Research Clinical Institute, Moscow Oblast | 17 |
| 8 | Granov Russian Research Center of Radiology and Surgical Technologies, St. Petersburg | 17 |
| 9 | Rostov Regional Clinical Hospital, Rostov-on-Don | 15 |
| 10 | Irkutsk Regional Clinical Hospital, Irkutsk | 14 |
| | **TOTAL** | **482** |
| | 86.2% of the total number of liver transplants performed in the Russian Federation (559) | |

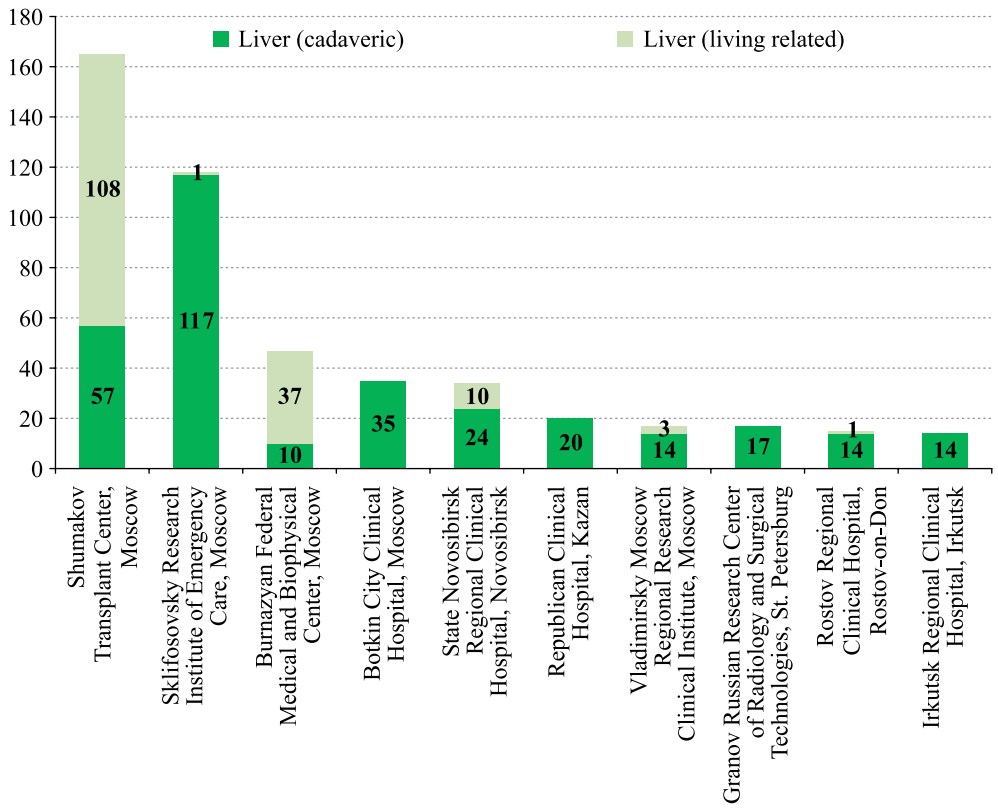


Fig. 11. Leading medical institution in terms of number of liver transplantations performed

The rating demonstrates the relative sustainability of the programs at the leading liver transplant centers presented; the negative impact of COVID-19 affected transplant activity at Sverdlovsk Regional Clinical Hospital No. 1 (Yekaterinburg) the most. The total transplant activity was 482, which was more than that of 2019 (474, +8).

Related liver transplants were performed at 9 centers. Living-related transplants accounted for 169 surgeries (30.2%). In 2019, there were 8 centers that performed 147 related liver transplants (25.2%).

In 2020, 131 pediatric (mostly young children) liver transplants were carried out; 113 in 2019. The liver transplants were performed at three centers: Shumakov Center (120), Petrovsky National Research Centre of Surgery (8), and State Novosibirsk Regional Clinical Hospital (3).

Pancreas transplantations in 2020 were performed at 3 centers: Shumakov Center (2), N.V. Sklifosovsky Institute (13), and Rostov Regional Clinical Hospital in Rostov-on-Don (1). A total of 16 pancreas transplant surgeries were performed (10 in 2019), all of them being kidney-pancreas transplants.

Thus, there were 836 extrarenal transplants performed in 2019 – 42.6% of the total number of transplants (1960). During the follow-up period from 2006 (106), the number of extrarenal organ transplants in Russia increased by 730 (7.9-fold). See Fig. 12. The proportion of extrarenal transplants in the total number of transplants increased by 26.7%.

Transplant centers in Moscow and Moscow Oblast accounted for 605 extrarenal organ transplants (72.4%) in 2020, which remains decisive.

Table 11 presents information on the number of organ transplants performed in Russia from 2006 to 2019.

## ORGAN TRANSPLANT RECIPIENTS

According to information from the Federal Registry, there were 19,097 organ transplant recipients in Russia in 2020 (130.6 per million population). Among these recipients, 12,563 (85.9 per million population) received kidney, 3,489 (23.9 per million population) received liver, while 1524 (10.4 per million population) were heart transplant recipients. Since 2013 (for 7 years of observation), the number of organ recipients in Russia has increased by 1,044 (123.3%).

Data on the number of organ recipients in Russia from 2013 to 2020 from the Federal Registry of the Russian Ministry of Health (see Order No. 2323-r of the Russian Government dated October 23, 2017; Resolution No. 404 of the Russian Government dated April 26, 2012) are presented in Table 12.

## CONCLUSION

Last year, transplant care came under the strong negative influence of a fundamentally new problem, the COVID-19 outbreak. The peculiarities and results of transplant centers in 2020 were influenced by this factor. The main conclusion to be drawn from 2020 results is that COVID-19 should not generally be considered as a reason to stop organ transplant and donor programs. The COVID-19 pandemic imposes certain limitations and complicates the activities of transplant surgeons, but does not make transplant activities impossible.

In 2020, the number of organ donors and the number of organ transplants in Russia declined. However, most centers did not stop their activities, organ transplants

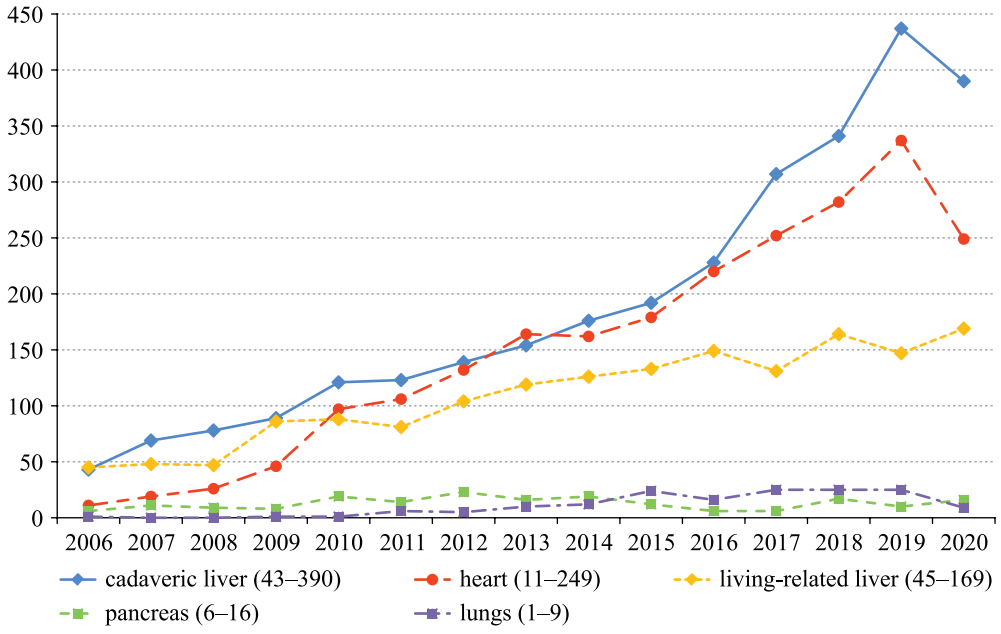


Fig. 12. Extrarenal solid organ transplantation in 2006–2020

Table 11

**Organ transplantation in the Russian Federation in 2006–2020**

| No. | Organ | 2006 | 2007 | | 2008 | | 2009 | | 2010 | | 2011 | | 2012 | | 2013 | | 2014 | | 2015 | | 2016 | | 2017 | | 2018 | | 2019 | | 2020 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Abs. number | Abs. number | Change over the year | Abs. number | Change over the year | Abs. number | Change over the year | Abs. number | Change over the year | Abs. number | Change over the year | Abs. number | Change over the year | Abs. number | Change over the year | Abs. number | Change over the year | Abs. number | Change over the year | Abs. number | Change over the year | Abs. number | Change over the year | Abs. number | Change over the year | Abs. number | Change over the year | Abs. number | Change over the year |
| 1 | Kidney (total) | 556 | 666 | +110 | 782 | +116 | 830 | +48 | 1037 | +207 | 975 | –62 | 941 | –34 | 935 | –6 | 1026 | +91 | 945 | –81 | 1084 | +139 | 1175 | +91 | 1361 | +186 | 1473 | +112 | 1124 | –349 |
| 2 | including cadaveric | 417 | 527 | +110 | 637 | +110 | 666 | +29 | 867 | +201 | 796 | –71 | 746 | –50 | 747 | +1 | 836 | +89 | 755 | –81 | 852 | +97 | 974 | +122 | 1161 | +187 | 1290 | +129 | 967 | –323 |
| 3 | from living related donor | 139 | 139 | 0 | 145 | +6 | 156 | +11 | 170 | +14 | 179 | +9 | 195 | +16 | 188 | –7 | 190 | +2 | 190 | 0 | 232 | +42 | 201 | –31 | 200 | –1 | 183 | –17 | 157 | –26 |
| 4 | Liver (total) | 88 | 117 | +29 | 125 | +8 | 175 | +50 | 209 | +34 | 204 | –5 | 243 | +39 | 272 | +29 | 302 | +30 | 325 | +23 | 378 | +53 | 438 | +60 | 505 | +67 | 584 | +79 | 559 | –25 |
| 5 | including cadaveric | 43 | 69 | +26 | 78 | +9 | 89 | +11 | 121 | +32 | 123 | +2 | 139 | +16 | 154 | +15 | 176 | +22 | 192 | +16 | 229 | +37 | 307 | +78 | 341 | +34 | 437 | +96 | 390 | –47 |
| 6 | from living related donor | 45 | 48 | +3 | 47 | –1 | 86 | +39 | 88 | +2 | 81 | –7 | 104 | +23 | 119 | +15 | 126 | +7 | 133 | +7 | 149 | +16 | 131 | –18 | 164 | +33 | 147 | –17 | 169 | +22 |
| 7 | Heart | 11 | 19 | +8 | 26 | +7 | 46 | +20 | 97 | +51 | 106 | +9 | 132 | +26 | 164 | +32 | 162 | –2 | 179 | +17 | 220 | +41 | 252 | 32 | 282 | +30 | 335 | +53 | 249 | –86 |
| 8 | Pancreas | 6 | 11 | +5 | 9 | –2 | 8 | –1 | 19 | +11 | 14 | –5 | 23 | +9 | 14 | –9 | 19 | +5 | 12 | –7 | 6 | –6 | 6 | 0 | 17 | +11 | 10 | –7 | 16 | +6 |
| 9 | Lungs | 1 | 0 | –1 | 0 | 0 | 1 | +1 | 1 | 0 | 6 | +5 | 5 | –1 | 10 | +5 | 12 | +2 | 14 | +2 | 16 | +2 | 25 | +9 | 25 | 0 | 23 | –2 | 9 | –14 |
| 10 | Heart-lungs | | | | | | | | | | 2 | +2 | 2 | 0 | 1 | –1 | 0 | –1 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | +3 | 2 | –1 | 2 | 0 |
| 11 | Small intestine | | | | | | | | | | | | | | 1 | +1 | 1 | 0 | 0 | –1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | +1 |
| | Total | 662 | 813 | +151 | 942 | +129 | 1060 | +118 | 1363 | +303 | 1307 | –56 | 1345 | +38 | 1400 | +55 | 1522 | +122 | 1485 | –37 | 1704 | +219 | 1896 | +192 | 2193 | +297 | 2427 | +234 | 1960 | –467 |

Table 12

**Number of organ recipients in Russia in 2013–2020**

| ICD-X code | Number of patients in the registry, persons | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2013 | 2014 | | 2015 | | 2016 | | 2017 | | 2018 | | 2019 | | 2020 | |
| | | abs. | change (%) | abs. | change (%) | abs. | change (%) | abs. | change (%) | abs. | change (%) | abs. | change (%) | abs. | change (%) |
| Z94.0 Kidney transplant status | 6651 | 7502 | 12.8 | 8164 | 8.8 | 9063 | 11.0 | 9658 | 6.6 | 10,851 | 12.4 | 11,880 | 9.5 | 12,563 | 5.7 |
| Z94.1 Heart transplant status | 416 | 520 | 25.0 | 639 | 22.9 | 803 | 25.7 | 952 | 18.6 | 1164 | 22.3 | 1355 | 16.4 | 1524 | 12.5 |
| Z94.2 Lung transplant status | 2 | 3 | 50.0 | 4 | 33.3 | 5 | 25.0 | 8 | 60.0 | 28 | 250.0 | 26 | -7.1 | 24 | -7.7 |
| Z94.4 Liver transplant status | 1150 | 1406 | 22.3 | 1649 | 17.3 | 1948 | 18.1 | 2152 | 10.5 | 2632 | 22.3 | 3032 | 15.2 | 3489 | 15.1 |
| Z94.8 Other transplanted organ and tissue status (bone marrow, intestines, pancreas) | 334 | 467 | 39.8 | 654 | 40.0 | 808 | 23.5 | 909 | 12.5 | 1135 | 24.9 | 1344 | 18.4 | 1497 | 11.4 |
| TOTAL | 8553 | 9898 | 15.7 | 11,110 | 12.2 | 12,627 | 13.7 | 13,679 | 8.3 | 15,810 | 15.6 | 17,637 | 11.6 | 19,097 | 8.3 |

were performed when possible. Waiting lists continued to be maintained, as well as follow-up for transplant recipients.

Moscow demonstrated the sustainability of the regional system of organ donation coordination to the COVID-19 factor, which largely mitigated the drop in the final indicators of donor and transplantation activity in the country as a whole.

Of the positive trends in the development of transplant care in Russia, which persisted in 2020, the following should be noted:

– Increase in the number of pediatric organ transplants (+13.6%);
– Increase in the proportion of effective brain-dead donors (97.0%);
– Increase in the number of living-related donor kidney transplants (+15.0%);
– Expansion of the geography of organ donor and transplantation programs (Tula Regional Clinical Hospital – deceased-donor kidney transplantation; Regional Clinical Hospital No. 1 in Tyumen – deceased-donor liver transplantation);
– Opening of a branch of Shumakov National Medical Research Center of Transplantology and Artificial Organs in Volzhsky (Volgograd Oblast).

As for the prognosis, it is obvious that implementation of organ donor and transplant programs in 2021 depends on the level of COVID-19 coronavirus infection and on the effect of vaccination of the population. The number of waitlisted candidates at transplantation centers continues to increase year by year. Therefore, the basic task for 2021 and subsequent years will be to restore and further increase the number of organ transplants in order to meet the needs of the population and the donor potential. It should be noted that departmental target program "*Organ Donation and Transplantation in the Russian Federation*", approved under Order No. 365 of the Russian Ministry of Health on June 4, 2019, continues to exist. The program provides for an increase in the number of organ transplants to 25.2 per million population by 2024, an increase in the number of regions where transplants are performed to 42, and an increase in the number of transplant centers to 80. The COVID-19 factor should

not be considered by health care organizers and health specialists as a reason to ignore this document.

Under such conditions, a special responsibility lies with chief freelance transplant surgeons working at healthcare bodies across Russia. It is their task to draw the attention of healthcare managers and leaders to the transplant program, justify its necessity and expediency, and protect the rights of potential organ recipients to affordable and quality healthcare.

In 2021, the Shumakov National Medical Research Center of Transplantology and Artificial Organs will continue to provide comprehensive methodological assistance to healthcare bodies and transplant centers, train specialists from across Russia, perform on-site audits, conduct telemedicine consultations 24/7, and monitor donor and transplantation programs.

*The authors declare no conflict of interest.*

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# FIRST RUSSIAN EXPERIENCE IN LIVER AND KIDNEY TRANSPLANTATION FROM DONORS WITH OUT-OF-HOSPITAL CARDIAC ARREST: 3 YEARS' RESULTS

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**Introduction.** In megacities, the use of organs obtained from those who died as a result of sudden out-of-hospital cardiac arrest (OHCA) for transplantation is one of the promising ways of addressing the problem of organ donor shortage. In St. Petersburg, the model of transition from life support via extracorporeal membrane oxygenation (ECMO) of patients after OHCA to ECMO life support for organs of potential donors was tested for the first time. **Materials and methods.** In order to implement the program, round-the-clock ECMO and transplantation teams were organized at the inpatient emergency ward of Pavlov First St. Petersburg State Medical University. Interaction with the St. Petersburg City Emergency Station, St. Petersburg was established. The protocol of work with potential donors brought to the hospital after a sudden circulatory arrest was developed, approved by the ethics committee, and implemented in clinical practice. This was the first in Russia and in international practice. Between 2017 and 2020, 67 patients with sudden OHCA were brought to the inpatient emergency ward. In 4 (5.97%) cases, advanced cardiovascular life support was successful, and 11 (16.42%) patients became effective donors. Mortality among this group of patients without subsequent postmortem donation was 77.61% (52 patients). **Results.** Liver transplantation from non-heart-beating donors (NHBDs) whose blood circulation was restored by ECMO (ECMO NHBD) was performed in 5 recipients who were in severe condition against the background of liver failure. In 1 (20%) case, there was severe liver allograft dysfunction for 33 days with subsequent complete restoration of function. Kidney transplantation was performed in 22 patients. Immediate graft function occurred in 10 (45.45%), while delayed function occurred in 12 (54.55%) patients. Kidney graft survival was 86.4%, kidney graft recipient survival was 95.5%, liver graft recipient survival was 80%, and the follow-up period was 24.1 ± 7.15 months. **Conclusion.** The use of ECMO to save the lives of patients with sudden OHCA can be implemented in conditions of a high degree of organization and synchronization of the work of the city emergency medical station and the emergency department of a multidisciplinary hospital. If cardiopulmonary resuscitation with ECMO (ECMO CPR) fails, it is possible to launch the ECMO NHBD donor program. Long-term outcomes of liver and kidney transplantation from ECMO NHBD are consistent with those using organs from brain-dead donors. Widespread implementation of the new organ donation model will increase the availability of transplant care.

*Keywords: out-of-hospital irreversible cardiac arrest donors, non-heart-beating donors, extracorporeal membrane oxygenation, ECMO CPR, ECMO NHBD, liver transplantation, kidney transplantation.*

## INTRODUCTION

The main focus of modern transplantology is the development of new strategies for solving the problem of donor organ shortage [1–3]. The use of organs obtained from those who died as a result of sudden out-of-hospital cardiac arrest (asystolic donors, ASD) is one of the promising directions in solving this problem [4, 5]. Our own [6] and European experience with the use of protocols for working with ASD have shown the effectiveness and no significant differences in the outcomes of transplants, compared to the results of kidney transplants obtained from brain-dead donors [7–10].

Extracorporeal membrane oxygenation (ECMO) for emergency restoration of blood circulation is an invasive method of extracorporeal cardiopulmonary resuscitation (ECPR), which was proposed for patients with cardiac

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arrest in order to restore and maintain blood circulation in the body during cardiac arrest [11, 12]. ECMO has been used in cardiac arrest since 1976 with the introduction of the battery-powered portable cardiopulmonary bypass machine [13]. The use of this circulatory restoration method for a number of years was limited to the use only in certain groups of patients: after open-heart surgery, subjected to profound hypothermia and drug overdose [14–16]. Miniaturization of extracorporeal circulatory restoration devices, the use of perfusion circuits with heparinized coating and methods of percutaneous cannulation of main vessels have made it possible to expand the clinical range of application of this method [17–21]. Clinical studies have shown the effectiveness of early ECMO to improve prognosis in patients with prolonged cardiac arrest occurring both in inpatient (in-hospital cardiac arrest) and out-of-hospital conditions (out-of-hospital cardiac arrest) [22–23].

Guidelines by the International Liaison Committee on Resuscitation explicitly state that ECMO can improve outcomes in patients with cardiac arrest, compared with the standard cardiopulmonary resuscitation (CPR) protocol, in cases of cardiogenic shock and cardiac arrest, where cardiac pathology is known from history to be amenable to immediate invasive correction [24]. The American Heart Association has proposed that ECPR should be considered as a care option for inpatients with cardiac arrest in period that there is no blood flow is minimal and the condition that led to cardiac arrest is reversible (for example, hypothermia or drug intoxication) or treatable by myocardial revascularization or heart transplantation [25]. In any case, the use of ECMO in clinical practice presupposes the adoption of rather complex medical decisions in situations where the patient is in critical condition and his rescue fully depends on the success of the medical team.

The effectiveness of ECMO in reviving patients with cardiac arrest explains the reason why it is also used for donor programs. The most famous is the so-called "Spanish protocol" – donors from the streets – successfully implemented in major cities of Spain [1, 27, 33].

Ischemia-reperfusion injury inevitably accompanies the process of obtaining donor organs from ASDs. Pathophysiological processes occurring during ischemia-reperfusion can be minimized by consecutive application of indirect automatic cardiac massage and switching to restoration and maintenance of blood circulation in the deceased patient using ECMO. This has been demonstrated in some of our previous works [6, 32, 50].

This paper presents the 3-year outcomes of organ transplantation from donors with out-of-hospital irreversible cardiac arrest.

## MATERIALS AND METHODS

Actions by ambulance teams were based on the organizational protocol primarily aimed at saving the life of a patient with sudden out-of-hospital irreversible cardiac arrest, which was approved by the local ethics committee of Pavlov University (Protocol No. 46 dated September 22, 2017). Discussion and approval of the protocol was agreed with City Ambulance Station, St. Petersburg. Taking into account the radical influence of time on outcomes of resuscitation measures, work involved those ambulance substations of Petrogradsky district of St. Petersburg, whose area of responsibility included the territory near Pavlov University.

According to the protocol, upon arrival, the ambulance team performed a set of resuscitation measures on the patient with sudden cardiac arrest, including the connection of Lucas II chest compression system (Jolife AB, Sweden), and mechanical ventilation (MV). Given the localization of the program within Petrogradsky district of St. Petersburg only, in cases of timely notification, the ambulance team was able to arrive to the patient and begin resuscitation measures within 7–10 (7.3 ± 4.1) minutes of receiving the call, and deliver the patient to the hospital within 50–70 (57 ± 12.8) minutes. On the background of continuing chest compressions with the help of Lucas II device, the resuscitated person was delivered to the inpatient emergency ward of Pavlov University, where in addition to the intensive care unit (ICU), an ECMO team set out for a 24-hour work to implement this program.

Each patient, depending on the specific clinical situation, was given the full range of life support procedures from the arrival of the ambulance team, and continued in the ICU of the hospital.

The ECMO protocol was initiated primarily to save the patient's life and as part of the "life support" procedures based on the criteria developed and accepted at Pavlov University (Table 1).

Under resuscitation conditions, the femoral artery and vein (arterial with "Luer-port" 17 Fr and bicaval venous 30/33 Fr cannulae) were cannulated by puncture, under ultrasound control or "openly" (Fig. 1). Connection to a prefilled perfusion circuit was performed. For ECMO, we used centrifugal pump RotaFlow (Maquet, Germany), a prototype of Ex-Stream for emergency blood circulation restoration (TransBioTech, Russia, the use of prototype portable perfusion device was approved by the local ethics committee of Pavlov University). We used perfusion tubes (Kewei, China), RotaFlow centrifuge head (both for Maquet, Germany, and for Ex-Stream, TransBioTech, Russia), membrane oxygenator (Kewei/Oxygenator Keweiadult, China), and leukocyte filter (LeukoGuard 6, Pall, Terumo, USA) (Fig. 2, 3). The circuit was initially filled with sodium bicarbonate solution, saline, colloidal solution, antibiotic, heparin sulfate, fibrinolytic, and methylprednisolone (Table 2). Accepted standard perfusion characteristics for ECMO: flow rate 2.5–3.5 L/min, mean arterial pressure 80–100 mm Hg, oxygen flow through oxygenator 3–4 L/min. Control

and correction of laboratory indicators were performed every hour.

After extended CPR measures, including ECPR, where the measures were not effective (atonic coma, wide pupils, no reaction to light, isoelectric line on an ECG when the Lucas device was stopped), death was stated according to the generally accepted criteria. In patients eligible for donation, after the 'no touch' period, 20 minutes, the deceased was injected with 10,000 U of heparin sulfate via central venous access and the ECMO procedure was resumed in order to maintain viability of the deceased organs. Thus, there was a transfer from life support procedure to the deceased donor's organ preservation procedure.

Decisions to perform organ transplantation from donors with out-of-hospital cardiac arrest and determine their suitability were based on the assessment of time parameters, ECMO effectiveness, and imaging and laboratory findings. Each patient underwent spiral computed tomography (SCT) with intravenous contrast (Ultravist 370 or Omnipack 350, 100 ml, bolus injection) to assess organ perfusion.

Absolute contraindications to the use of medical technology were taken as follows:

1. Presence of infections: HIV, HBs, HCV, RW (hemotransmissible infections were tested on Abbott Architect i2000sr automatic immunofluorescent analyzer with Abbott diagnostic kits).

Table 1

**Criteria for initiating the ECMO Protocol**

| Pre-hospital stage | Inpatient Emergency Ward (IEW) | ECMO team |
|---|---|---|
| Age (18–75 years) | Re-assessment of the pre-hospital criterion set | Diameter of femoral vessels not <5.5–6 mm according to ultrasound examination |
| Ventricular fibrillation or ventricular tachycardia (VF/VT) without electro-pulse therapy effect (at least three discharges) | Exhaled $CO_2$ (on arrival at the IEW) >10 mmHg | Time from the moment the ambulance team is called to the moment the patient on the IEW table is about 60 minutes |
| Received Amiodarone 300 mg | $PaO_2$ >50 mmHg<br>or $O_2$ Sat >85% | Synchronization with Lucas machine if IEW criteria do not meet the setting up and connection of femoral cannulas against the background of CPR |
| Technical ability to connect the Lucas device | Lactate <15 mmol/L | Transfer to the Department of X-ray surgical methods of diagnosis and treatment, assessment of angiography results |
| Absence of incurable diseases (Stage 4 cancer, end-stage liver, kidney, heart disease, etc.) | | |
| Information about refusal to do intubation and CPR | | |
| No ongoing bleeding<br>No injuries | | |


Fig. 1. Connection of ECMO device to the donor femoral vessels (prototype of portable perfusion pump Ex-Stream) in the conditions of X-ray endovascular diagnostics and treatment room

2. Information about kidney disease, liver disease, malignant tumors, purulent inflammatory processes in abdominal cavity and retroperitoneal space, generalized infections;
3. Presence of direct or indirect evidence of drug use (traces of injections, tattoos, anamnestic indications, etc.);
4. Severe atherosclerosis of the donor's peripheral vessels, which precludes adequate perfusion;
5. Violation of the integrity of the donor's vascular bed, ongoing bleeding, massive blood loss;
6. More than 4 days of ECMO procedure after CPR (signs of multiple organ failure);
7. Macroscopic changes in the donor organ, excluding its subsequent use for transplantation (signs of thrombosis, marked cyanosis, stony density, etc.);
8. Lack of satisfactory organ perfusion in SCT angiography (Fig. 3).

Relative contraindications to donation were developed and adopted in the form of a point system (5 or more points – donor refusal):

1. The period from cardiac arrest to the beginning of resuscitation measures by the emergency medical team (more than 20 minutes) (1 point);
2. Warm ischemia period ≥120 min (mechanical chest compressions before the onset of ECMO), $pO_2$ less than 50 mmHg (1 point);
3. Presence of expanded donor criteria: age (over 50 years old), diabetes mellitus, hypertension, history of arterial hypertension, systemic diseases (psoriasis, autoimmune diseases), etc. (1 point for each condition);
4. Diagnosed damage to abdominal organs, retroperitoneal space (1 point);
5. Proteinuria, pyuria, macrohematuria, bacteria, fungal spores in urine sediment (1 point);
6. Absence of diuresis for 2 or more hours (1 point);
7. Serum creatinine level – 0.25 mmol/L or higher (1 point);
8. Being in the "red zone" for more than 6 hours and in the ICU for more than 72 hours (1 point).

After the donor's organs were found to be preliminarily suitable for explantation, and permissions were obtained from a forensic medical expert and the hospital management, the donor was taken to the operating room. Under continuing ECMO, laparotomy was performed, the aorta, iliac vessels, and inferior vena cava were isolated. The isolated abdominal region was cold perfused with Custodiol solution (Dr. Franz Kohler Chemie GmbH, Germany, temperature 4 °C, 15 liters) through luer-port of arterial cannula previously used for ECMO. Then the kidneys and liver were removed according to standard technique.

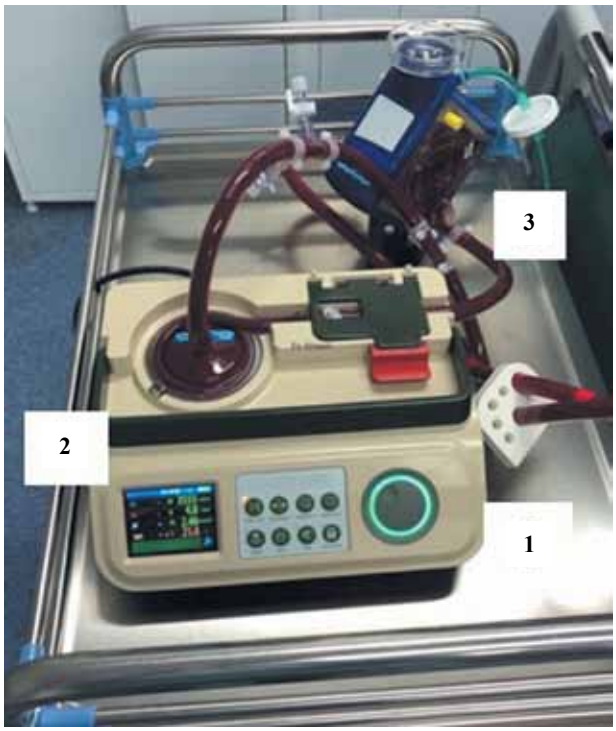


Fig. 2. General view of ECMO donor perfusion circuit (1 – prototype of Ex-Stream, 2 – centrifugal head, 3 – oxygenator and arterial filter) in the "red zone" of the inpatient emergency department

Kidney allotransplantation and orthotopic liver transplantation from donors with sudden out-of-hospital circulatory arrest were performed using standard hospital-accepted surgical techniques, medication and immunosuppressive therapy regimens.

Table 2

**Primary filling of the EKMO contour**

| Group of drugs | Drugs used according to the protocol | Dosage (volume) |
|---|---|---|
| Crystalloid solutions | Sodium chloride 0.9% | Contour filler base |
| | Sodium bicarbonate 5% | 200 ml |
| Colloidal solution | Gelofusine | 500 ml |
| Fibrinolytic | Streptokinase | 1.5 million units |
| Antibiotic | Cefazolin | 2.0 g |
| Anticoagulant | Heparin sulfate | 10000 U |
| Methylprednisolone | Solu-Medrol | 1000 mg |

## CLINICAL EXAMPLE

*Female patient N., 27 years old, at 00:00 at a metro subway station, suddenly lost consciousness, and suffered a cardiac arrest. Chest compression was performed until the arrival of an ambulance team. At the time of arrival of the medical team (00.15), she was in a state of clinical death. Prolonged CPR (electro-pulse therapy 200 J #5, connection of Lucas 2 (Sweden) resuscitation machine for chest compression, MV, adrenaline 12 ml total, cordarone 400 mg) was performed. Against the background of continuing mechanical chest compression, the patient was delivered to the in-patient emergency ward, 62 minutes after cardiac arrest. The patient's initial data: height 165 cm, weight 60 kg, lactate – 15 mmol/L, hemoglobin 107 g/L, $pO_2$ – 37 mmHg, $pCO_2$ 55.6 mm Hg, atonic coma, isoelectric line in ECG (when the Lucas II machine was off). Given persistent asystole, atonic coma, lactate value over 15 mmol/L in accordance with the ECMO protocol, CPR was not included in the scope of measures. After a full CPR complex within 30 minutes, chest compressors were turned off, and biological death was stated (01.32).*

*After 20 minutes from the moment of confirmation of biological death, taking into account the possibility of implementing the organ donation protocol, the Lucas II device was resumed. Cannulation of the right femoral vessels was performed under ultrasound control, ECMO perfusion was initiated. Perfusion characteristics: flow rate 2.5–3.5 l/min, mean arterial pressure 85–105 mmHg, oxygen flow through oxygenator 3–4 l/min.*

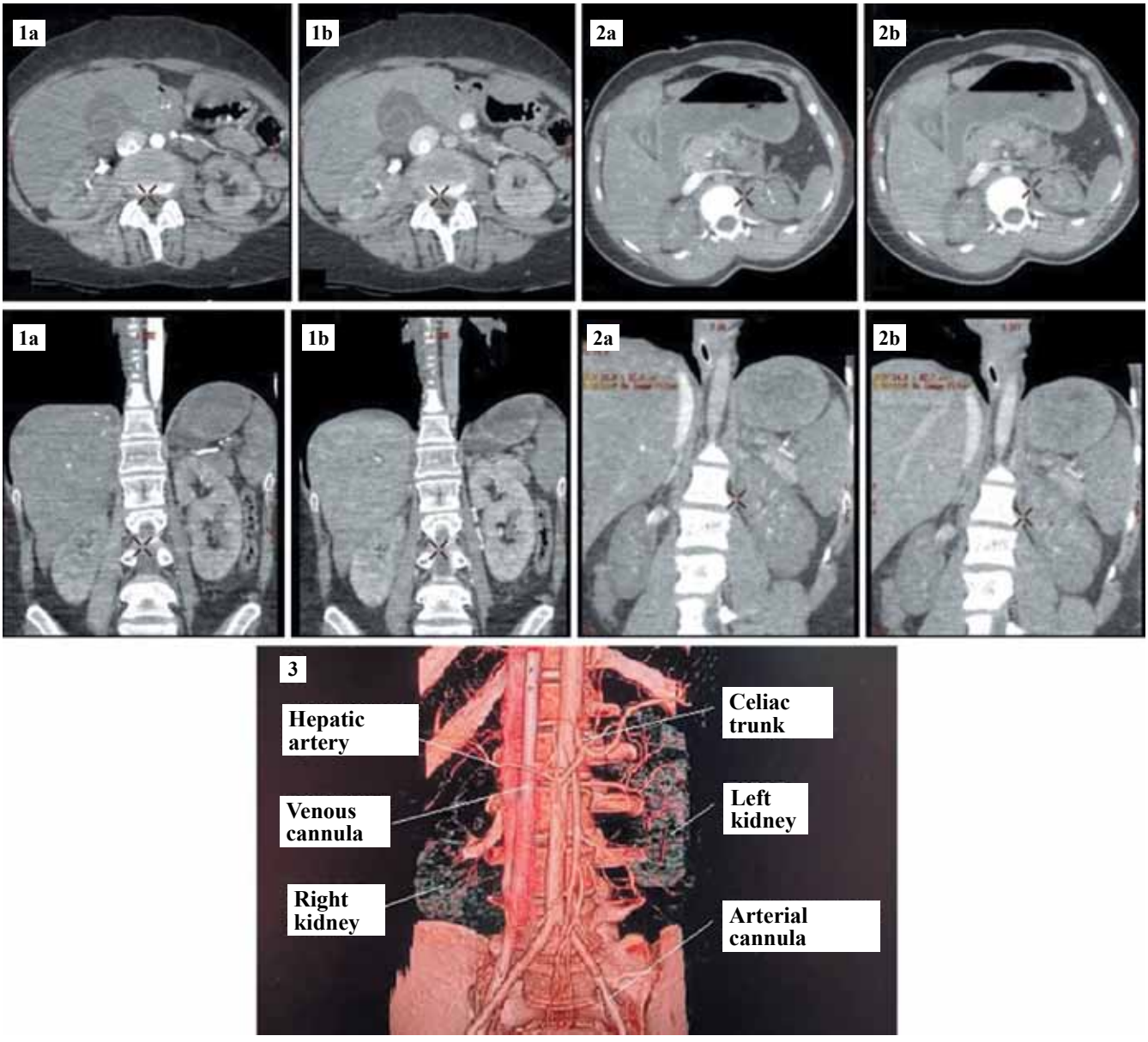


Fig. 3. Results of intravenous contrast-enhanced CT in a potential donor during ECMO (1 – satisfactory perfusion of the liver, kidneys (1a – arterial phase, 1b – venous phase); 2 – no perfusion (1a – arterial phase, 1b – venous phase); 3 – 3D reconstruction, arterial phase, satisfactory perfusion of abdominal region)

*Examination against the background of ECMO revealed no contraindications to donation (2 points according to the accepted relative contraindication scale), the organs were found to be preliminarily suitable for transplantation. After obtaining the permission of a forensic medical expert and hospital administration, the liver and two kidneys were extracted in the operating room according to the accepted technique. ECMO lasted for a total of 343 minutes.*

*A 55-year-old female patient M. became the liver recipient. She was diagnosed with chronic unverified hepatitis with an outcome in liver cirrhosis. Child-Pugh class C. MELD 19. Portal hypertension, grade 2 esophageal varices. Ascites. Hepatic encephalopathy. The operation was performed according to the standard Piggy-Back technique. Liverless period – 50 min, secondary warm ischemia time – 30 min, cold ischemia time – 435 minutes. Graft function was immediate. Drains were removed on days 4 and 5 after the operation. She was transferred to the ward on day 5 after transplantation. Duration of hospitalization was 23 bed-days, the patient was discharged with a satisfactorily functioning graft. Laboratory indicators 18 months after transplantation: AST 35 U/L, ALT 41 U/L, bilirubin 19 μmol/L.*

*Transplantation operations for the obtained kidneys were performed sequentially in immunologically compatible recipients.*

1. *Patient R., 27 years old, diagnosed with "chronic glomerulonephritis, morphologically unverified. Nephrosclerosis. Renal replacement therapy by long-term hemodialysis since 2016". There was delayed graft function, 6 hemodialysis procedures were performed. The patient was discharged on day 24 with a satisfactory functioning graft. Laboratory indicators 18 months after transplantation: creatinine 0.189 mmol/L, urea 10.8 mmol/L.*
2. *Patient P., 34 years old, diagnosed with "chronic glomerulonephritis, morphologically unverified. Nephrosclerosis. Renal replacement therapy by long-term hemodialysis since 2007". There was delayed graft function, 1 hemodialysis procedure was performed. The patient was discharged on day 24 with a satisfactory functioning graft. Laboratory indicators 18 months after transplantation: creatinine 0.169 mmol/L, urea 11.2 mmol/L.*

## STUDY RESULTS

Between 2017 and 2020, the program delivered 67 patients with out-of-hospital cardiac arrest, who were brought to the emergency ward within the framework of the program. In 4 (5.97%) cases, life-saving extended CPR and ECMO interventions were effective (patients were discharged without neurological deficit), 11 (16.42%) became effective donors. The mean age of the patients with out-of-hospital cardiac arrest was 50.3 ± 16.7 years. The pattern of outcomes of extended CPR and ECMO is shown in Fig. 4.

Kidney transplantation from the mentioned donors was performed in 22 patients. The mean age of the recipients was 46.8 ± 11.6 years. The time from the onset of cold perfusion to graft reperfusion was 570.4 ± 179.6 minutes, secondary warm ischemia lasted for 34.2 ± 6.42 minutes. Immediate graft function occurred in 10 (45.45%) cases. There was an average of 4.2 ± 3.2 hemodialysis procedures in cases of delayed graft function. Kidney graft function was restored on day 12 to 15. In 2 (9.1%) cases of kidney transplantation, the graft was removed due to acute rejection crisis and renal vein thrombosis during the first 3 days after transplantation.

Liver transplantation was performed in 5 patients with cirrhosis of various etiologies (autoimmune hepatitis, primary sclerosing cholangitis, chronic viral hepatitis C, etc.) in severe condition caused by end-stage liver failure and its complications (severe hepatic encephalopathy, portal hypertension, etc.). The mean age of the patients was 55.4 ± 10.1 years, MELD value 18.6 ± 7.9, Child-Pugh class C. The average cold and warm ischemia times were 380 ± 36.7 and 52 ± 10.3 minutes, respectively. There was 1 (20%) case of severe graft dysfunction within 33 days after transplantation, followed by complete restoration of graft function. One death was due to total thrombosis of the portal vein and hepatic artery on day 2 after transplantation in a patient

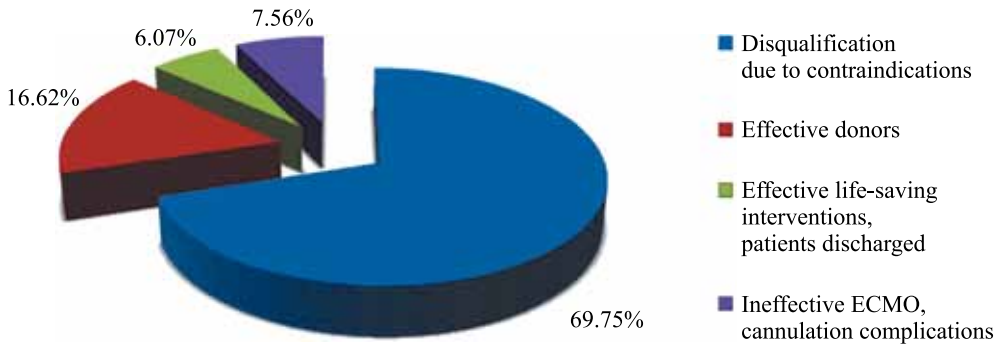


Fig. 4. Structure of results of advanced ECMO CPR

with hepatocellular cancer against the background of chronic viral hepatitis C.

The follow-up period of the organ recipients from ASD was from 14 to 34 (24.1 ± 7.15) months. Graft function during the follow-up period was satisfactory. The dynamics of the main laboratory indicators reflecting the graft function are shown in Figs. 5–8.

Among late complications (in the period from 1 to 34 months after transplantation), the frequency of kidney transplant and liver transplant rejection crises were 10% (2) and 25% (1), respectively. Liver rejection crisis was caused by the patient's violation of immunosuppressive therapy regimen; it was successfully treated with glucocorticoids. Among the late complications of liver transplantation, ischemic stricture of biliary anastomosis also occurred in one case. Endoscopic stenting of the anastomosis area was performed. Early and late complications of kidney and liver transplantation are presented in Table 3.

The survival rates of kidney grafts, renal transplant recipients, and liver transplant recipients were 86.4%, 95.5% and 80%, respectively (Fig. 9). One kidney transplant recipient died 23 months after transplantation due to severe COVID-19.

## DISCUSSION

In Spain, asystolic donors account for 24% to 28% of donor activity, depending on the center [33]. In Russia and other countries, patients with sudden out-of-hospital cardiac and respiratory arrest for whom resuscitation

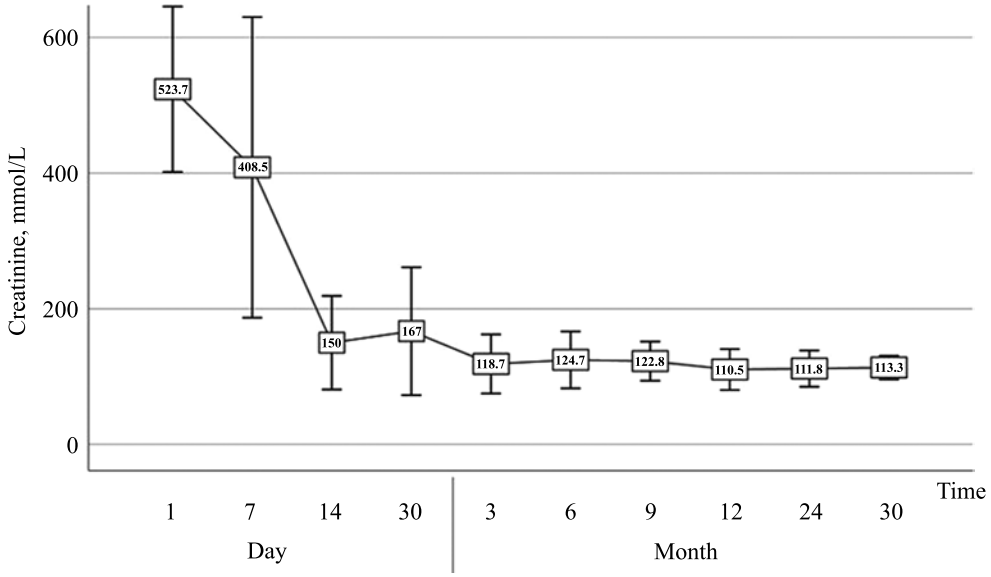


Fig. 5. Dynamics of mean creatinine levels during the follow-up period for kidney transplant recipients (95% CI)

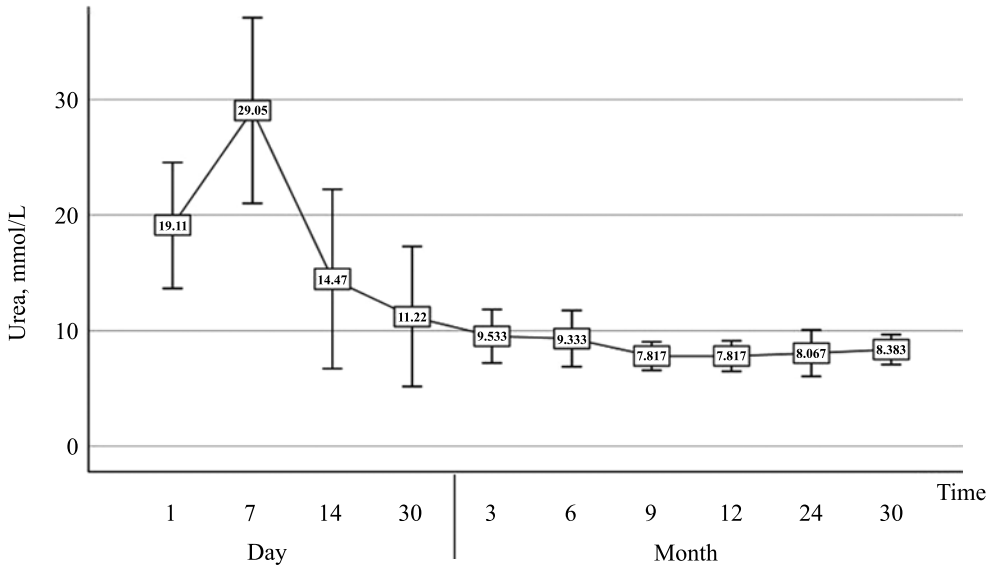


Fig. 6. Dynamics of mean urea levels during the follow-up period for kidney transplant recipients (95% CI)

attempts have been unsuccessful can also be considered as potential donors [34]. The novelty of donor service is in rapid interaction between the transplantation team and specialists trying to save the life of patients with sudden out-of-hospital cardiac arrest using a combination of conventional resuscitation and emergency use of ECMO. On one hand, having a life-support system in the emergency ward can increase the survival of patients whose resuscitation would not even have been performed in the past, and in cases of ineffective life-saving measures, minimize ischemic injury to the organs of those who have thus become donors, and initiate a transplantation program [6, 26–27].

In our case, the most significant seems to be the problem of determining the possibility of transition from "resuscitation measures" to the "donor protocol" (for example, how to ascertain the death of a person whose heart is not working, but blood circulation is preserved due to the use of an external mechanical support device; can we use the classical "brain death" criteria to establish the death of a patient who is on "assisted" circulation). It is necessary to further improve perfusion programs to save the lives of patients, both in cases of sudden cardiac arrest, and waitlisted patients with end-stage chronic diseases [26, 27].

Out-of-hospital asystolic donors are subjected to at least two critical non-circulation periods (from cardiac

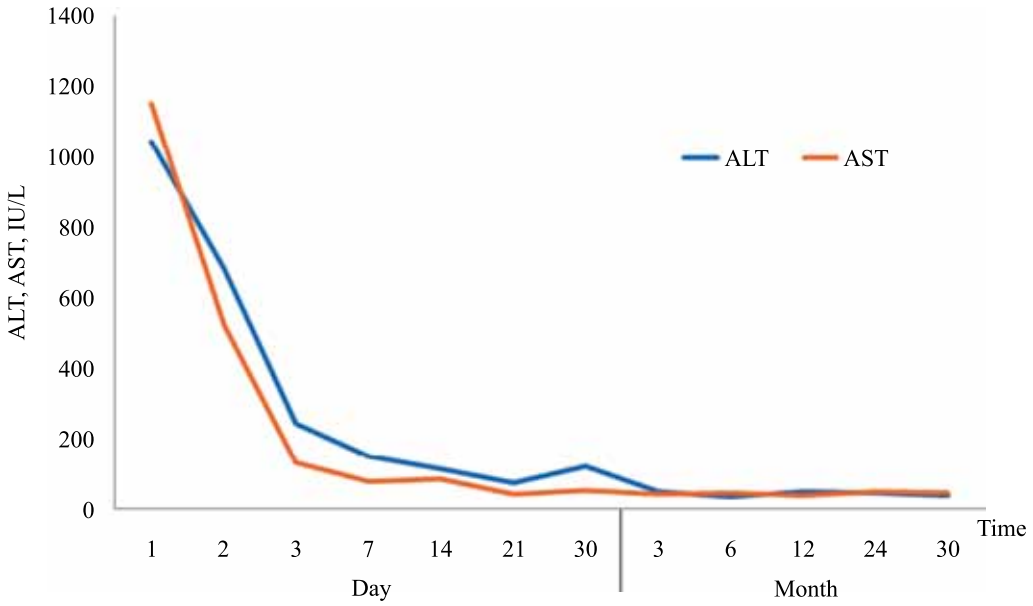


Fig. 7. Dynamics of mean values of ALT, AST (U/L) during the follow-up period for liver transplant recipients

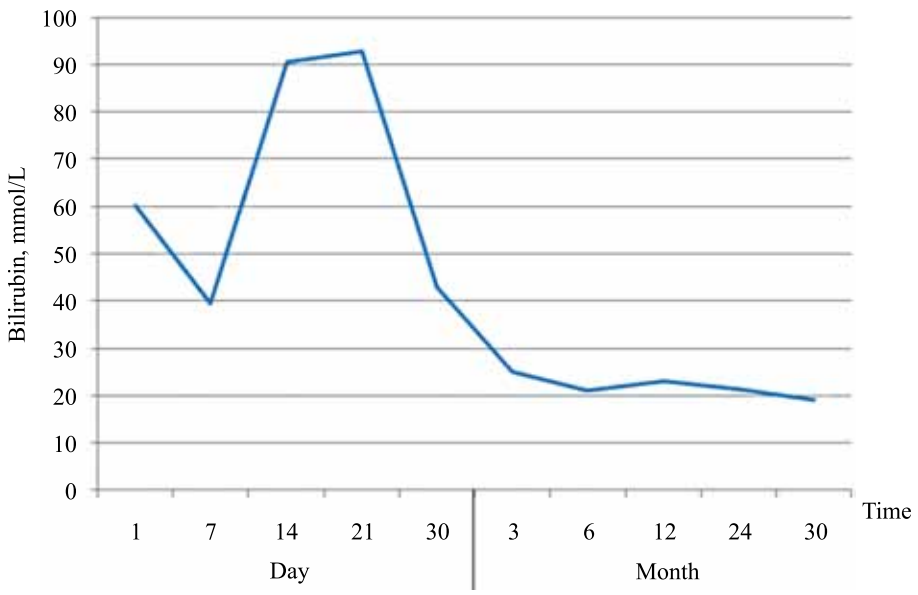


Fig. 8. Dynamics of mean bilirubin levels (μmol/L) during the follow-up period for liver transplant recipients

arrest before resuscitation and during the 'no touch' period, or the so-called "inactivity" period), whose duration, in turn, determines the fundamental possibility of using their organs and has a significant impact on outcomes of subsequent transplants [35].

All mechanical techniques for blood circulation resumption in the donor's body can be called by a common term "extracorporeal membrane oxygenation" (ECMO), or, more precisely, "extracorporeal life support", in our case – *restoration and maintenance of organ life in the body of a deceased person*.

There is a steady tendency to use devices for stage-by-stage ex vivo perfusion for diagnosis, selection and treatment for organs obtained from this category of donors [28–31].

The time interval from the moment of cardiac and respiratory arrest to the beginning of resuscitation measures should be considered as one of the most important factors influencing the effectiveness of both the programs of care for patients with sudden out-of-hospital circulatory arrest and out-of-hospital organ donation. Only CPR performed by emergency physicians or using external mechanical systems for chest compressions (device-assisted CPR) should be considered. Thus, if this interval was more than 20 minutes or its duration was not known, such patients were not considered as potential donors in the Pavlov University protocol. According to our data, the average duration of such an interval was 7.5 ± 2.4 minutes. In the Italian and Spanish ASD protocols available to us, this period was not more than 15 minutes [36].

In some countries, implementation of this donor protocol is limited by the very possibility of performing any manipulations with the body of the deceased after confirmation of biological death. In Spain and the United

Table 3

**Complications of kidney, liver transplantation from NHBDs**

| | Liver transplant (n = 5) | Kidney transplant (n = 22) |
|---|---|---|
| Graft function: | | |
| Delayed | 1 (20%) | 10 (45.45%) |
| Immediate | 3 (60%) | 10 (45.45%) |
| Early complications (within 1 month after transplantation): | n = 5 | n = 22 |
| Graft vascular thrombosis | 1 (20%) | 1 (4.55%) |
| Acute graft rejection crisis | – | 1 (4.55%) |
| Long-term early graft dysfunction | 1 (20%) | 1 (4.55%) |
| Mortality | 1 (20%) | – |
| Late complications (1–34 months after transplantation) | n = 4 | n = 20 |
| Graft rejection crisis in time | 1 (25%) | 2 (10%) |
| Biliary anastomotic stricture | 1 (25%) | – |
| Mortality | – | 1 (5%) |

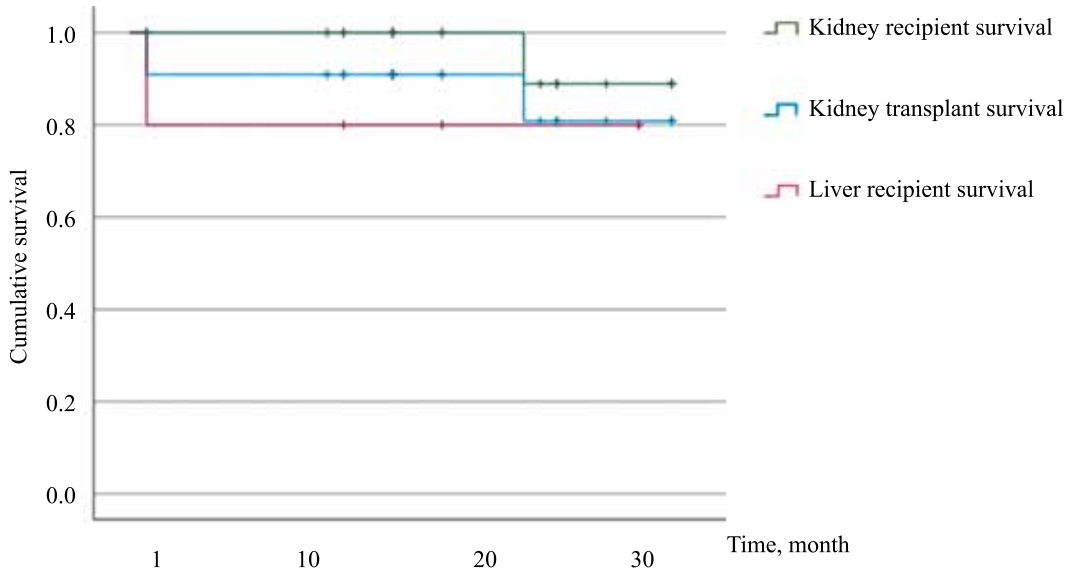


Fig. 9. Cumulative survival of kidney transplants and recipients who received donor organs from NHBDs (95% CI)

States, it is required to obtain the consent of relatives to perform femoral cannulation for the purpose of donation after the death of the person, which in some cases significantly complicates the implementation of the protocol (inability to contact relatives or lack of communication with relatives for a long period of time) [37]. Thus, in the so-called New York protocol, transportation of a potential donor to a donor hospital was planned to be performed by a separate team [38]. However, in Italy, part of the manipulations, namely, puncture of the femoral vessels of the potential donor and administration of anticoagulants, can be performed already at the stage of transportation to the hospital from the scene [39]. The situation is complicated by implementation of two protocols at once: CPR using auto-mechanical systems of assisted circulation and oxygenation (ECPR) and out-of-hospital asystolic donation program. The use of additional external assisted circulatory devices in CPR until death is confirmed often creates additional difficulties in the "validation" of the potential donor, and if there are signs of resuscitation failure, it is required to confirm brain death against the background of continuing assisted circulation [40]. Although recent experience in Portugal has shown that both programs can coexist successfully [41].

Another, no less important factor is the 'no-touch' time (period of inactivity), the period from the moment cardiac arrest occurs to the moment biological death is confirmed. The 'no-touch' time varies from 2 to 20 minutes without any ECG activity on the monitor [42]. As for the Pavlov University protocol for asystolic donors, such interval is not defined and not regulated. Therefore, we accepted the highest permissible time interval of 20 minutes, which, in our opinion, excludes ethical contradictions in the issue of establishing and irreversibility of biological death of a patient when all means of resuscitation have been exhausted.

In most protocols available to us from foreign literature, the total time determined as insufficient perfusion of internal organs against the background of an automatic indirect heart massage machine is limited to 150 minutes [43, 44]. In general, analysis of international results of organ transplants from out-of-hospital donors have shown that the permissible total warm ischemia time for kidneys is up to 360 minutes, liver – up to 140 minutes, lungs – up to 240 minutes [45]. Given the insufficiency of organ perfusion during chest compressions, we designated its time as the primary warm ischemia time, it was considered as an aggravating factor, and was limited to the "permissible" 120 minutes of automatic cardiac massage.

General heparinization and cannulation of vessels for perfusion have a special place in implementation of these protocols. According to Italian authors, as mentioned earlier, the use of heparin and installation of introducers (but not cannulas) in the lumen of the femoral vessels before the patient's death is allowed [39]. Injection of anticoagulants before the moment of death is confirmed when working with this category of donors (at the stage of potential donor transportation to the hospital) is associated with possible fatal complications (intracerebral bleeding, hemothorax, intra-abdominal bleeding, etc.), which itself can stop initiation of donor protocol. Based on our experience, heparin administration before the moment of death can be justified if CPR is performed using the emergency use of ECMO assisted circulation to save the lives of patients with sudden cardiac death. At the same time, installation of introducers in the vascular lumen should be performed as early as possible, since absence of pulsation in the arteries increases the likelihood of technical errors at the stage of donor vascular cannulation. The use of mechanical cardiac compression systems and a ventilator can significantly reduce total warm ischemia time and increase graft utilization and survival [46].

Vascular cannulation prior to death makes it possible to reduce the warm ischemia time and. At the same time, this raises some ethical concerns among the foreign professional transplant community because there is the probability of restoring the patient's cerebral blood flow after biological death has been confirmed [47]. In this regard, almost everywhere abroad, one or another method of isolating the abdominal perfusion region is used (in normothermic regional (abdominal) perfusion (NRP)) is used – balloon inflation in the aortic lumen above the diaphragm or application of a ligature on the aorta, after performing rapid laparotomy.

We did not use balloon insertion into the aortic lumen, because we consider the "inactivity" period of 20 minutes to be sufficient to ensure confirmation of irreversibility of cardiac arrest, if any, and to exclude the possibility of recovery of some part of the brain function. Exclusion of occluding aortic balloons from the protocols allows us to safely assume that in the nearest future, it will be possible to use heart and lungs from asystolic donors for clinical transplantation [48].

Despite repeated experiments on animals where the efficacy of thrombolytics was shown, as well as in our clinical experience of thrombolytics use (Streptokinase, Alteplase) in abdominal normothermic perfusion in situ, there are currently no reports on inclusion of this group of drugs in out-of-hospital donor protocols [6, 49, 50]. The same situation is observed with the use of mechanical and pharmacological leukoreduction in the donor body. Routine use of leukocyte filters has been described in isolated lung perfusion, where it has been shown that leukoreduction is associated with incidence of primary pulmonary graft dysfunction [51]. In our opinion, today these integral components of perfusion protocols are underestimated; they have significant potential to improve the functional status of organs obtained from asystolic donors to the moment of transplantation; in fact, without

their use, the donor therapeutic potential of ECMO is reduced to zero.

To summarize the discussion, it has to be said that out-of-hospital donors are a full-fledged donor resource, not only for kidney transplants, but also for such donor organs as liver, pancreas, lungs, when extracorporeal life support is used. In a recent European study evaluating the effectiveness of various post-mortem organ donation pathways in 2016, the use of out-of-hospital asystolic donors was 75%, compared with 91% of "controlled" asystolic donors and 93% of brain-dead donors [35]. Thus, over a 15-year period, 1,713 kidney transplants, 158 liver transplants, and 86 lung transplants from these donors were performed in Spain. These results were achieved with a rather high rejection rate (45% of kidneys and 62% of livers) mostly due to ineffective perfusion. At the same time, the actual one-year survival rate of kidney transplants was 87%, despite an increased rate of delayed graft function (78%) and early graft dysfunction (7%) compared with standard brain-dead donors. These results were later confirmed by several individual Spanish centers as well as by French and Italian studies [52].

## CONCLUSION

Constant technologization of the medical industry leads to introduction of high-tech methods in the practice of critical care and life support medicine. The state of the art is the implementation of technologies for the use of mechanical cardiac compressing systems and ECMO in emergency medical care primarily in order to improve the efficiency and effectiveness of resuscitation measures. Possession of resuscitators and wide use of portable perfusion devices for ECMO, in combination with advanced resuscitation measures, can achieve survival of 5.92% of patients with sudden out-of-hospital circulatory arrest. In the case of ineffectiveness of life-saving measures, such medical technology allows switching over to the procedure for preserving the organs of a deceased donor and implementing the donor program in 16.47% of cases. The long-term outcomes of organ transplantation from asystolic donors, whose blood circulation was restored using ECMO, practically do not differ from the outcomes of transplantation from brain-dead donors. Thus, the across-the-board widespread technologization of resuscitation care, the use of perfusion technology and portable perfusion equipment as part of life support procedure, can potentially save the lives of patients with sudden out-of-hospital cardiac arrest and has the impressive potential to expand the pool of donor organs.

*The authors declare no conflict of interest.*

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# LIVER TRANSPLANTATION IN PEDIATRIC PATIENTS UNDER 15-KG; DUCT-TO-DUCT VS. ROUX-EN-Y HEPATICOJEJUNOSTOMY BILIARY ANASTOMOSES

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**Back ground.** Liver transplantation is an effective treatment for acute or chronic liver failure and metabolic liver disease, which is associated with good quality of life in over 80 percent of recipients. We aimed to evaluate outcome of duct-to-duct vs. Roux-en-Y hepaticojejunostomy biliary anastomoses in pediatric liver transplant recipients below 15-kg. **Methods.** In this single-center retrospective study, all children less than 15 kg that have undergone liver transplantation at Nemazee Hospital Organ Transplant Center affiliated with Shiraz University of Medical Sciences from 2009 till 2019, were enrolled. Over a 10-yr period, 181 liver transplants were performed in patients with two techniques including duct-to-duct (Group 1) vs. Roux-en-Y hepaticojejunostomy biliary anastomoses (Group 2). All data was collected from patients' medical records, operative notes, and post-transplant follow up notes. Data was analyzed by SPSS software V21. **Results.** Overall, 94 patients had duct to duct anastomosis (group 1) and 87 cases had Roux-en-Y hepaticojejunostomy (group 2). The mean age of the patients was 2.46 ± 1.5. The most common underlying diseases was biliary atresia (32%). The most prevalent complication after the surgery was infection in both groups. cardiopulmonary problems were significantly higher in group 2 (24.1% vs 4.3%) ($p < 0.001$). The rate of infection was significantly higher in group 2, as well. **Conclusion.** Our study showed a relatively high rate of post-operative infection which was the most among patients who had undergone Roux-en-Y hepaticojejunostomy. Except from biliary complications which were mostly observed in DD group, other complications were more common among Roux-en-Y group.

*Keywords: liver transplantation, duct-to-duct biliary reconstructions, Roux-en-Y hepaticojejunostomy, Biliary complications.*

## INTRODUCTION

Liver transplantation (LT) is an established curative therapy for acute or chronic liver failure or liver metabolic disease. Survival after LT has improved significantly in developed countries and this has increased the awareness of this treatment modality in the developing world. The development of newer immunosuppressive drugs, refinements in operative technique, improved organ preservation, advances in allied service specially anesthesia and intensive care have contributed to a dramatic improvement in the survival of these patients. It is essential to encourage both the child and the family to return to a normal life as far as possible. Most of the children who survive liver transplantation will achieve nutritional rehabilitation and a normal lifestyle. Most will enter puberty normally, and fertility is normal. Pediatric liver transplantation has been evolving over recent decades. The developments in reduced sized grafts and living donor liver transplants have significantly reduced mortality rates for pediatric patients suffering from end-stage liver disease [1, 2]. However, despite continuous improvements in surgical techniques, biliary complications including leakage from biliary radicals, anastomotic leaks, and strictures are still associated with significant morbidity and mortality occurring with an incidence of 10% to 50% [3, 4]. Although transplant-related biliary complications are not associated with decreased patient survival in pediatrics, these complications do cause considerable morbidity, increased length of stay, need for increased operative and non-operative interventions, and occasionally re-transplantation. Historically, the biliary anastomosis has been termed the Achilles heel of orthotopic liver transplantation [5]. Complications such as leakage, stricture, fistula, infection, and cast formation led to the evolution of new technical methods. Early biliary reconstructions were performed using loop choledochojejunostomy and Roux-en-Y hepaticojejunostomy,

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as well as using the gallbladder as a conduit. In the 1980s, duct-to-duct choledocho-choledochostomy drainage over a T-tube became the most popular technique. During the past two decades, duct-to-duct anastomosis without a T-tube has become the primary method of reconstruction. With the advent of living donor liver transplantation in the 1990s, reconstruction without enteric anastomosis has been performed (5). Although Roux-en-Y hepaticojejunostomy has been the standard procedure in LDLT, the disadvantages of this technique are a comparatively long operative time and higher risk of contamination due to construction of the Roux-en-Y limb. Theoretically, Duct-to-duct biliary reconstruction has some benefits when it is compared with the Roux-en-Y procedure, namely no manipulation of the gastrointestinal tract, a shorter operative time and allowing for easy endoscopic access to the anastomotic site if complications arise. It is the standard technique of choice for biliary anastomosis in cadaveric liver transplantation. When the duct-to-duct technique can be employed for LDLT, an extra-intestinal anastomosis can be avoided, the continuity is more physiologic than that of RYHJ, and preservation of the sphincter function of the lower bile duct may reduce the risk of enteric reflux into the biliary tract. Also, Duct-to-duct biliary reconstruction is mostly the standard technique in adult liver transplantation. On the other hand, the predominance of biliary atresia along with the technical challenges related to the small size and fragility of recipient's duct in the pediatric age population have made Roux-en-Y hepaticojejunostomy far more common. There are still a lot to learn and there are future challenges to improve liver transplantation techniques. The evolution of the field of liver transplantation has resulted in improved patient outcomes. A paucity of data exists for the outcomes of these two techniques [10]. The aim of this study was to compare the outcomes of duct-to-duct vs. Roux-en-Y hepaticojejunostomy biliary anastomoses in pediatric liver transplant recipients below 15-kg.

## METHODS

In this single-center retrospective study, all children less than 15 kg that have undergone liver transplantation at Nemazee Hospital Organ Transplant Center affiliated with Shiraz University of Medical Sciences from 2009 till 2019, were enrolled. Over a 10-yr period, 181 liver transplants were performed in patients with two techniques including duct-to-duct (Group 1) vs. Roux-en-Y hepaticojejunostomy biliary anastomoses (Group 2). The electronic medical record was used to collect patients' characteristics including age, medical history, details of transplantation surgery, and postoperative hospital course. Postoperative information collected included patient and graft survival. Data were expressed as means ± standard deviation. Fisher's exact test, unpaired *t* tests, and the log-rank test were used for statistical analysis. *P* values less than 0.05 were regarded as significant. All liver recipients, of a whole or partial liver graft, weighting less than 15 kg were included. All data were collected from patients' medical records, operative notes, and post-transplant follow up notes. Demographic data such as age, sex, weight, primary diagnosis, PELD (pediatric end-stage liver disease) score, previous biliary tract surgeries, type and weight of graft, type of donor, date of transplantation, graft ischemia time, operative time, biliary and vascular complications were collected. Patients were divided into two groups based on the type of biliary anastomosis done during surgery: duct-to-duct biliary reconstruction and Roux-en-Y hepaticojejunostomy biliary reconstruction. Duct-to-duct biliary anastomosis was preferably performed whenever it was thought to be technically feasible by the operating surgeon. Biliary complications including biliary leaks, strictures, or development of cholangitis were recorded. Biliary leaks from anastomotic sites were diagnosed based on cholangiograms and were considered as significant complications if any further intervention was deemed to be necessary. Biliary strictures were diagnosed based on cholestatic trend of liver enzymes suggestive of a stricture in addition to a confirmatory cholangiogram demonstrating the presence of a stricture. Cholangitis was considered in any patient developing fever or raised level of white cell count with elevated bilirubin. Vascular complications consisted of hepatic artery thrombosis or stricture. Both vascular complications were initially diagnosed by Doppler ultrasound with further confirmation of arterial stenosis by angiogram. Mortality rate of two groups were also considered. Student's t-test and Pearson's chi-square were primarily used to compare both groups. Further statistical analysis was performed using Fisher's exact test with the Freeman–Halton extension when appropriate.

## RESULTS

### Pre-operative characteristics of the patients

Overall, 94 patients had duct to duct anastomosis (group 1) from which 46 expired and 87 cases had Roux-en-Y hepaticojejunostomy (group 2) from which 43 cases passed away. The mean age of the patients was 2.468 ± 1.5711 year (0.5–10) and their mean weight was 10.403 ± 2.5501 Kg (4–15). The most common underlying diseases were biliary atresia (32%), PFIC (17.1%) tyrosinemia (16%) and Crigler-Najjar (10.5%). The most common complication before the surgery was ascites with a prevalence of 8.8% in our patients. The mean PELD scores in group 1 and 2 before surgery were 15.19 ± 8.9 and 19.64 ± 6.9 respectively. Out of 94 patients in group 1, 44 underwent partial liver transplantation, 23 experienced split transplantation and 27 had whole organ transplantation. The corresponding numbers in the group 2 were 61, 13, and 12 patients. The most common donor relation

in group one was DD and in group 2, mothers were the most donors. The complete pre-operative characteristics of patients are listed in table 1.

## Post-operative characteristics

Prednisolone and Tacrolimus were the most required immunosuppressive used in both groups. Our data showed that the patients who need Prednisolone, Tacrolimus and Mycophenolate in group 1 were significantly more than group 2 (P value <0.05). The most prevalent complication after the surgery was infection in both groups. cardiopulmonary problems were significantly higher in group 2 (24.1% vs 4.3%) ($p < 0.001$). The rate of infection was significantly higher in group 2, as well. Although the rate of biliary complications was higher in the group 1, this difference was not statistically significant. The least needed re-operation was drainage of the collection and the most prevalent surgical procedures were ERCP and Roux-en-Y Hepaticojejunostomy after Duct to Duct, both with the prevalence of 7%. The most common causes of death were infection (9.2%), sepsis (8.3%) and rejection (6.1%) (table 2).

## DISCUSSION

Despite the fact that the most common type of biliary anastomosis is duct to duct hepaticojejunostomy biliary anastomosis, there is still discussion about the safety of this method in pediatrics [11–13]. Some studies suggested Roux-en-Y hepaticojejunostomy as the method of choice as it is associated with lower stricture formation and higher graft survival. There are not enough reports on using these methods in children. As the most prevalent type of complication followed by this procedure is biliary complication, some studies focused on this type of complication. The biliary complication rate after optimizing duct to duct anastomosis has been reported to be around 28.6–47.7% in children based on some earlier small reports [14, 15]. Meanwhile, the corresponding rate for adults was reported 26.9% [16]. However, A more recent study indicated a 12.7% rate of biliary complication in a group of 298 LDLT pediatrics, 75% of which had

Table 1

**Pre-operative characteristics of patients**

| Pre-operative characteristics | | Group 1 | Group 2 |
|---|---|---|---|
| Male/Female | | 54/40 | 47/40 |
| Age | | 2.829 ± 1.5551 (0.5–8) | 2.078 ± 1.5018 (0.5–10) |
| Weight | | 11.178 ± 2.3343 (6–15) | 9.567 ± 2.5209 (4–15) |
| PELD score | | 15.19 ± 8.9 | 19.64 ± 6.9 |
| Donor Relation | Mother | 26 | 29 |
| | Father | 16 | 27 |
| | DD | 49 | 25 |
| | Uncle or Aunt | 3 | 6 |
| Graft type | Partial | 44 | 61 |
| | Split | 23 | 13 |
| | Whole organ | 27 | 12 |
| Underlying disease | PFIC | 31 (17.1%) | |
| | Intrahepatic Cholestasis | 2 (1.1%) | |
| | Crigler-Najjar | 19 (10.5%) | |
| | Tyrosinemia | 29 (16%) | |
| | Biliary Atresia | 58 (32%) | |
| | Cryptogenic | 9 (5%) | |
| | HCC | 3 (1.7%) | |
| | Autoimmune Hepatitis | 4 (2.2%) | |
| | Hypercholesterolemia | 6 (3.3%) | |
| | Neonatal Hepatitis | 6 (3.3%) | |
| | Choledochal Cyst | 2 (1.2%) | |
| | Other* | 12 (6.6%) | |
| Complications | Encephalopathy | 3 (1.7%) | |
| | GI bleeding | 4 (2.2%) | |
| | Ascites | 16 (8.8%) | |
| | Hepatorenal Sx | 1 (0.6%) | |
| | Hepatopulmonary sx | 1 (0.6%) | |

*Note.* * Other: the underlying diseases which contain problems like hepatitis, Caroli, glycogen storage disease, Hepatoblastoma, Alagille syndrome, MSUD and Methylmalonic acidemia.

undergone duct to duct anastomosis [17]. The prevalence of this complication was lower in patients who have undergone Roux-en-Y surgery (12.6%) compared with the duct to duct anastomosis group (20.2%). However, this difference was not significant.

In this study, in addition to biliary complication, which is the most common complication discussed in previous studies, other complications related to these procedures were also discussed and compared. The most prevalent complication in both techniques was infection. Despite the fact that most of our immunosuppressive agents were mostly used in the DD group, the rate of infection was higher among Roux-en-Y patients which can be related to the higher length of the operation time. Our study indicated that around 38% OF DD group and 56% of Roux-en-Y group developed infection after surgery. More specifically, about 15% of our patients died either from infection or sepsis. Acute and chronic rejections both showed higher rates among DD group. However, these differences were not significant. One of the most common vascular complications in liver transplant patients is Hepatic artery thrombosis (HAT), which has been identified as a cause of biliary complication. Some studies have shown that the incidence of HAT in Roux-en-Y reconstruction was higher than DD anastomosis in adult liver transplantation and have identified Roux-en-Y

Table 2

**Post-operative characteristics of patients**

| Post-operative characteristics | | Group 1 | Group 2 |
|---|---|---|---|
| Immunosuppressive treatment requirement | Prednisolone | 91 (96.8) | 70 (80.5) |
| | Cyclosporine | 3 (3.2) | 4 (4.6) |
| | Tacrolimus | 87 (92.6) | 67 (77) |
| | Mycophenolate | 22 (23.4) | 9 (10.3) |
| | Sirolimus | 1 (1.1) | 1 (1.1) |
| Complications | Biliary Complications | 19 (20.2) | 11 (12.6) |
| | Vascular Complications | 9 (9.6) | 11 (12.6) |
| | Infection | 36 (38.3%) | 49 (56.3%) |
| | Ascites | 3 (3.2%) | 7 (8%) |
| | Convulsions | 4 (4.35) | 8 (9.2%) |
| | Renal problems | 2 (2.1%) | 6 (6.9%) |
| | Cardiopulmonary problem | 4 (4.3%) | 21 (24.1%) |
| | Acute Rejection | 23 (24.5%) | 16 (18.4%) |
| | Chronic rejection | 2 (2.1%) | 1 (1.1%) |
| Bile duct dilatation requirement | No Need | 167 (92.3%) | |
| | PTC | 4 (2.2%) | |
| | ERCP | 7 (3.9%) | |
| | Missing | 3 (1.7%) | |
| Need for surgical repair | No Need | 171 (94.5%) | |
| | Duct to Duct repair | 1 (0.6%) | |
| | Roux-en-Y Hepaticojejunostomy after Duct to Duct | 7 (3.9%) | |
| | Missing | 2 (1.1%) | |
| Need for drainage of the collection | No Need | 179 (98.9%) | |
| | Missing | 2 (1.1%) | |
| Cause of death | Bleeding | 3 (1.7%) | |
| | PNF | 6 (3.3%) | |
| | Liver Necrosis | 2 (1.1%) | |
| | Pulmonary Problems | 5 (2.8%) | |
| | Rejection | 11 (6.1%) | |
| | Sepsis | 15 (8.3%) | |
| | Renal Failure | 3 (1.7%) | |
| | Convulsion | 1 (6%) | |
| | Pneumonia | 7 (3.9%) | |
| | Cardiac arrest | 9 (5%) | |
| | Infection | 4 (9.2%) | |
| | PTLD | 9 (5%) | |
| | Other | 12 (6.6%) | |
| | Missing | 4 (2.2%) | |

surgery as a risk factor for HAT [18]. Studies in children have also found the higher rates of HAT in Roux-en-Y reconstruction (9%) than its prevalence in DD anastomosis group (3.3%), which was consistent with our study [19]. The prevalence of vascular complications in Roux-en-Y surgery group was 12.6% compared with 9.6% in DD group. One of the reasons that might cause this difference is that Roux-en-Y construction leads to compression of the arterial anastomosis [19]. The cardiopulmonary complication was also one of the significant complications in this study that has been less noticed in previous studies. Some studies have shown a prevalence of respiratory failure following Roux-en-Y hepaticojejunostomy is about 10% [20]. Kawachi et al. also showed in their study that one patient (10%) had idiopathic cardiac failure following Roux-en-Y surgery and pulmonary complications observed in one patient (10%) who have undergone duct to duct anastomosis [21]. In this study, cardiopulmonary complications were higher in Roux-en-Y surgery group (24.1%) compared with the duct to duct anastomosis group (4.3%). This difference was statistically significant ($p < 0.001$). Our finding is supported by Stilling et al's study on 139 adult patients who had bile duct injury during cholecystectomy and underwent repaired Roux-en-Y hepaticojejunostomy. They reported that 12% of patients developed cardiopulmonary complications in less than a month after surgery [22]. S.V. McDiarmid et al. in 2003 investigated the liver transplantation in children. Their study revealed that a long-term outlook for children receiving liver transplantation is likely to be limited by donor supply, the side effects of immunosuppressive drugs and the potential development of post transplantation lymphoproliferative disease or other tumors [1]. T. Kimura et al. in their study which was performed in 2006 about Feasibility of duct-to-duct biliary reconstruction in pediatric living related liver transplantation (Report of three cases) demonstrated that duct-to-duct biliary reconstructions in pediatric seemed to be feasible and safe [6]. Y. Shirouzu et al. compared the outcome of Roux-en-Y hepaticojejunostomy and duct-to-duct anastomosis in 2008. Their results showed that their surgical technique using DD in recipients weighing no more than 10 kg produced excellent outcomes with a low incidence of biliary complications, including leakage and stricture [2].

## CONCLUSION

Our study showed a relatively high rate of post-operative infection which was the most among patients who had undergone Roux-en-Y hepaticojejunostomy. Except from biliary complications which were mostly observed in DD group, other complications were more common among Roux-en-Y group.

*The authors declare no conflict of interest.*

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# LAPAROSCOPIC URETERAL RECONSTRUCTION IN A KIDNEY TRANSPLANT: SUCCESSFUL CLINICAL CASES

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Treatment of ureteral strictures in the long-term post-transplant period is a complex surgical procedure. We present successful clinical cases of developed laparoscopic ureteral stricture reconstruction methods at three levels (in the pelvis, along the ureter, in the anastomosis area). These methods have shown their clinical efficacy: they are less traumatic, there are no adverse events in the early and long-term postoperative periods, and there is accelerated rehabilitation of recipients after surgery.

*Keywords: ureteral stricture, laparoscopy, kidney transplantation, urology.*

## INTRODUCTION

Ureteral stricture is a common urological complication following kidney transplantation (3–12.6%), especially in the long-term postoperative period [1].

According to various sources, the causes of ureteral stricture are excessive length, insufficient blood supply to the ureter, fibrosis in the surrounding tissues, donor age over 65, cold ischemia time, presence of several feeding renal arteries, delayed graft function, and formation of vesicoureteral anastomosis without stenting [2, 3]. There have been individual cases of ureteral strictures in a renal graft in combination with tubulointerstitial nephritis caused by cytomegalovirus infection [4].

Depending on the location and extent of strictures (distal part in 73% of cases, middle part in 12%, and proximal part in 15%), the most common methods of ureteral stricture treatment are bougienage, endoscopic cold knife dissection, balloon dilation or surgical correction of graft ureteral stricture zone [2, 5].

Currently, laparoscopic correction of urological complications in a kidney transplant is rarely used in clinical practice due to the complexity of this technique. Despite this, it has significant advantages over other methods of treatment: better visualization, ability to accurately identify all structures, which in turn leads to accelerated rehabilitation of kidney recipients after surgery [5, 6, 7].

Development of laparoscopic technologies in transplantology has provided new opportunities for the treatment of urological complications following kidney transplantation. Our accumulated long-term unique experience at Shumakov National Medical Research Center of Transplantology and Artificial Organs allows us to offer various types of laparoscopic surgeries to correct urinary tract obstructions in a transplanted kidney.

**Objective:** to present successful clinical cases of laparoscopic ureteral reconstruction techniques in a kidney transplant.

## CLINICAL CASES

### Clinical Case #1: Laparoscopic correction of ureteropelvic junction stricture in a transplanted kidney

*In 2013, 42-year-old female patient P., diagnosed with chronic glomerulonephritis, had a deceased-donor kidney transplant surgery. Kidney graft rotation by compression of the ureter at the level of the ureteropelvic junction (peculiarity – high ureteral insertion from the graft pelvis) was detected in the early postoperative period (Fig. 1). The patient repeatedly had nephrostomies and ureteral stent replacements. These were accompanied by persistent recurrent urinary tract infection and required combined antibiotic therapy. In December 2017, laparoscopic correction of ureteropelvic junction stricture in the transplanted kidney was performed.*

*Under general anesthesia, a 12 mm trocar is inserted into the abdominal cavity in the right paraumbilical region, then a laparoscope is inserted, and the abdominal cavity is examined. Next, 10 mm and 5 mm trocars are inserted in the left paraumbilical and right iliac regions. The peritoneum over the graft is dissected using the apparatus Harmonic© (USA). With a precision technique, the ureteropelvic junction with high ureteral insertion and acute bend of the ureter in this area is highlighted and visualized. As carefully as possible, taking into account the close location of the vascular pedicle of the*

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*graft, the pelvis and the proximal ureter of the kidney graft are mobilized. The pelvis of the graft is dissected for 1.5–2 cm, with the incision passing to the ureter, which is also dissected in its upper third for 1.5–2 cm. The posterior wall of the renal pelvis is sutured to the posterior wall of the ureter using a monofilament suture. A ureteral stent is placed into the lumen of the graft ureter in an antegrade fashion. The anterior wall of the renal pelvis is stitched to the anterior wall of the ureter with a monofilament suture. Hemostasis control is performed at the end of the operation. The abdominal cavity is drained.*

*The ureteric stent was removed 6 weeks after ureteropelvic junction plasty. There were no postoperative complications. No recurrent stricture or anastomotic leaks were detected 41 months after operation.*

## Clinical Case #2: Laparoscopic correction of mid-ureteral stricture in transplanted kidney

*In 2016, 24-year-old male patient G., diagnosed with extracapillary glomerulonephritis, had a deceased-donor kidney transplant surgery without ureteral stenting with delayed graft function. Increased dilatation in the pelvicalyceal system two weeks after surgery required drainage of the graft with a nephrostomy tube. Antegrade ureteral stenting was unsuccessful. In February 2017, laparoscopic correction of the middle ureter of the graft was performed (Fig. 2).*

*Under general anesthesia, a 12 mm trocar is placed in the abdominal cavity in the left/right paraumbilical region, then a laparoscope is inserted, and the abdominal cavity is examined. Next, 10 mm and 5 mm trocars are*


Fig. 1. Patient P. Graft rotation and high ureteral insertion from the: A) renal artery; B) nephrostomy tube balloon; C) pelvis, D) ureter; E) ureteropelvic junction (stricture area)

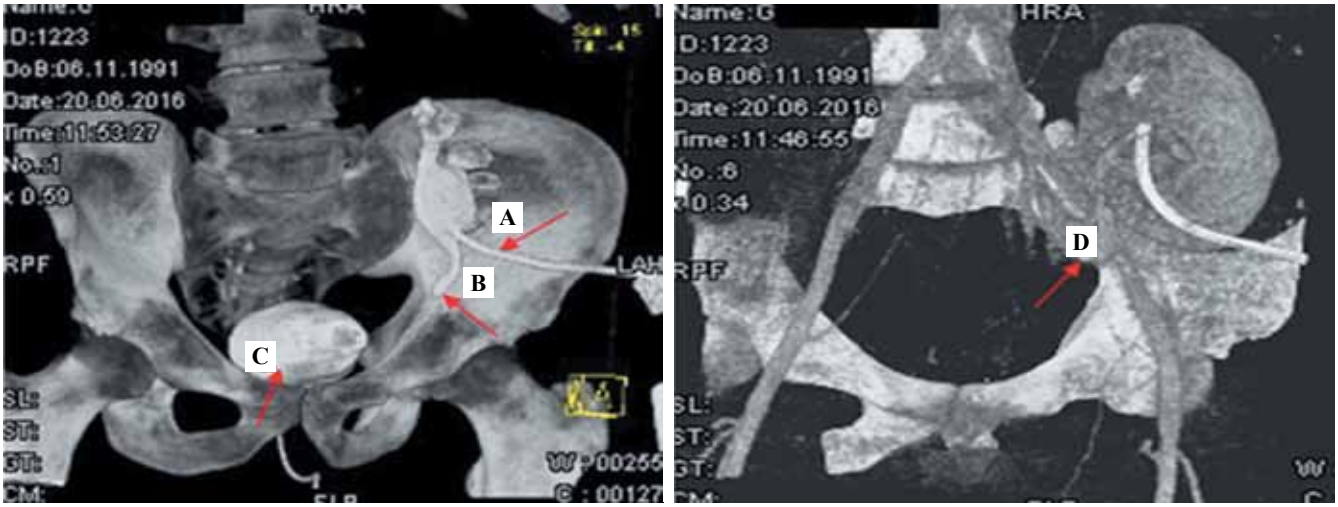


Fig. 2. Patient D. Mid-ureteral stricture in kidney graft: A) nephrostomy tube (antegrade pyeloureterography); B) graft ureter (stricture area); C) bladder (cystography); D) renal artery

*placed in the right/left paraumbilical region and left/right iliac region.*

*The graft ureter is mobilized with an apparatus (Harmonic©, USA) within healthy tissues and the distal end is clipped. Next, ipsilateral nephrectomy is performed with preservation of the distal ureter. Uretero-ureteroanastomosis is formed with ureteral stenting. Hemostasis control is performed at the end of the operation. The abdominal cavity is drained.*

*After reconstruction, the stent stayed in the ureter for 42 days. There were no postoperative urological, infectious complications. The patient was followed up for 52 months after the operation, and no recurrent stricture was detected.*

## Clinical Case #3: Laparoscopic correction of uretero-cysto-anastomotic stricture in transplanted kidney

*In 2016, 55-year-old patient K., diagnosed with chronic kidney disease of unknown etiology, had a deceased-donor kidney transplant surgery. During outpatient follow-up six months later, increased hydronephrosis was noted, and graft dysfunction developed, which required drainage of the kidney graft with a nephrostomy tube. Next, bougienage and stenting of the kidney graft ureter were carried out. Recurrent vesicourethral anastomotic stricture occurred three months after stent removal (Fig. 3). There was a need to reinsert the nephrostomy tube due to graft dysfunction. In November 2016, laparoscopic correction of the vesicourethral anastomosis was performed.*

*Under general anesthesia, a 12 mm trocar is placed in the abdominal cavity in the left/right paraumbilical region, then a laparoscope is inserted, and abdominal cavity is examined. Next, a 5 mm trocar is placed in the left/right iliac region and a 10 mm trocar is placed in the right subcostal area.*

*In the graft area, the peritoneum is dissected with an apparatus (Harmonic©, USA). The bladder is mobilized in the projection of the iliac vessels, the graft ureter is isolated from the scar tissue and its fibrously altered part is cut off. Uretero-cysto-anastomosis is formed with ureteral stenting. Hemostasis control is performed at the end of the operation. The abdominal cavity is drained.*

*After reconstruction, the stent stayed in the ureter for 28 days. There were no postoperative urological, infectious complications. The patient was followed up for 55 months after the operation, and no recurrent stricture was detected.*

## CONCLUSION

Despite accumulated experience, kidney transplantation comes with urological complications including ureteral strictures, which adversely affect graft survival and recipient's quality of life.

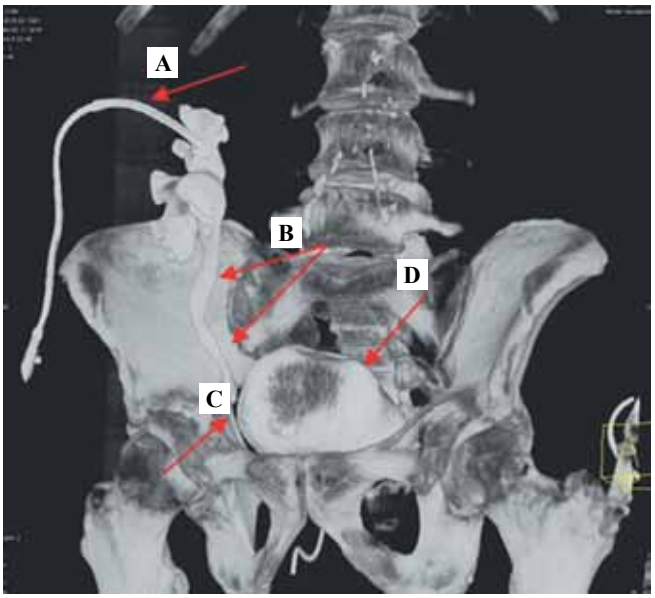


Fig. 3. Patient K. Uretero-cysto-anastomotic stricture: A: nephrostomy tube (antegrade pyeloureterography); B) graft ureter; C) bladder-ureteroanastomosis (stricture zone); D) bladder (cystography)

Laparoscopic ureteral reconstruction on the urinary tracts of a kidney transplant at different levels (in the pelvis, along the ureter, in the anastomosis area) compared to open surgical interventions are less traumatic, the patient requires minimal anesthetic drugs in the postoperative period, the patient is activated early, hospitalization period is less, and there is lower risk of adverse postoperative complications (postoperative wound infection, postoperative ventral hernia) [8–10].

The presented clinical cases of the use of laparoscopic ureteral reconstruction in kidney transplants, created and being used at Shumakov National Medical Research Center of Transplantology and Artificial Organs, have shown to be clinically efficient. Therefore, we recommend them for treatment of urethral strictures in transplanted kidneys at different levels.

*The authors declare no conflict of interest.*

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# NEUROPSYCHOLOGICAL DEVELOPMENT OF CHILDREN WITH BILIARY ATRESIA AFTER LIVER TRANSPLANTATION

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**Background.** In young children, the most common liver disease leading to transplantation is biliary atresia. Liver transplantation has fundamentally improved the survival rate of children with biliary atresia. Studies on developmental outcomes in children are mostly limited to small samples; there are no such studies in the Russian Federation. **Objective:** to determine the cognitive outcomes in children undergoing one-stage or two-stage surgical treatment of biliary atresia. **Materials and Methods.** 83 children were divided into groups: 36 children underwent transplantation without previous surgical interventions (group 1), 47 children underwent the Kasai palliative portoenterostomy (group 2). Inclusion criteria: 24 months of age or younger at the moment of transplantation, no medical history of neurological pathology. All children were examined before transplantation and at 1, 3, 6 and 12 months after liver transplantation. Psychomotor development was assessed using the Griffiths Psychomotor Development Scale for children under 24 months (translated by E.S. Keshishian), the Griffiths Intellectual Development Scale for children aged 2 to 8 years, and the Modified Checklist for Autism in Toddlers, Revised, for children 16-30 months old. **Results.** All children had developmental delays at the time of transplantation. Up to 50% of the children had signs of cachexia, with a shoulder circumference of less than 3 percentile. Only two children showed obvious hepatic encephalopathy in the form of depressed consciousness. After liver transplantation, 94% of group 1 children recovered their preoperative psychomotor development levels, and only 68% in group 2 made these gains. At 3 and 6 months after transplantation, about 80% of group 1 children showed normal psychomotor development, whereas in group 2, only 61% did. By 12 months after liver transplantation, the difference between the groups was more evident: 83.3% of group 1 children and only 53.2% of group 2 children were developing according to age. The difference between the groups was statistically significant ($p < 0.05$). **Conclusion.** Children who received one-stage treatment of biliary atresia and underwent liver transplantation have better neuropsychological development within a year after surgery than children with two-stage surgical treatment.

*Keywords: liver transplantation, biliary atresia, Kasai portoenterostomy, neuropsychological development, cognitive development.*

Over the past decades, liver transplantation has become the gold standard treatment for end-stage liver disease in pediatric patients [1]. Among the indications for liver transplantation, biliary atresia is the most common in childhood [2] [1]. Before surgical treatment, the life expectancy of children with biliary atresia did not exceed 2 years [3]. According to Russian sources, the 1-year and 5-year survival of children with native liver after portoenterostomy is 82.7% and 42.1%, respectively [4]. This is consistent with evidence from Marie-Odile Serinet 2009 about the 5-year survival rate of children after Kasai surgery – 37.9% [3].

The first liver transplantation for biliary atresia in a 3-year-old child was performed in 1953 by the pioneer of orthotopic human liver transplantation Thomas E. Starzl [5]. This event fundamentally changed the survival rate of children with biliary atresia.

Children with biliary atresia develop biliary cirrhosis in the first year of life, during active myelination of the conductive pathways of the central nervous system, which is associated with the risks of impaired psychomotor development. Existing complications of cirrhosis, such as portal hypertension, hepatic cell failure, and hepatic encephalopathy (HE) promote impaired neuropsychiatric development in children. Survival rates are improving every year, whereas there is little information about child development and cognitive outcomes. Studies are conducted on small samples, for which it is difficult to judge objectively development prognosis. There are no such studies in the Russian Federation.

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## MATERIALS AND METHODS

The study involved 94 children with biliary atresia operated on at Shumakov National Medical Research Center of Transplantology and Artificial Organs from January 2019 to May 2020. Of these children, 87 met the inclusion criteria – under 2 years of age at the time of related orthotopic liver transplantation (ROLTx), and an unburdened neurological history.

Five children older than 2 years and two children with spontaneous intracerebral hemorrhage in the preoperative period were excluded from the study. See Table 1.

Four patients died during the observation period, which was 4.16% of the total number of children in the study. Among the causes of death were multiple organ failure syndrome in 3 cases and infectious toxic shock syndrome in 1 case.

Thus, 83 children completed the study, including 36 children transplanted without prior surgery and 47 children who underwent palliative Kasai portoenterostomy at the age of 1–3 months. The distribution of children by groups is presented in Table 2.

The level of neuropsychological development was assessed in all children before surgery and 1, 3, 6 and 12 months after liver transplantation. The following scales were used for assessment: the Griffiths Scale of Psychomotor Development (translated by Keshishian E.S.) for children under 24 months [6], the Griffith Mental Development Scale (GMDS) from 2 to 8 years [7], and the Modified Autism Test for Children, Revised (M-CHAT-R, 2009 D. Robins, D. Fein, M. Barton, Russian translation: A. Steinberg, I. Shpitsberg) for children 16–30 months [8].

In accordance with the Griffiths scales, five subscales were scored: "motor skills", "social adaptation", "hearing and speech", "eyes and hands", and "ability to play". Each of the subscales was assigned a certain number of scores according to the level of development of a particular area. The sum of the scores from the five subscales reflected the child's overall development at the time of assessment. The level of development could be both age-appropriate and have abnormalities. For objective comparison of the degree of neuropsychological development of children of different ages deviating from the norm, we identified three categories: general developmental level corresponds to age, development is delayed within 3 months of the norm, development is delayed by more than 3 months of the norm. In further assessments of development at 1, 3, 6, and 12 months after ROLTx, we also used these categories to form statistical data sets.

The Modified Test for Autism in Children, Revised (M-CHAT-R) was administered to all children 16–30 months of age, according to test guidelines. Using a scoring algorithm, a final score was given, corresponding to a child's low, medium, or high risk for autism spectrum disorders.

Table 1

**Children with biliary atresia who had intracerebral hemorrhage before liver transplantation**

| | Patient 1 | Patient 2 |
|---|---|---|
| Age of hemorrhage | 3 months | 4 months |
| Type of hemorrhage | Nontraumatic intracerebral hemispheric | Traumatic intracerebral hemispheric, brain contusion, linear fracture of the left parietal bone |
| Treatment | Surgical | Surgical |
| Consequences | Spastic hemiparesis, structural epilepsy, psychomotor retardation | Spastic hemiparesis |
| Age at OLT | 9 months | Psychomotor retardation |
| Anticonvulsants at OLT | Yes | 6 months |
| OLT complications | No | Yes |

Table 2

**Characteristics of patients in group 1 (no palliative Kasai portoenterostomy) and in group 2 (with palliative Kasai portoenterostomy)**

| | Group 1 | Group 2 |
|---|---|---|
| Total | 36 | 47 |
| Male | 20 | 27 |
| Female | 16 | 20 |
| Mean age at surgery | 6.5 months | 9.1 months |
| Number of children with shoulder circumference <3 percentile | 17 (47%) | 23 (48%) |
| PELD (mean) | 23 | 28.5 |
| Preoperative average developmental level | Development delay of 1–3 months | Development delay of >3 months |
| Hepatic encephalopathy | 1 | 1 |

Statistical analysis using nonparametric data processing methods, such as Pearson's goodness-of-fit test (chi-square) and correlation coefficient was performed using the STATISTICA 12 program.

## RESULTS

All children, regardless of undergoing palliative portoenterostomy, had developmental delays at the preoperative stage of liver transplantation due to the severity of biliary cirrhosis and its complications. Before surgery, the children were underweight and had critically low shoulder circumference. Almost half of the children in each group showed decreased shoulder circumference length below the 3rd percentile, suggesting cachexia and sarcopenia. Only two children showed signs of apparent HE in the form of depressed consciousness.

After related orthotopic liver transplantation, the first assessment of psychomotor development was performed 1 month after surgery. Most children, 94% from group 1, recovered their preoperative developmental level within 2–4 weeks, whereas only 68% of children from group 2 recovered their preoperative developmental level. The level of postoperative complications was higher in the group of children who underwent palliative Kasai portoenterostomy surgery. However, this did not statistically affect the developmental outcomes of children 1 month after liver transplantation ($p < 0.05$).

Three months after liver transplantation, the increase, first of all, in motor skills was evaluated. In group 1, there was a rapid increase in children's motor skills, 80.5% of them demonstrated normal development. In group 2, only 61% achieved normal development.

Six months after transplantation, children in both groups had a slowdown in the rate of skill gain. Group 2 children showed no improvement in development, the same 61% of children fell within the normal intervals of motor and psycho speech development. In Group 1, 88.9% of the children corresponded to their age developmental rates.

At 12 months after liver transplantation, differences between the groups increased significantly. Group 1 children were confidently gaining motor and psychoretic skills, and 83.3% demonstrated normal development. Whereas Group 2 children lagged behind their peers in almost half of the cases. Only 53.2% of the children were assessed within the developmental age range.

At 12 months after liver transplantation in group 1, 6 children (16.7%) had a less than 3 months developmental delay, of which 1 child showed psycho-speech developmental delay without signs of autism, 2 children had signs of autism (scores 4 and 9 on the M-CHAT-R scale, medium and high risk, respectively), and 1 child showed non-autistic developmental regression, the remaining children had delayed speech development.

In group 2, 8 children demonstrated developmental delays of more than 3 months and 14 children had delays less than 3 months – a total of 22 children (46.8%). Of these, 7 children (14.9%) had intermediate or high risk of autism on the M-CHAT-R scale, 4 children (8.5%) had low risk of autism spectrum disorders (less than a score of 2 on the M-CHAT scale), the remaining 11 children (23.4%) exhibited different degrees of speech impairment: from delayed speech development to underdeveloped speech. Figs. 1 and 2 show the dynamics of children's development in Group 1 and Group 2, respectively.

In group 2, the number of children with developmental disorders was statistically significant compared to group 1 children who had better cognitive outcomes ($p < 0.05$).

## DISCUSSION

Most studies on the cognitive status of children with biliary atresia, survivors of Kasai portoenterostomy, sug-

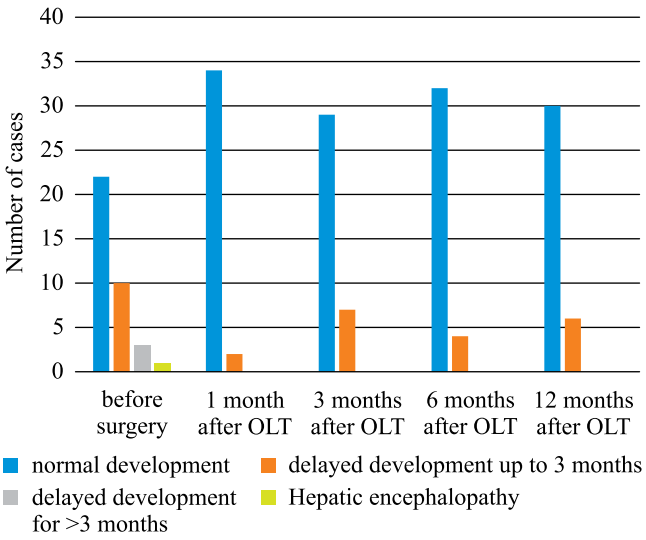


Fig. 1. Development of children, group 1

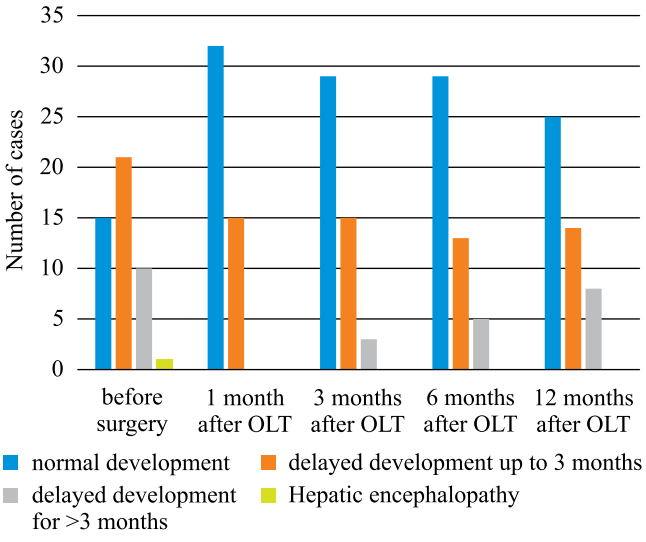


Fig. 2. Development of children, group 2

gest the existing problem of developmental disorders. At different times, the following have been claimed to be predictors of developmental disorders: duration of disease, vitamin E deficiency, protein-energy deficiency [9] [10], serum albumin and bilirubin [11], height and shoulder circumference [12]. None of the predictors have proven to be superior.

Children with biliary atresia who survived with a native liver after hepatoportoenterostomy, in the Squires et al. 2020 study involving 148 children, were at increased risk of neurodevelopmental delay at 12 and 24 months of age. Ascites was a significant predictor of developmental delay. Patients with failed portoenterostomy were more than 4 times more likely to have neuropsychiatric developmental delays than patients with successful portoenterostomy [13]. Portal hypertension was a predictor of decreased IQ in children 3–12 years old with biliary atresia who survived with a native liver. In addition, being male and having high gamma-glutamyl transferase (GGT) levels were predictors of reduced IQ on Wechsler and Wechsler-III tests [13].

Mental disorders play a major role among the consequences for children who have undergone surgical treatment for bilateral atresia. Attention deficit hyperactivity disorder in 31% [14], moderate and severe decrease in IQ 71–85 and IQ <70 in 26% and 4%, respectively [15], developmental disorders associated with sensorineural hearing loss in 5% [16], learning disability in 17%, which is almost 3 times higher than in the general population [10]. The disadvantage of such studies was the small sample size – less than 30 participants.

The spectrum of causes of mental disorders among children with biliary atresia is diverse. HE, which obviously affects the cognitive abilities of patients, is rarely seen in young children. The criteria for diagnosis are blurred in this category of patients. Whereas other causes of developmental delay come to the fore: sarcopenia, anorexia, insulin-like growth factor 1 (IGF-1) deficiency, increased proinflammatory markers and cytokines.

Malnutrition and growth insufficiency before liver transplantation are important risk factors for delayed cognitive development [10].

The brain neurotrophic factor gene is expressed in muscles, adipose tissue, and in the ventromedial nucleus of the hypothalamus and ventral tegmental region. In the brain, a brain-derived neurotrophic factor is responsible for eating behavior and adequate control of food intake. In children with liver cirrhosis resulting from biliary atresia, Wilasco et al. 2016 showed an anorexigenic effect of decreased concentration of brain neurotrophic factor [17].

IGF-1 is synthesized mainly in the liver under the influence of growth hormone. IGF-1 takes a central place in regulation of anabolic processes in the body throughout life. The blood-brain barrier is permeable to IGF1, and peripheral IGF1 enters the brain, where it binds to the receptor via a tyrosine kinase intermediate, triggering the metabolic cascade [18]. IGF1 in in vitro and in vivo studies demonstrates the effects of neuronal dendrite growth and branching, induces apoptosis [19] [20], increases glucose uptake by neurons [21], and determines postnatal neurogenesis and myelination. IGF-1 production in the liver is reduced in the background of cirrhosis [22], while the levels of pituitary somatotropic hormone in the population of children with cirrhosis were high in studies [10].

In patients with cirrhosis, HE is a manifestation of decompensation, along with bleeding from dilated esophageal veins or ascites [23]. HE pathogenesis is currently not entirely clear. It is suggested that HE development is associated with ammonia accumulation, increased circulating and tissue concentrations of tissue glutamine, which leads to cytotoxic cerebral edema and imbalance in the excitatory and inhibitory neurotransmitter systems of the brain [24] [25].

## CONCLUSION

Palliative Kasai portoenterostomy performed prior to liver transplantation, negatively affects cognitive outcomes. Children who have undergone two-stage treatment need early intervention programs to correct cognitive, communication, and speech impairments. Children who received single-stage treatment for biliary atresia have better neuropsychiatric development within a year following liver transplantation.

*The authors declare no conflict of interest.*

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# DIAGNOSTIC VALUE OF MICRORNA-27 AND -339 IN HEART TRANSPLANT RECIPIENTS WITH MYOCARDIAL FIBROSIS

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Myocardial fibrosis plays a key role in the pathogenesis of heart failure. A family of small non-coding signaling molecules, microRNAs (miRNAs), has been identified as promising profibrogenic biomarkers capable of signaling a possible risk of adverse events after heart transplantation. **Objective:** to identify and evaluate the diagnostic significance of miRNAs, as well as comprehensive miRNA-based tests in heart recipients with graft myocardial fibrosis. **Materials and Methods.** The study included 83 heart recipients aged 16 to 64 (48.4 ± 13.1) years. The expression levels of five microRNAs (miR-27, -101, -142, -339, -424) in venous blood plasma were measured by quantitative real-time polymerase chain reaction; galectin-3 serum levels were determined by enzyme immunoassay. **Results.** Morphological signs of graft myocardial fibrosis were verified in 48 recipients. The miR-27 and miR-339 expression levels were significantly higher in heart recipients with myocardial fibrosis than in those without myocardial fibrosis ($p = 0.018$ and $p = 0.043$, respectively). Diagnostically significant threshold levels of miR-27 and miR-339 for detection of myocardial fibrosis in heart transplant recipients were determined (–4.33 and –5.24 units, respectively). The relative risk of detecting graft myocardial fibrosis in recipients with miR-27 expression value above the threshold level was RR = 1.5 ± 0.157 [95% CI 1.104-2.039], $p = 0.009$; for miR-339, RR = 1.31 ± 0.130 [95% CI 1.018-1.692], $p = 0.036$. When miR-27 expression levels and galectin-3 serum levels simultaneously exceeded their estimated thresholds, the risk of transplanted heart myocardial fibrosis increased to RR = 2.7 ± 0.456 [95% CI 1.090-6.524], $p = 0.032$; when miR-339 and galectin-3 simultaneously exceeded threshold values, the risk was RR = 2.0 ± 0.316 [95% CI 1.076-3.717], $p = 0.028$). **Conclusion.** The miR-27 and miR-339 expression levels are associated with the presence of fibrotic changes in the graft myocardium. The combination of molecular-genetic and proteomic biomarkers in one test improves the diagnostic characteristics of these expressions with respect to post-transplant complications in heart recipients.

*Keywords: heart transplantation, myocardial fibrosis, microRNA-27, microRNA-339, galectin-3.*

## INTRODUCTION

Despite significant advances in heart transplantation (HTx), post-HTx recipients are at risk of developing subclinical chronic heart failure (CHF) due to graft fibrosis caused by accumulation of fibrillary collagen in the myocardium. Examination of endomyocardial biopsy specimens can detect graft pathology, but is severely limited by the invasiveness of this intervention.

In recent years, there has been an active development of minimally invasive methods for diagnosing post-transplant complications that will detect the presence of acute rejection, as well as other forms of cardiac graft pathology [1, 2].

Promising candidates for the role of such biomarkers are microRNAs – a family of small endogenous non-coding single-stranded RNAs acting as post-transcriptional gene regulators that play a key role in many biological processes [3].

To date, over 2000 microRNAs have been identified and their involvement in the functions of healthy and damaged cells has been confirmed. MicroRNAs are mostly tissue-specific and regulate expression of over 30% of genes. The diagnostic potential of evaluation of expression level of some microRNAs in blood samples of patients in relation to development and course of CHF has been shown [4]. Recent studies have shown that measuring the expression levels of certain types of microRNAs in solid organ recipients can be used to improve diagnosis of post-transplant complications, including fibrosis of heart, kidney, liver, and lung grafts [5, 6].

Our objective in this work is to identify and assess the diagnostic value of microRNAs, as well as compre-

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hensive tests based on them in heart recipients with graft myocardial fibrosis.

## MATERIALS AND METHODS

The study included 83 randomly selected patients aged between 16 and 64 years (48.4 ± 13.1), who underwent HTx between 2013 and 2018 at Shumakov National Medical Research Center of Transplantology and Artificial Organs, Moscow, of whom 64 (77.1%) were male.

According to the protocol of patient management at Shumakov Center and the National Clinical Guidelines of the Russian Transplant Society, all recipients underwent routine examinations after HTx, which included clinical evaluation of their condition, general and biochemical blood tests with determination of tacrolimus levels, and repeated myocardial biopsies.

The expression of five microRNAs (miR-27, -101, -142, -339, -424), presumably playing a role in the development of cardiac graft pathology, was examined in venous blood plasma (1 to 3 samples from each recipient, mean 1.2). Blood samples were collected in disposable tubes with anticoagulant, centrifuged and the resulting plasma was frozen at –20 °C. Total RNA was isolated from 100 μL of blood plasma using Serum Plasma kits (Qiagen, USA) with preliminary addition of $1.6 \times 10^8$ copies of synthetic cel-miR-39 microRNA (Qiagen) after plasma incubation with Qiazol phenolic mixture. Cel-miR-39 was used as an internal control for RNA isolation efficiency, complementary DNA (cDNA) synthesis and real-time polymerase chain reaction (PCR). The expression intensity was calculated by the $2^{-\Delta\Delta CT}$ method [7] and expressed in relative units equivalent to $\log_2(2^{-\Delta\Delta Ct})$, where ΔCt is the working values of the change in the product cycle relative to the internal control of cel-miR-39 microRNA expression.

Galectin-3 levels were measured by enzyme-linked immunosorbent assay using the Human Galectin-3 Platinum ELISA reagent kits (Bender MedSystems GmbH, Austria) according to instructions accompanying them.

Signs of fibrotic changes in the graft myocardium were determined by histological examination of biopsy material. Endomyocardial biopsy (EMB) in recipients was performed during routine examination or as indicated in accordance with the protocol.

The magnitude of expression was assessed using Spearman's correlation and Mann–Whitney U test to compare independent variables. Differences in the compared groups were considered significant at $p < 0.05$. Sensitivity and specificity were determined by ROC analysis. A log-rank comparison of Kaplan–Meier survival curves for non-adverse events was performed in the recipients. The relative risk ratio (RR) was used to assess the diagnostic significance. The Youden index was calculated to determine the threshold of microRNA expression [8]. Sensitivity, specificity as well as the positive predictive value (PPV) and negative predictive value (NPV) of the tests were evaluated. Data were statistically processed using Statistica v.13.0, StatSoft Inc. (USA).

## RESULTS

A series of studies on endomyocardial biopsy specimens obtained from 48 heart recipients revealed graft myocardial fibrosis.

Table 1 presents a comparative analysis of the expression levels of the investigated microRNAs in heart recipients with and without myocardial fibrosis.

When five microRNAs were examined, the expression levels of miR-27 and miR-339 were higher in patients with myocardial fibrosis than in recipients without fibrosis ($p = 0.018$ and $p = 0.043$ respectively).

The diagnostic significance of miR-27 and -339 was assessed by calculating the area under their ROC curves (AUC). Fig. 1 shows the ROC curves of miR-27 and -339 expression in heart recipients with graft myocardial fibrosis.

The area under the ROC curve for miR-27 was 0.69 ± 0.072 [95% CI 0.545–0.828] and significantly differed from 0.5 ($p = 0.010$); for miR-339, it also significantly differed from 0.5 – 0.67 ± 0.072 [95% CI 0.528–0.812] ($p = 0.019$).

The optimal combination of sensitivity and specificity values, corresponding to the highest Youden Index, determined the diagnostically significant threshold of miR-27 and miR-339 for detection of graft myocardial fibrosis (–4.33 and –5.24 relative units, respectively).

The log-rank method was used to assess the survival rate without adverse events in recipients with myocardial fibrosis and miR-27 and miR-339 expression levels above and below the calculated thresholds. Taken as

Table 1

**MicroRNA expression levels in heart recipients with and without myocardial fibrosis**

| MicroRNA | Recipients with fibrosis | Recipients without fibrosis | p |
|---|---|---|---|
| miR-27 | –5.414 [–6.430; –4.330] | –3.742 [–5.738; –1.576] | 0.018 |
| miR-101 | –7.629 [–8.732; –5.913] | –5.844 [–7.452; –4.467] | 0.105 |
| miR-142 | –6.925 [–8.297; –5.863] | –6.226 [–8.036; –5.152] | 0.409 |
| miR-339 | –9.907 [–11.603; –7.784] | –7.925 [–10.132; –3.543] | 0.043 |
| miR-424 | –6.532 [–7.779; –5.288] | –7.006 [–7.883; –5.617] | 0.579 |

adverse events were death, heart retransplantation or balloon angioplasty.

Recipients with miR-27 expression levels below the threshold were shown to have significantly higher event-free survival than those with higher expression levels (log-rank p = 0.04, Fig. 2).

There was no significant difference in survival between heart recipients with miR-339 expression above and below the threshold (log-rank p = 0.34).

The relative risk of detecting graft myocardial fibrosis in heart recipients with miR-27 expression above the threshold was RR = 1.5 + 0.157 [95% CI 1.104–2.039], p = 0.009; for recipients with miR-339 expression above threshold, the risk of myocardial fibrosis was RR = 1.31 + 0.130 [95% CI 1.018–1.692], p = 0.036.

When miR-27 and miR-339 expression levels were simultaneously high (above threshold), the risk of developing graft heart myocardial fibrosis increased 1.93-fold (RR = 1.93 + 0.245 [95% CI 1.191–3.111], p = 0.007).

Results obtained indicate that miR-27 and miR-339 expressions are associated with development of myocardial fibrosis in transplanted heart, but the practical value of the tests is limited by their lack of sensitivity (not more than 60%). Therefore, a study was undertaken to investigate the combination of microRNA and galectin-3, a proteomic biomarker with proven efficacy in myocardial fibrosis.

Serum galectin-3 levels in recipients with myocardial fibrosis were significantly higher than those in recipients without fibrosis (p = 0.009, Fig. 3).

The diagnostic significance of galectin-3 in identifying patients with fibrosis was assessed. Fig. 4 shows the ROC curve of galectin-3 levels in heart recipients with graft myocardial fibrosis.

The area under the ROC curve for galectin-3 was 0.73 ± 0.077 [95% CI 0.574–0.879] and was significantly different from 0.5 (p = 0.004).

The highest Youden index was used to determine the diagnostically significantly galectin-3 threshold with respect to detection of myocardial fibrosis in the transplanted heart, which was 21.66 ng/mL; The relative risk of detecting graft myocardial fibrosis in recipients with galectin-3 levels above the threshold was RR = 1.46 + 0.157 [95% CI 1.071–1.978] (p = 0.016) with sensitivity and specificity of 60.9% and 78.6% respectively.

A comparative analysis of the survival of heart recipients with galectin-3 levels above and below the calculated thresholds showed a significant difference (Fig. 5).

In the group of patients with galectin-3 levels below 21.66 ng/mL, event-free survival was significantly higher than in recipients with galectin-3 levels higher than that (log-rank p = 0.003).

Evaluation of the diagnostic characteristics of tests involving combined measurement of miR-27 expression and galectin-3 levels showed the following results: with simultaneous expression level of miR-27 and galectin-3 levels above the calculated thresholds, the risk of myocardial fibrosis in the transplanted heart increases up to 2.7-fold (RR = 2.67 + 0.456 [95% CI 1.090–6.524], p = 0.032 (Fig. 6).

When miR-339 expression levels and serum galectin-3 levels exceeded their threshold at the same time, the risk of developing myocardial fibrosis increased by up to 2-fold (RR = 2.00 + 0.316 [95% CI 1.076–3.717], p = 0.028) as compared to individual tests.

Table 2 presents the main diagnostic characteristics of miR-27, miR-339, galectin-3 and their combinations in relation to development of myocardial fibrosis in the transplanted heart.

The combined measurement of miR-27 expression levels and serum galectin-3 levels in heart recipients has the best diagnostic characteristics in detecting myocardial fibrosis in transplanted hearts.

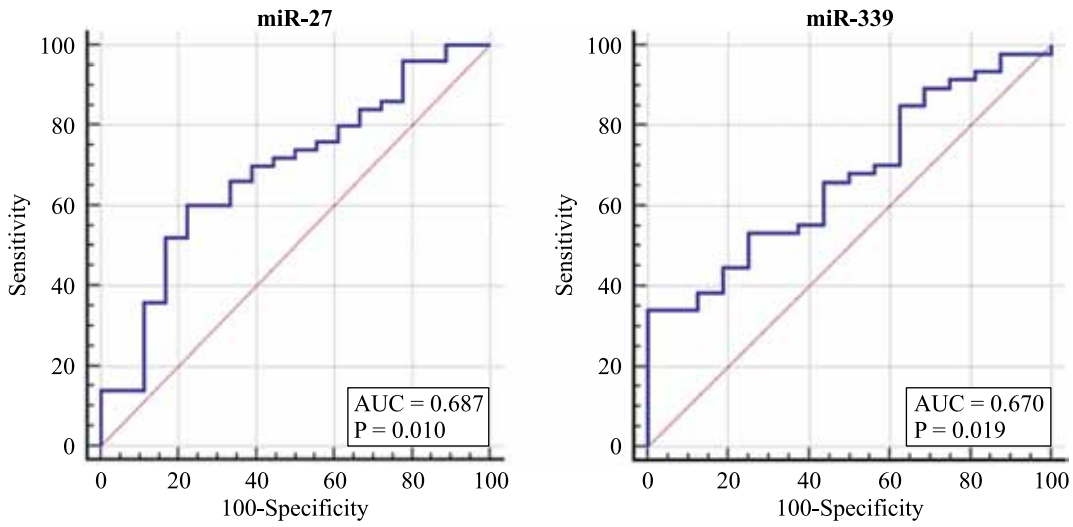


Fig. 1. ROC curves of miR-27 and miR-339 in heart recipients with graft myocardial fibrosis

## DISCUSSION

Myocardial fibrosis plays an important role in the development of subclinical post-transplant heart failure. The formation of fibrous tissue in the intercellular space promotes structural and functional graft remodelling. Pathological factors such as arterial hypertension, acute

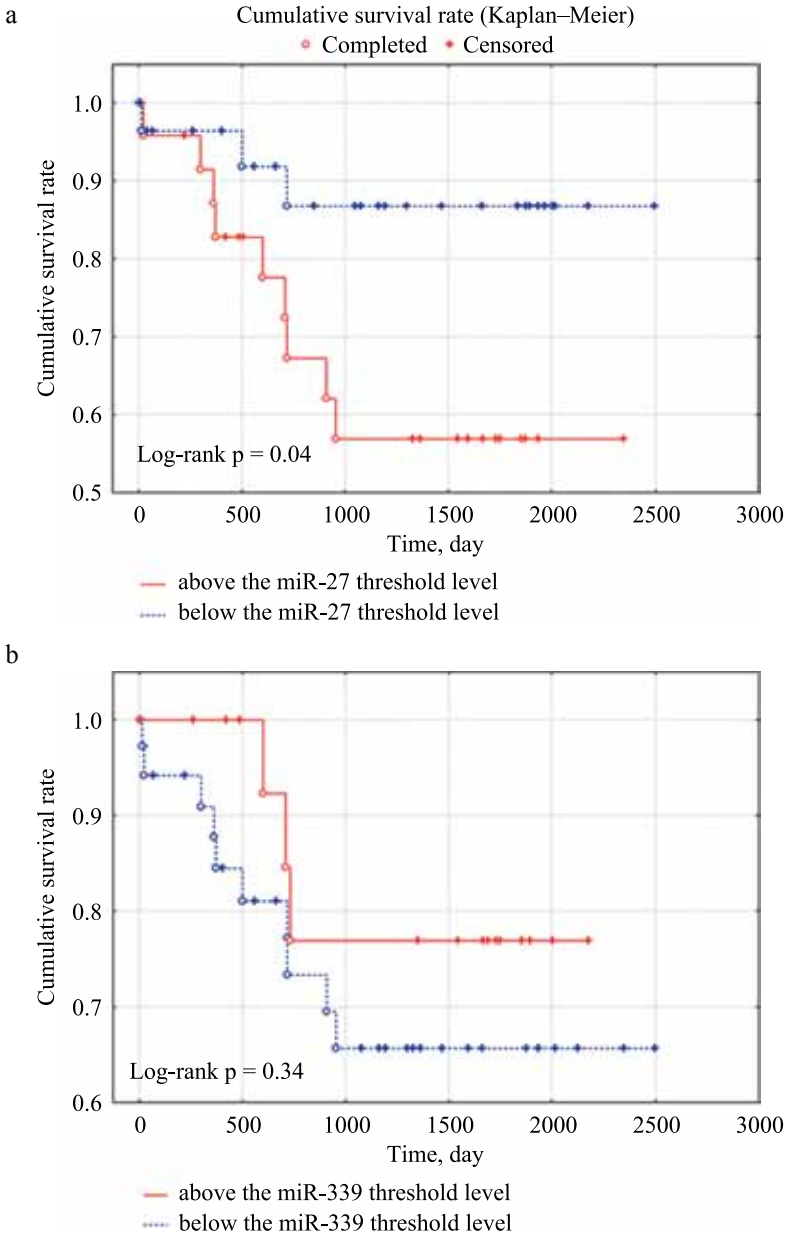


Fig. 2. Survival curves without adverse events in heart recipients as a function of miR-27 (a) and miR-339 (b) expression levels

Table 2

**Diagnostic characteristics of miR-27, miR-339, and galectin-3 in relation to detection of myocardial fibrosis in heart recipients at levels above thresholds**

| Tests | Sensitivity | Specificity | PPV | NPV |
|---|---|---|---|---|
| miR-27 | 60.0% | 77.8% | 88.2% | 41.2% |
| miR-339 | 36.2% | 87.5% | 89.5% | 31.8% |
| galectin-3 | 60.9% | 78.6% | 90.3% | 37.9% |
| miR-27 + miR-339 | 52.4% | 91.7% | 91.7% | 52.4% |
| miR-27 + galectin-3 | 66.7% | 100.0% | 100.0% | 62.5% |
| miR-339 + galectin-3 | 54.5% | 100.0% | 100.0% | 50.0% |

PPV – positive predictive value of the test; NPV – negative predictive value of the test.

rejection and graft vasculopathy lead to myocardial fibrosis in the transplanted heart [9, 10].

Myocardial fibrosis is verified by morphological analysis of myocardial tissue obtained during EMB, and its quantitative characterisation is performed by determining the collagen formation index – collagen volume fraction (CVF). CVF makes it possible to estimate the content and ratio of type I and type III collagen in the myocardium. However, the diagnostic value of this analysis can be significantly limited by possible errors when taking the examined biopsy material due to uneven distribution of collagen in tissues [11].

Over the past decade, the understanding of how fibrosis develops has significantly expanded. Fibroblasts play a key role in maintenance of extracellular matrix, they also regulate collagen synthesis and degradation. Transforming growth factor TGF-β1 and angiotensin II are the best known profibrogenic factors. Response to the action of angiotensin is manifested in the form of expression of galectin-3, an active stimulator of fibroblast proliferation. Similar stimulation of fibroblasts is observed in the activation of the immune system caused by oxidative stress or due to mechanical damage to the atria [12].

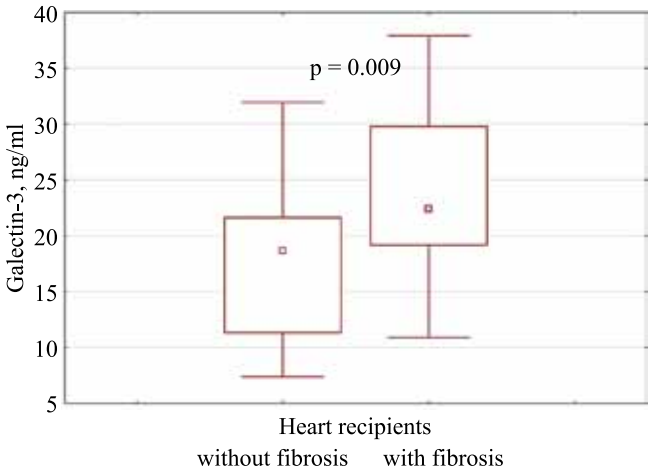


Fig. 3. Galectin-3 serum concentrations in heart recipients with and without myocardial fibrosis

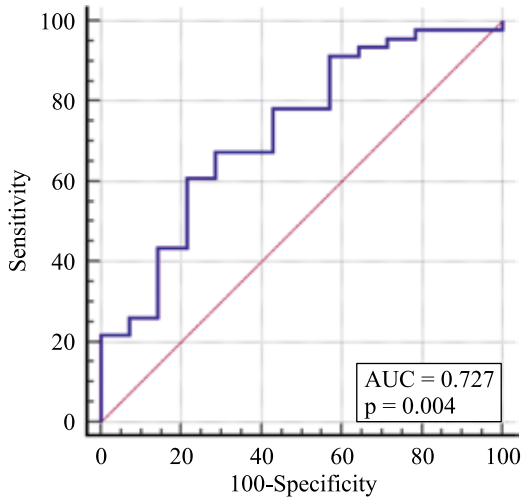


Fig. 4. ROC curve of galectin-3 serum concentrations in heart recipients with myocardial fibrosis

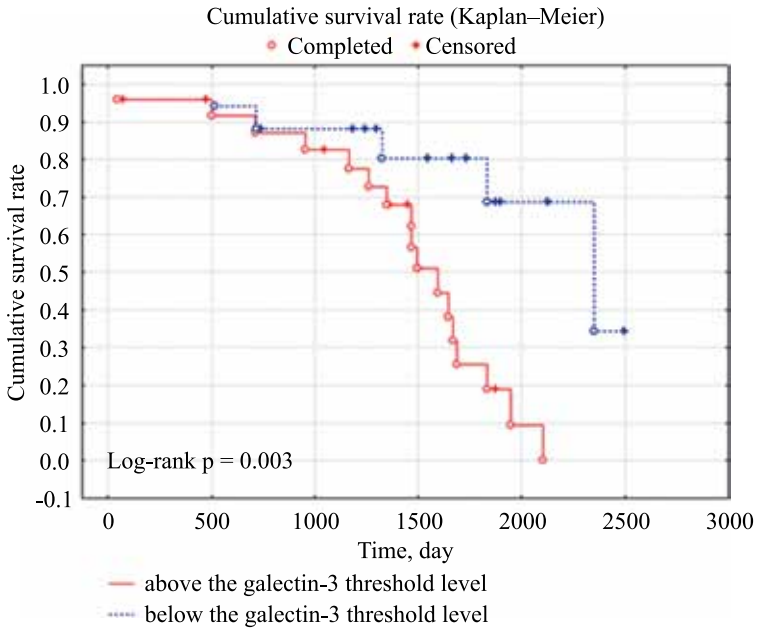


Fig. 5. Survival curves without adverse events in heart recipients with galectin-3 serum concentrations above and below threshold levels

Circulating microRNAs play an important role in heart remodelling and other important biological processes. Huang YM et al. evaluated the diagnostic potential of a number of microRNAs in relation to the development and course of heart failure in patients with coronary heart disease and dilated cardiomyopathy [4].

The function of miRNA molecules is associated with regulation of gene expression, namely with their inhibitory effect on RNA at the level of transcription processes. The regulatory role of miR-30 and -133a in myocardial fibrosis mechanisms through inhibition of connective tissue growth factor has been shown [13]. In experiments on mice, it was found that increased miR-133 expression leads to decreased collagen synthesis, and consequently decreased myocardial fibrosis, whereas absence of miR-133 is associated with high susceptibility to heart failure (HF) and fibrosis. In addition, miR-21 is involved in regulation through one of the profibrotic pathways and has a protective effect against oxidative stress. Finally, miR-29 is associated with deposition of collagen types I and III. Increased miR-29 leads to decreased synthesis of these proteins and vice versa.

Our findings with respect to the association of miR-27 and miR-339 with fibrosis are consistent with reports from foreign authors. There is evidence of the inhibitory effect of miR-27 and miR-101 on the development of fibrotic processes in heart and other organs [14–16]. A number of studies have noted the involvement of miR-27 as an inhibitor of myocardial inflammatory responses [17], and that miR-27b, as a member of microRNA-27 family, can play antifibrotic role in left atrium and be considered as a novel therapeutic target for heart failure. The mechanism of antifibrotic action of miR-27 is attributed to its inhibitory effect on TGF-β [18]. Meanwhile, there is little evidence on the profibrotic effect of miR-27 through inhibition of the synthesis of transcriptional protein FBW7 [19]. It was found that miR-27 is important in the development of atherosclerosis: by suppressing lipoprotein lipase-induced lipid accumulation and inflammatory response, miR-27 reduces progression of atherosclerosis [20]. Cruz L.O. et al. reported that miR-27 can disrupt Treg cell differentiation, thereby reducing Treg-mediated immunological tolerance [21]. Along with that, recent studies have shown that miR-339 is involved as an inhibitor of cell proliferation [22, 23]. In coronary heart disease, miR-339 is able to increase oxidative stress by inhibiting the Nrf2/FOXO3 signaling pathway via specific protein Sirt2 [24]. A study by Chen J. et al. showed that miR-339 suppresses pulmonary artery smooth muscle cell proliferation through inhibition of FGF signaling pathway [25]. Our results suggest that heart recipients with the development of myocardial fibrotic processes have significantly higher expression levels of miR-27 and -339.

Another new biomarker for the development of severe heart failure is galectin-3, a member of the lectin family. It plays an important role in regulation of inflammation, immune response and nerve tissue degeneration, and has also been identified as a profibrogenic factor. Galectin-3 is secreted into the extracellular space at the site of injury and activates previously resting fibroblasts [26]. Coromilas et al. found that increased galectin-3 levels were associated with development of heart failure, whereas lower galectin-3 levels were associated with lower severity of the disease [27]. The prognostic significance of galectin-3 in the risk of adverse events in patients with heart failure has thus been demonstrated.

We have previously shown the diagnostic potential of galectin-3 in relation to myocardial fibrosis in a transplanted heart [28, 29]. In the present study, we have evaluated variants of tests of its combination with microRNAs miR-27 and miR-339, potentially significant in the development of heart failure [29].

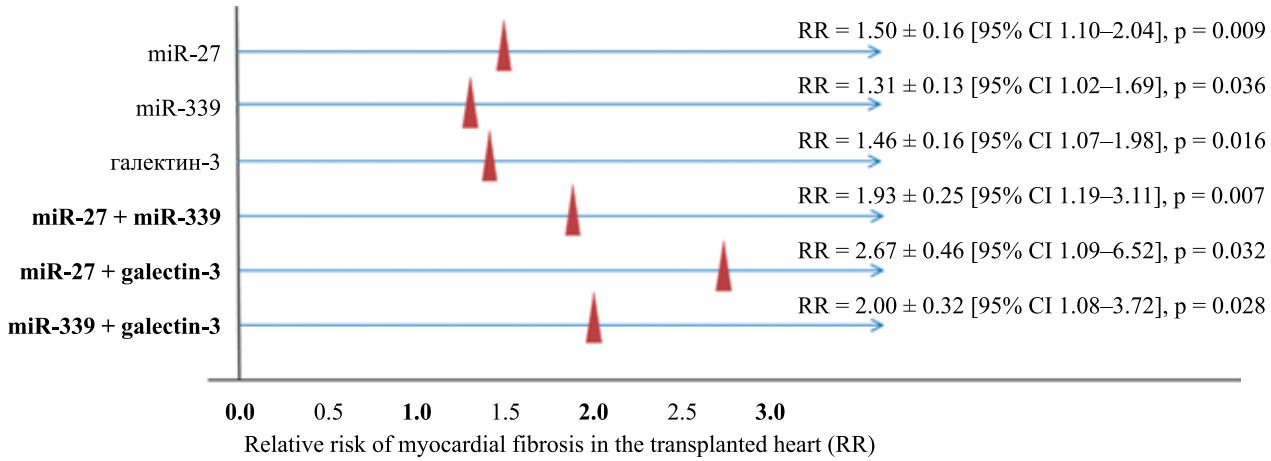


Fig. 6. Relative risks of myocardial fibrosis in heart recipients with miR-27, miR-339 expression levels and galectin-3 serum concentrations above threshold levels, and when they are determined together

The best diagnostic characteristics in detecting myocardial fibrosis in transplanted hearts was found to be the combined test for miR-27 expression level and serum galectin-3 levels in heart recipients.

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*The authors declare no conflict of interest.*

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# EVALUATION OF THE EFFICACY OF A NOVEL PERFUSION SOLUTION FOR NORMOTHERMIC *EX VIVO* LUNG PERFUSION COMPARED WITH STEEN SOLUTION™ (ANIMAL EXPERIMENTAL STUDY)

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Respiratory diseases, together with infectious complications and hereditary lung diseases, rank third in international mortality statistics. Today, lung transplantation is a recognized method of treating end-stage lung diseases. However, the number of transplant surgeries performed is not much. This is down to the high requirements on the condition of a potential lung donor and directly on the quality of the donor lung. This has significantly limited the number of optimal donors. Rehabilitation of donor lungs to optimal gas exchange indicators can be achieved and objectively assessed in the course of *ex vivo* lung perfusion (EVLP). The EVLP procedure is widespread in leading transplantation centers in Europe and North America. It allows to significantly expand the pool of donor lungs, thereby serving a greater number of patients in need of lung transplantation. The possibility of EVLP procedure using publicly available perfusion equipment was demonstrated. The optimized protocol fully demonstrated its reliability and efficiency. The developed perfusion solution had no statistically significant differences in comparison with the Steen SolutionTM, which in the future will serve as an alternative for EVLP procedure.

*Keywords: lung transplantation, donation, ex vivo lung perfusion.*

## INTRODUCTION

Respiratory diseases, together with infectious complications and hereditary lung diseases, rank third in international mortality statistics. Progression of respiratory diseases often confronts physicians and patients with the need for lung transplantation. Chronic obstructive pulmonary disease (COPD), cystic fibrosis, interstitial pulmonary fibrosis, primary pulmonary hypertension, and mucoviscidosis are the most frequent indications for lung transplantation, and the effectiveness of transplantation in malignant tumors is reported [1]. According to reports from the World Health Organization, about 3,973 lung transplantations are performed annually [1]. In the Russian Federation, 164 lung transplantations were performed from 2009 to 2019. The small number of lung transplants is due to the complexity of the surgical intervention and, to a greater extent, to the high requirements imposed on the condition of the potential lung donor and directly on the quality of the donor organ. This ultimately leads to significant limitations in the number of optimal donors. In order to provide the majority of patients in need of lung transplantation, the possibility of using lungs from suboptimal donors is being considered [4]. The main requirement for lungs from suboptimal donors is having the functional capacity to provide sufficient oxygenation of the recipient's blood [11]. Rehabilitation of donor lungs to the level of optimal values of gas exchange parameters can be achieved and objectively assessed during ex vivo lung perfusion (EVLP) [10, 12]. EVLP is widely used in leading transplant centers of Europe and North America, it allows to significantly expand the pool of donor lungs and thus provide more patients in need of lung transplantation [2, 4]. The first prospective, non-randomized clinical trial of EVLP efficacy was the HELP study, published in 2011. A team led by Cypel (Toronto, Canada) performed 4-hour extracorporeal perfusion of 23 donor lungs. The donor lungs in the study group were classified as high-risk with $PaO_2/FiO_2$ values <300 mmHg. In 20 cases, EVLP achieved satisfactory gas exchange and oxygenation, which allowed to transplant the rehabilitated lungs. When compared with the control group of recipients who underwent optimal donor lung transplantation during the same period, there were no differences in 30-day mortality, duration of postope-

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rative ventilation, bronchial complications, or length of stay in the intensive care unit [3].

Thus, today EVLP can be of great interest as an effective way to increase the number of lung transplants through rehabilitation of suboptimal donor organs.

Taking into account the necessity of wide implementation and active use of this technique within the framework of national clinical transplantology, it seems advisable to study and master it in experimental conditions on big animals.

**Objective of the study:** to evaluate the possibility of using ex vivo donor lung perfusion in experiment using routine components of extracorporeal perfusion, artificial ventilation, and invasive monitoring on a biological lung model of a large experimental animal (ram); to evaluate the effectiveness of our own perfusion solution in an EVLP experiment.

## MATERIALS AND METHODS

Male Romanov rams weighing 50 kg were used in the experiments. The experiment program was approved by the biosafety and bioethics committee. The experiments were performed in compliance with the rules of the European Convention for the Treatment of Laboratory Animals and the 2010/63/EU Directive.

The study design involved two series of experiments: Group 1 (control) consisted of lungs of rams perfused with Steen Solution TM (n = 5), Group 2 (experiment) included lungs of rams perfused with our own solution (n = 5). Functional capacity of the lungs was assessed according to the following parameters: oxygenation index, dynamic compliance, pulmonary vascular resistance, histological changes.

The experiment included stages of donor anesthesia, lung explantation, static hypothermic storage for 4 hours, EVLP initiation. The rams were kept in standard pens with adlibitum water supply under a 12-hour day-night cycle.

*Donor anesthesia stage:* on the day of the experiment, the animal was sedated in the pen 60 minutes before surgery with Zoletil 100™ solution at a 15 mg/kg dose. During sedation, the animal was taken to the operating room and the surgical field and vascular access points were shaved. The animal was positioned on the operating table in the supine position and ECG monitoring was connected in standard leads. Aseptic catheterization of the external jugular vein with a 7 Fr dual-lumen central catheter and the common carotid artery with a 5 Fr catheter was performed for invasive arterial pressure (AP) monitoring. Hemodynamics were monitored via a Philips™ monitoring system. After setting vascular access and providing invasive AP monitoring, central venous pressure (CVP) monitoring, intravenous premedication was performed: lornoxicam 8 mg, metoclopromide 10 mg, chloropyramine 20 mg, infusion of 4.2% sodium bicarbonate solution 200 ml at 200 ml/hr, infusion of Sterofundin isotonic solution at 100 ml/hr. Anaesthetic induction: atropine 1 mg intravenously, methylprednisolone 500 mg, Zoletil 100™ 10 mg/kg, tracheal intubation with an 8.0 intubation tube. Mechanical ventilation was performed using Draeger Fabius plus anesthetic breathing apparatus in the volume control mode at 8–10 ml/kg, peak inspiratory pressure did not exceed 25 cm $H_2O$, positive end expiratory pressure did not exceed 5 cm $H_2O$, breathing rate was 20 d/min, anesthetic depth was controlled using isoflurane vaporizer, optimum anesthesticization for explantation surgery was achieved at 3 vol% vaporizer setting. To maintain hemodynamics, a 160 ng/kg norepinephrine solution was infused via a syringe dispenser.

*Donor lung procurement.* Surgical access through median sternotomy. The pericardium was opened longitudinally, and the aorta and pulmonary artery were divided bluntly. After administering sodium heparin at a 300 units/kg dose, purse-string sutures were applied on the aorta and the pulmonary artery. The aorta was cannulated with a 7 Fr catheter to collect donor blood. The pulmonary artery was cannulated with a 20 Fr cannula. The first stage was preparation of the animal's autologous blood into a hemocontainer with citrate preservative. Erythrocyte mass was obtained by centrifugation of whole blood purified from leukocytes. Upon completion of blood banking, prostaglandin E1 solution ("Vasoprostane", Bayer Schering Pharma, Germany) was injected into the pulmonary artery in a 20 μg dose. Before preservation was started, mechanical ventilation parameters were changed with a 4–5 ml/kg tidal volume. In order to decompress pulmonary circulation, the left atrial appendage was widely dissected, then 1 liter of cold 0.9% NaCl 4 °C with addition of 25,000 units of sodium heparin was injected anterogradely through the pulmonary artery. Preservation was performed with 2 liters of Celsiore™ 4 °C solution. After the perfusion of the preservation solution was completed, the lungs were explanted. For ease of removal, the donor heart was removed while maintaining the maximum length of the pulmonary artery to connect it to the EVLP circuit. Intubation tube #8 was inserted into the trachea at the middle third level, followed by fixation with a synthetic tape. Above the tracheal intubation site, extracorporeal suture was applied. After tracheal intubation, several recruitment maneuvers were performed with subsequent clamping of the intubation tube with a main clamp in order to keep the lungs in a flattened state. Upon completion of explantation, the lungs were placed in a sterile plastic bag filled with 500 ml Celsior preservative solution (manufactured by Ganzyme, France). The bag was hermetically sealed and placed in a thermocontainer for static cold storage for 4 hours.

*EVLP perfusion circuit assembly included* $^1/_4$ size trunk lines connected to a cardiotome reservoir and a Terumo Corp. membrane oxygenator CapioxRX15™.

A hydrocirculatory heat exchange element, a deoxygenating mixture containing 86% $N_2$, 8% $CO_2$, 6% $O_2$, and an oxygen-air mixture were connected to the oxygenator. A Stockert Sorin 5™ circulatory machine roller pump was installed in the piping system between the cardiotomy reservoir and the oxygenator. The tubing after the oxygenator was connected to a cannula installed in the pulmonary artery. Pressure in the trunk system was measured by installing two invasive sensors: the first was installed after the oxygenator to measure pressure in the proximal part of the perfusion circuit, the second was directly in the pulmonary artery cannula to measure perfusion pressure in the pulmonary artery. Upon completion of the prescribed cold preservation period, the graft was removed from the sterile bag and placed in a prone position in a sterile container equipped with a recess with a drainage hole communicating with the cardiotomy reservoir. Perfusion of the graft was performed through the pulmonary artery; perfusate was drained passively through the pulmonary veins. Perfusate was collected by gravity into the cardiotomy reservoir.

The EVLP circuit diagram is shown in Fig. 1.

In the control group, Steen Solution™ with the addition of red blood cell mass was used as perfusion solution, in the study group – our own solution with the addition of albumin and red blood cell mass in specified proportions. The volume of perfusate in both groups was 2 liters. Erythrocytic mass was harvested by centrifugation of whole leukoreduced auto blood at 3500 rpm for 15 minutes. The following were added to the perfusate: cefepime 1000 mg, methylprednisolone 1000 mg, insulin 4 units, and glucose 40% 5 ml. Target hemoglobin level was maintained at 30 g/L, hematocrit index was 10%–15% in both groups.

The maximum perfusion duration did not exceed 180 minutes.

*EVLP initiation:* The initial temperature was 15 °C, target pulmonary artery pressure was 4–10 mmHg. Perfusion rate was adjusted based on pulmonary artery pressure, at the beginning of perfusion it was 150–200 ml/min. Gas-air mixture flow where $FiO_2$ <0.5 was set, corresponding to target minimum $pO_2$ values >100 mmHg. A deoxygenating mixture was required to achieve gas homeostasis, where $pCO_2$ 40 to 50 mmHg, the flow rate corresponded to a 1:1 perfusion rate. The ionic and gas composition of the perfusion solution was monitored using an ABL 800™ gas analyzer. When all parameters were stabilized, the perfusion rate was gradually increased to 1200 ml/min for 20 minutes, and the perfusate was warmed to 32 °C. After the temperature reached 340 °C, mechanical ventilation (MV) was started. The MV parameters were protective in nature and consisted of tidal volume of 6 to 8 ml/kg, positive-end expiratory pressure (PEEP) of 5 cm $H_2O$, respiratory rate (RR) – 16/min. The main goal of protective MV was to optimize the volume and pressure in the airways, while avoiding damage to atelectasized sections of the alveoli. Recruitment maneuver was performed not more frequently than once every 10 minutes to prevent ventilator-associated graft damage of the graft. Respiratory oxygen fraction did not exceed 50% ($FiO_2$ <0.5). Perfusate gas composition was monitored at a preset frequency. After reaching the target temperature of 37 °C for 20 minutes, the volumetric perfusion rate was increased to 100% of cardiac

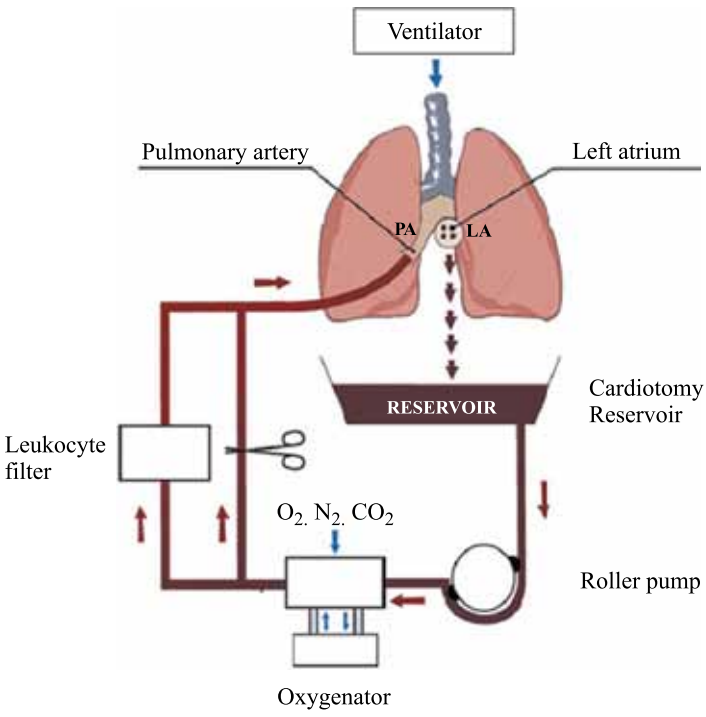


Fig. 1. Diagram of EVLP open circuit

output – 1700–1900 ml/min. Perfusion lasted no more than 180 minutes.

## Graft assessment after EVLP procedure

After the graft was warmed up to 37 °C and parameters of gas and ionic perfusate composition stabilized, oxygen fraction on inspiration decreased to 21% ($FiO_2$ = 0.21), clinical, imaging and laboratory assessment of gas composition was performed. The surgeon palpated and visually assessed the homogeneity of the parenchyma and the absence of lung infiltrative changes. X-ray examination was performed to assess residual atelectasis and pulmonary parenchymal edema. Bronchoscopy was performed to sanitize the tracheobronchial tree and identify indirect signs of pulmonary edema. Gas and ionic composition of perfusate was assessed every 15 minutes. At the end of the EVLP procedure, biopsy specimens were taken for morphological study.

## RESULTS

In both groups, the mean $PaO_2/FiO_2$ value at the time of explantation was 220 ± 25 mmHg.

$PaO_2$ values were similar in both groups before donor lung explantation (control: 220 ± 20.25 mmHg vs. experiment: 230 ± 10.20 mmHg, P = 0.606). Similarly, there were no statistically significant differences in $PaO_2$ values after lung perfusion 350 ± 20.53 mmHg, P = 0.348). Both groups showed significant improvement in lung function after EVLP procedure (Fig. 2).

Both groups demonstrated significant improvement in dynamic compliance in perfusion compared with baseline (P < 0.0001). The dynamics of the studied index in the comparison groups were comparable. However, after three hours, the final compliance in the experimental group tended to be higher than in the control group (Experimental 38.8, Control 29.3, P = 0.22) (Fig. 3).

According to perfusion results, both groups demonstrated decrease in pulmonary vascular resistance (PVR). There was no statistically significant difference between the experimental and control groups (P = 0.39) (Fig. 4).

## Morphological examination data

In both groups, histological examination showed no significant differences and structural damage to the pulmonary parenchyma (Fig. 5–6).

In both groups, the lung parenchyma had histological signs of functioning tissue without pathological changes. Well swollen alveoli were noted in most sections. Microatelectatic areas were distributed non-uniformly in both groups and were found only in some sections.

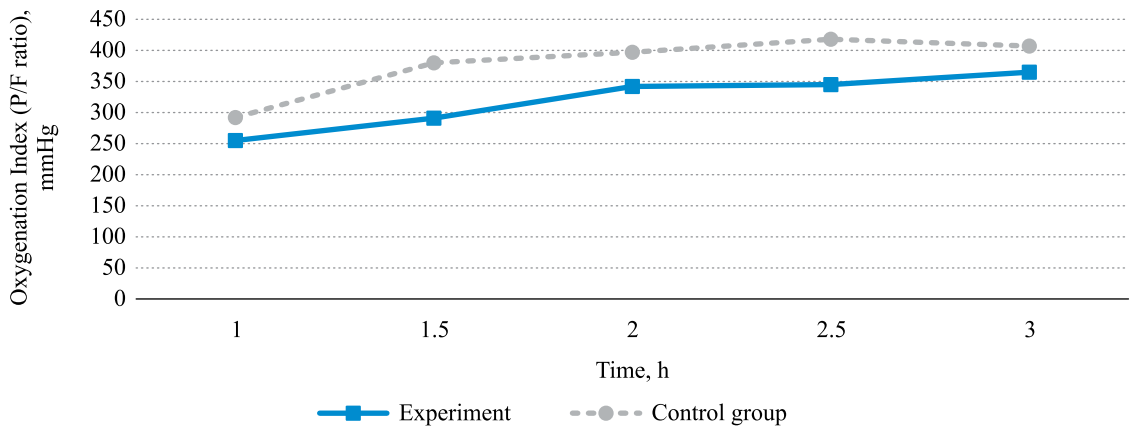


Fig. 2. Oxygenation Index

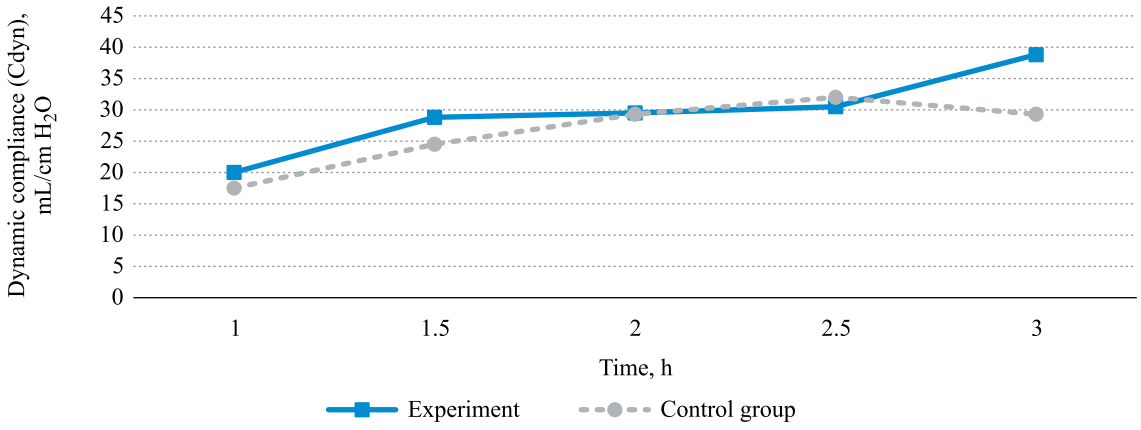


Fig. 3. Dynamic compliance

## DISCUSSION

Over the years, lung transplantation has established itself as a radical and effective treatment for patients with end-stage respiratory diseases [13, 14]. Despite the development of technology and improvements in immunosuppressive therapy protocols, long-term survival rates in lung transplant recipients remain the lowest among all solid organ recipients [12, 15]. The reason is high susceptibility of transplanted lungs to the influence of both external and internal negative factors [9]. The same circumstance is also relevant for lungs of a potential donor at the conditioning stage. Strict ventilation modes, contamination by hospital flora, consequences of ineffective hemodynamics, aggressive cardiorespiratory resuscitation and other circumstances lead to donor lung damage. As a result, about 70% of potential lung transplants are rejected due to their unsuitability, and only 20–30% are considered suitable for transplantation [4, 8, 16]. The emergence of a procedure for normothermic perfusion of donor lungs ex vivo has opened up new horizons in the development of lung transplantation worldwide [17, 18, 23]. In 2006, Stig Steen et al. (Lund University Hospital, Sweden) reported the first results of successful single lung transplantation after EVLP procedure. In 2009, Cypel et al. (Toronto, Canada) presented their own protocol, which later became the most physiological and successful, allowing long-term perfusion over 12 hours [5, 15, 22]. The EVLP procedure allowed to increase the pool of donor lungs several times, thereby increasing the number of transplants [4, 17].

Several patented perfusion systems have been developed, including XVIVO perfusion system (XVIVO Perfusion AB, Gothenburg, Sweden), Vivoline LS1 system (Vivoline Medical, Lund, Sweden) and organ care system (OCS lung) (Transmedics, Andover, Mass, USA) [6, 17, 18]. XVIVO perfusion system uses Steen cell-free solution (Vitrolife, Gothenburg, Sweden), and Vivoline system uses a mixture of Steen solution and erythrocyte mass. OCS Lung uses a combination of OCS Lung Solution and erythrocyte suspension [7, 14, 18]. Steen Solution is a solution containing dextran and albumin that mimics extracellular electrolyte concentrations [ 8, 14, 15]. OCS Lung Solution is a similar buffer solution containing dextran instead of albumin [5, 8, 20, 21].

Our study was based on the perfusion protocol developed by Steen et al. in Sweden for lung assessment

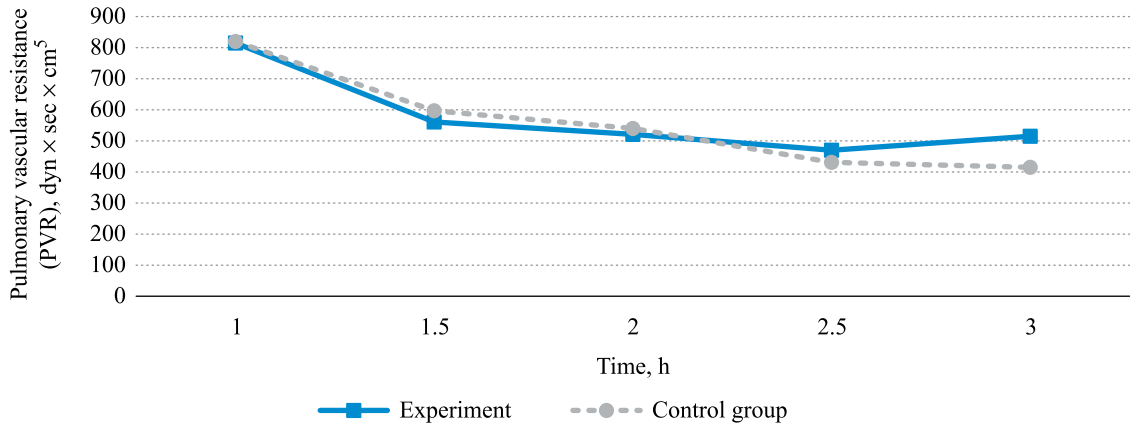


Fig. 4. Dynamics of changes in pulmonary vascular resistance

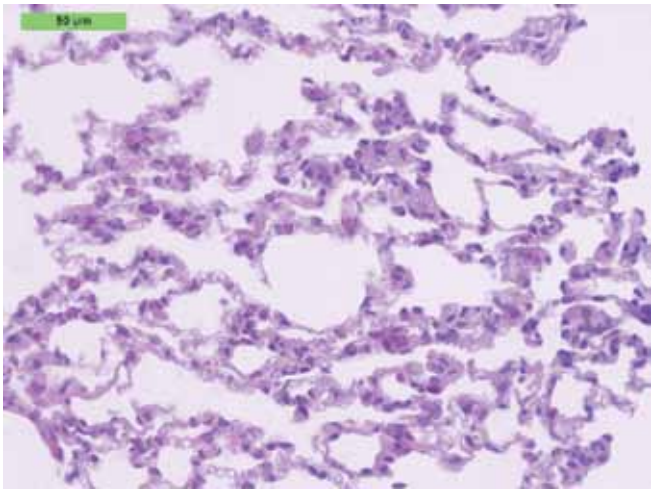

Fig. 5. Experiment

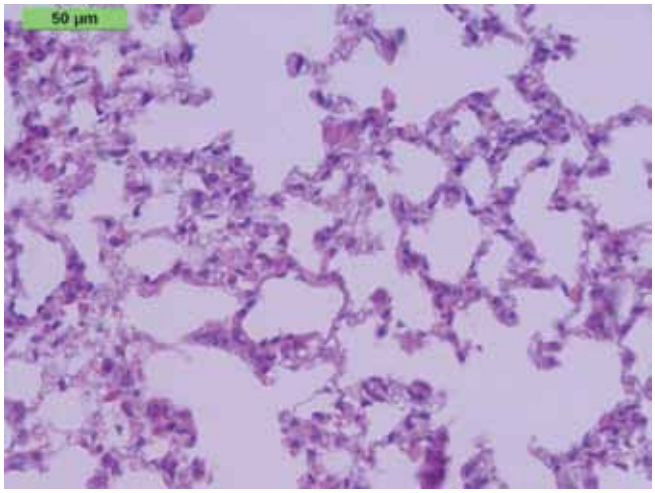


Fig. 6. Control

and rehabilitation. The study managed to demonstrate feasibility of the procedure and reproducibility of EVLP results in an experiment on ram model using routine technical components widely used in specialized medical institutions. The experimental conditions and the adapted perfusion protocol were as close to clinical practice as possible. Evaluation of the efficacy of the proprietary perfusion solution was performed within the standard ex vivo perfusion protocol undertaken in both groups. The perfusion solution, Steen Solution™, considered the gold standard in EVLP procedures worldwide, was used as a control. The results obtained in both groups show no statistically significant differences. The lungs of rams in the comparison groups were similar in functional and histological characteristics, and therefore the differences found after perfusion completion can be attributed to the lung preservation quality. For histological examination, a score based on semi-quantitative analysis of the changes observed in conventional light microscopy was used. The comparison groups showed the same degree of tissue injury after cold ischemia and at the end of perfusion. This may indicate that the developed solution is effective when compared with that of Steen Solution™.

## CONCLUSION

Results of our pilot study demonstrate that performing the EVLP procedure using commonly available perfusion equipment is feasible. The optimized protocol fully demonstrated its reliability and efficiency. The developed perfusion solution had no statistically significant difference with Steen Solution™ solution. This will allow it to be used as an alternative for EVLP procedure in the future.

*The authors declare no conflict of interest.*

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# COMPARATIVE STUDY OF CHONDROGENESIS OF HUMAN ADIPOSE-DERIVED MESENCHYMAL STEM CELLS WHEN CULTURED IN COLLAGEN-CONTAINING MEDIA UNDER *IN VITRO* CONDITIONS

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In terms of method of production, collagen carriers are subdivided into materials obtained on the basis of extracellular matrix (ECM) components, particularly collagen-containing hydrogels and decellularized tissue. **Objective:** to compare in vitro the ability of biopolymer microheterogeneous collagen-containing hydrogel (BMCH) and tissue-specific matrix from decellularized porcine articular cartilage (DPAC) to support adhesion, proliferation and chondrogenic differentiation of human adipose-derived mesenchymal stem cells (hAMSCs). **Materials and methods.** For cartilage decellularization, we carried out treatment with surfactants (sodium dodecyl sulfate, Triton X-100) followed by exposure in DNAase. The metabolic activity of hAMSCs was assessed by PrestoBlue™ (Invitrogen, USA) staining. The morphological study of cell-engineered constructs (CECs) formed by culturing hAMSCs in the presence of matrices was performed using histological staining and scanning electron microscopy (SEM) with lanthanide contrasting. **Results.** The number of cells on the surface of both BMCH and DPAC increased within 14 days. Mitochondrial activity of the cells was 1.7, 1.7, and 1.3 times higher on days 3, 10, and 14 when cultured on DPAC compared to BMCH, respectively. On day 14 of cultivation in the chondrogenic culture medium, hAMSCs formed cell layers on the DPAC surface and on the BMCH surface. Cytoplasm of the cells included numerous granules, which, when stained, resembled the matrix itself. On the DPAC matrix surface, cells were more evenly distributed, whereas in the case of BMCH, cell adhesion and proliferation were observed only in certain areas. The ECM produced by the cells contained collagen and glycosaminoglycans (GAGs). **Conclusion.** The ability of DPAC obtained according to the developed protocol to form CECs with hAMSCs with uniform distribution of cells and their production of specific collagen- and GAG-containing ECM suggests that DPAC is effective in regeneration of damaged cartilage. Chondrogenic differentiation of hAMSCs was observed both when cultured with BMCH and with DPAC. When creating a tissue equivalent of cartilage in vitro, the advantage of using tissue-specific matrix over BMCH should be considered.

*Keywords: cartilage tissue, chondrogenic differentiation, mesenchymal stem cells, decellularized matrix, tissue engineering.*

## INTRODUCTION

Articular cartilage is an avascular dense tissue with limited self-regeneration capacity, thus indicating the feasibility of developing therapeutic approaches to its repair using cell therapy and tissue engineering [1].

Today, autologous chondrocyte transplantation is the gold standard cell therapy for joint diseases. However, this method has a number of disadvantages, which primarily include traumatic biopsy and high probability of chondrocyte dedifferentiation during expansion [2]. Mesenchymal stem cells (MSCs) are considered as an alternative to autologous chondrocytes. By their nature, these cells are capable of directed differentiation into various mesenchymal tissues, including the cartilage, and have immunomodulatory properties. Note that due to its high chondrogenic potential, ease of isolation and minimal traumaticity, human adipose-derived tissue is seen as a promising source of MSCs for cartilage tissue engineering [3, 4].

To increase the efficiency of cell therapy of affected joints, MSCs are injected into the intra-articular capsule on biodegradable carriers – extracellular matrix (ECM) mimetics, which help maintain the viability and chondrogenic differentiation of MSCs [5].

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Collagen, the main structural protein of natural ECM cartilage, is used in most biomedical cell products for the treatment of cartilage defects [6]. Among Russian innovations, we can single out a biopolymer microheterogeneous collagen-containing hydrogel (BMCH) from the linear series of implantable heterogeneous gel composition *Sfero*®GEL (Biomir Service, Russia). The efficacy of using BMKG as a matrix in biomedical cellular products for regeneration of damaged articular cartilage, liver and pancreas, has been proven [7, 8]. From our point of view, of great interest for cartilage tissue engineering are decellularized carriers – biological ECM mimetics, obtained by removing cells and their fragments from tissue with maximum preservation of the structure and composition of natural ECM [9, 10]. It was shown that decellularized cartilage (DC) not only supports cell adhesion and proliferation, but also stimulates MSCs differentiation into chondrocytes [11–13]. A comparative assessment of the abilities of the obtained BMCH and DC to support adhesion, proliferation and chondrogenic differentiation of MSCs in vitro will make it possible to select a matrix that has the greatest potential for use in regenerative medicine to restore damaged cartilaginous tissue, which was the goal of this work.

## MATERIALS AND METHODS

### Heterogeneous gel *Sfero*®GEL

To create a cell-engineered construct (CEC), a composition of heterogeneous implantable gel from the *Sfero*®GEL linear series (Biomir Service, Russia) was chosen 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;
– swellability: not less than 86.6 ± 3.0 wt.%.

### Obtaining microparticles of articular cartilage

Pork femur and knee joints were obtained from a slaughterhouse (Promagro, Stary Oskol) after the slaughter of healthy animals (weight about 120 kg) in accordance with the European Directive 64/433/EEC. After refrigerated transportation (+4 °C), the cartilage was removed from articular surfaces with a scalpel and cut into 0.5 × 0.5 × 0.1 cm fragments. The resulting fragments were micronized using CryoMill (Retch GmBH, Germany). The fraction of microdispersed particles in the range of 100–250 μm was isolated by sieving the milling through a set of sieves with appropriate pore sizes.

### Decellularization mode

Decellularization was performed by treating the microparticles in three changes of phosphate buffer (138 mM NaCl, 2.67 mM KCl, 1.47 mM $KH_2PO_4$, 8.1 mM $Na_2HPO_4$, pH 7.4) containing 0.1% sodium dodecyl sulfate and an increasing Triton X100 concentration (1%, 2% and 3%, respectively) at room temperature and occasional stirring. The samples were then placed in a buffer solution (10 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.5 mol $CaCl_2$; pH = 7.6) containing 50 U/ml type I DNAase (New England Biolabs Inc., USA) for 48 hours at 37 °C. The matrix was washed in bidistilled water. It was sterilized by gamma irradiation at 1.5 Mrad dose.

### Cell isolation

The source of human adipose-derived mesenchymal stem cells (hAMSCs) was the subcutaneous adipose tissue of a healthy donor taken from him with informed voluntary consent. A 2–3 g sample of the subcutaneous adipose tissue was shredded with a scalpel, subjected to double washing with cold (+4…+6 °C) Hanks' solution, and then incubated in 0.1% collagenase type I solution (Gibco, USA) at 37 °C for 20 minutes.

All cells were precipitated by centrifugation, resuspended in complete DMEM/F12 (1:1) growth medium supplemented with 10% bovine fetal serum, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 2 mM L-glutamine (Gibco, USA) and cultured until monolayer formation, changing the medium twice a week. The cells were transferred into suspension by treating with Versene solution at 37 °C for 1 minute, followed by addition of TrypLe™ dissociating agent (Invitrogen, USA). Third passage cells were taken for the experiments.

### Cell differentiation

To confirm the presence of multipotent cells in the culture, experiments on its multidirectional differentiation were performed. Chondrogenic cell differentiation was performed in microspheres obtained by deposition of $2 \times 10^5$ cells in 96-well plates with a conical bottom. After 2 weeks of cultivation in chondrogenic culture medium (DMEM HG supplemented with GlutaMAX™ (Gibco, USA), 10% ITS+ (Corning, USA), 1% sodium pyruvate (Gibco, USA), 0.25% ascorbate-2-phosphate (Sigma-Aldrich, USA), 0.0001% dexamethasone (Sigma-Aldrich, USA), 0.002% TGF-β1 (PeproTech, USA) and 1% culture antibiotic-antimycotic (Gibco, USA)), the preparations were fixed in a 10% buffered formalin and embedded in paraffin. Sections were dewaxed, rehydrated, and stained with alcian blue. Adipogenic differentiation of the studied cultures was performed in DMEM/F12 medium containing 10% horse serum, 0.5 mM isobutyl methylxanthine and 60 mM indomethacin for 7 days. The preparations were then fixed in 4% paraformaldehyde and stained with oil red O. Differentiation of the obtained cultures into bone tissue was performed in serum-free DMEM/F12 medium supplemented with 0.2 mM ascorbic acid, 10 mM b-glycerophosphate calcium, 10-7 M dexamethasone (Sigma), 100 units/mL penicillin, 100 μg/mL streptomycin sulfate, and 2 mM L-glutamine (Gibco). The cells were cultured for 3 weeks,

changing the medium twice a week. Upon completion, the preparations were fixed in 4% formaldehyde solution and stained with alizarin red.

Previous studies [14] have shown that MSC cultures from all sources had a similar phenotype CD29, CD34, CD44, CD49b, CD45, CD73, CD90, HLA-DR, indicating a high content of multipotent mesenchymal cells. Most of these macromolecules are included in the list of markers recommended by the International Society for Cellular Therapy for characterization of MSC cultures [15].

## Method for determining metabolic activity

The metabolic activity of cells was determined using a test with PrestoBlue™ reagent (Invitrogen, USA) according to the manufacturer's instructions. The method was based on the dehydrogenase activity of cells converting resazurin to resorufin, which can also be determined spectrophotometrically. Spectrophotometric analysis was performed on a Tecan Spark10 plate reader (Tecan, Austria). When studying metabolic activity, $2 \times 10^4$ hAMSCs were added per 5 mg of decellularized cartilage (DC). In the metabolic activity study, $2 \times 10^4$ hAMSCs were added per 100 μl of BMCH. Absorbance measurements were used to calculate the metabolic activity coefficient (K) using the formula:

$$K = \frac{117{,}216 \times Abs_{570} - 80{,}586 \times Abs_{600}}{155{,}677 \times Abs_{600} - 14{,}652 \times Abs_{570}} \times 100\%,$$

where $Abs_{570}$ – absorbance at 570 nm wavelength, $Abs_{600}$ – absorbance at 600 nm wavelength.

## Creation of cell-engineered constructs

The CEC consisted of $1 \times 10^6$ cells and 5 mg of decellularized cartilage (DC) or 0.25 ml BMCH. Matrices were populated with cells by rotating them in tubes with culture medium on a Multi Bio 3D (Biosan, Latvia) ballerina type shaker platform. CECs were cultured in a growth culture medium for the first 5 days. Then the culture medium was replaced with a chondrogenic differentiation medium. The medium was replaced every third day. Samples were analyzed on days 14 and 42 in differentiation culture medium using histological staining methods.

## Scanning electron microscopy

The morphology of the surface and the nearest subsurface layer of samples was studied together with employees of the Laboratory of Basic Research in Oph-

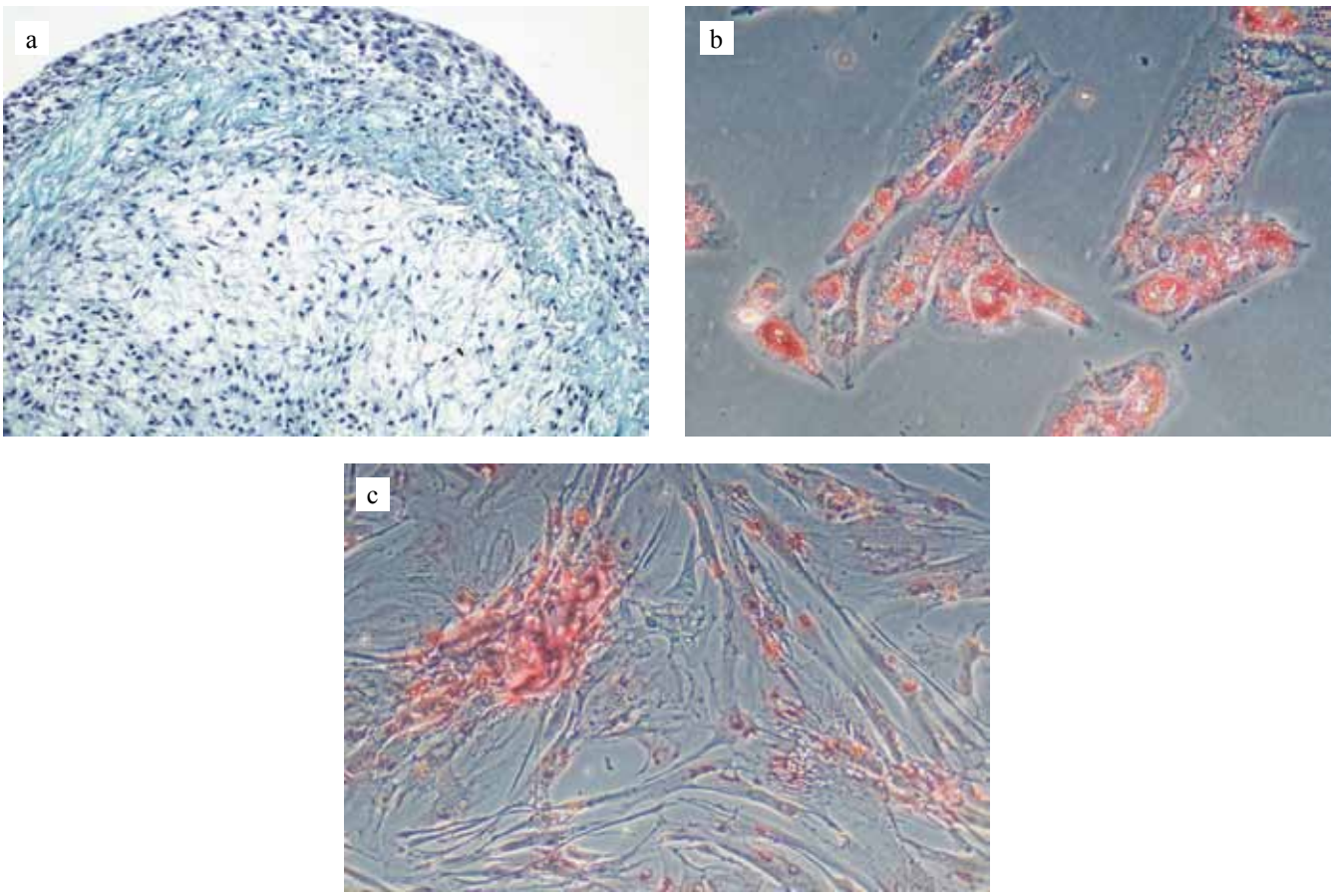


Fig. 1. Differentiation of hAMSCs in chondrogenic (a, alcian blue stain), adipogenic (b, Oil Red O stain) and osteogenic (c, Alizarin Red stain). 200× magnification

thalmology at the Research Institute of Eye Diseases in Moscow by scanning electron microscopy (SEM) using lanthanide contrasting [16].

Sample preparation of heavily watered samples for SEM is difficult because it requires their dehydration and sputtering of the conductive layer, and this leads not only to strong structural changes in such objects, but also complicates differentiation of cellular elements from the substrate. The lanthanide contrasting method allows observation of non-dehydrated biological samples in a low vacuum after keeping them in a saturated solution of a rare-earth metal. In this case, the native state of the studied object is preserved as much as possible, and the image obtained in backscattered electron detection mode carries extended information about intracellular structures [16].

The processing protocol included an initial wash, holding for 45 minutes in a BioREE contrast solution (Glaucon, Russia), and a final wash with distilled water. After contrasting, excess moisture was removed from the specimen surface using an airbrush and placed on the slide of an EVO LS10 microscope (Zeiss, Germany). Observations were performed in low vacuum (EP, 70 Pa), at an accelerating voltage of 20 kV.

## Methods of histological analysis of samples

The specimens were fixed in a buffered 10% formalin solution, washed in running water and dehydrated in

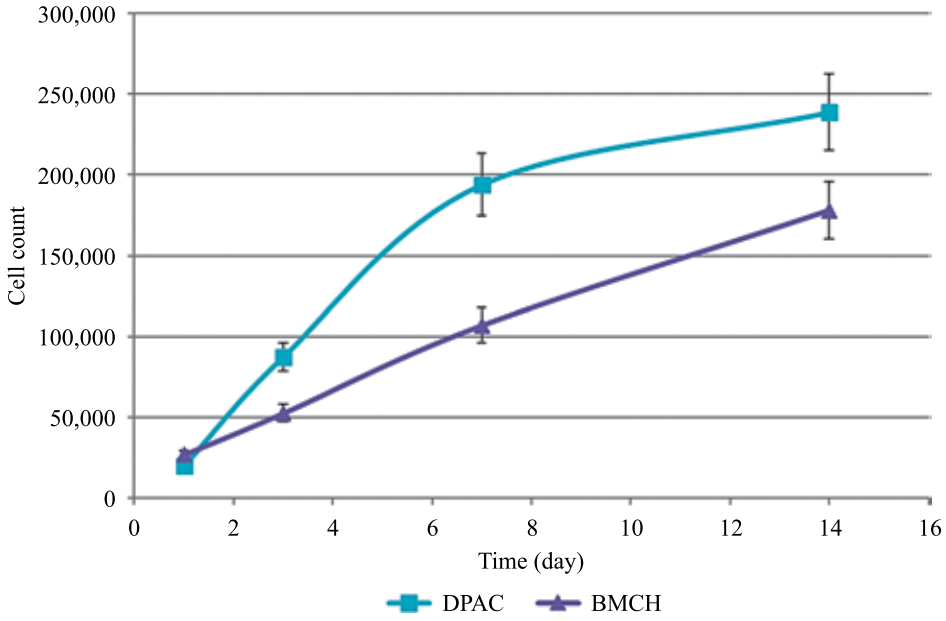


Fig. 2. Growth curve of hAMSCs when cultured in DPAC and BMCH

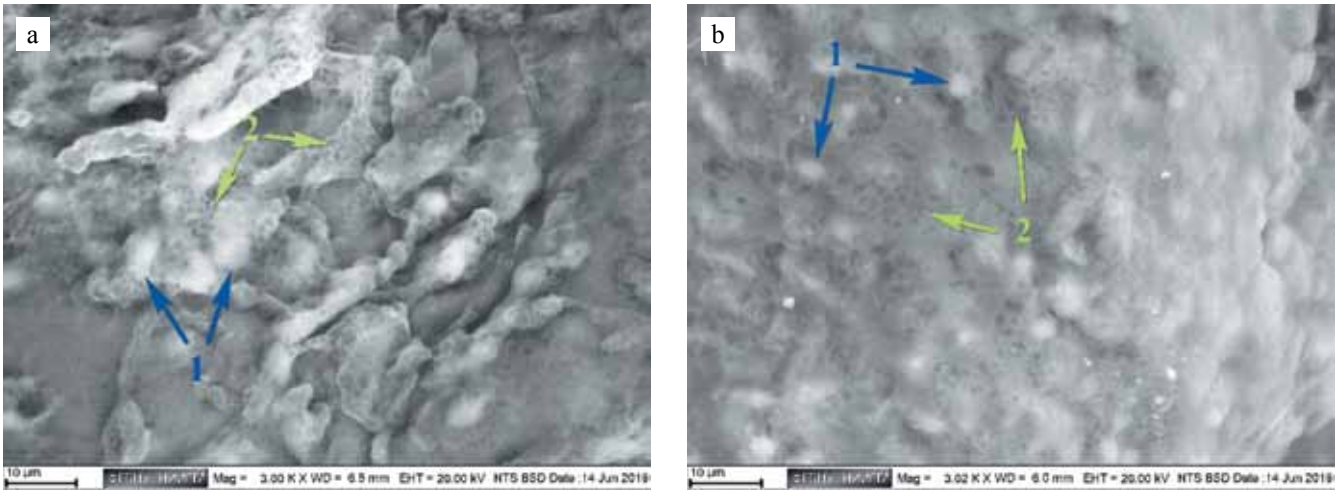


Fig. 3. Microphotographs of the CEC surface structure, including hAMSCs, cultured in DPAC (A) and in BMCH (B) in a chondrogenic differentiation medium for 14 days. SEM using lanthanide contrasting BioREE. The scale bar size is 10 μm. 1 – nuclei, 2 – intracellular vesicles

alcohol of ascending concentrations (in two portions of 70%, 80%, 96% ethanol), incubated in a mixture of ethanol and chloroform, in chloroform and embedded in paraffin. Sections 4–5 μm thick, obtained with a Leica RM3255 microtome, were dewaxed, rehydrated and stained by standard techniques, with hematoxylin and eosin, alcian blue to detect glycosaminoglycans (GAGs) and using Masson's method for connective tissue according to standard techniques.

Analysis and photography of the obtained preparations was performed using a Nikon Eclipse microscope equipped with a digital camera.

## RESULTS AND DISCUSSION

Ability of hAMSCs to differentiate in chondrogenic, adipogenic and osteogenic directions is shown in Fig. 1.

The growth curves show that the number of cells on the surface of both BMCH and DC increased over 14 days (Fig. 2). On the first day measurements, optical density of the samples did not differ, indicating a similar cell population on the BMCH and DC surfaces at the start of the experiment and probably the same adhesive capacity of the carriers for MSCs in the amount initially deposited in CEC. However, a significant difference in the volume of the cell population from day three onwards revealed the effect of the carriers on the proliferative capacity of hAMSC culture. The mitochondrial activity of cells ensuring the conversion of blue resazurin into pink resorufin was 1.7, 1.7 and 1.3 times higher on days 3, 10 and 14, respectively, when MSCs were cultured on DC compared to BMCH. The shape of the growth curves was similar; however, in the case of BMCH, we observed an approach towards the stationary phase by day 14, whereas in the case of BMCH, no signs of slowing down in hAMSCs growth were visualized by day 14. Apparently, the slowdown in cell growth was associated with the limited area of the carrier for colonization, as the optimal amounts of growth factors and nutrients were maintained by regularly replacing the culture medium. Note that the results obtained allow us to estimate the count of hAMSCs with metabolic activity, whose presence in the samples is important for cell function in

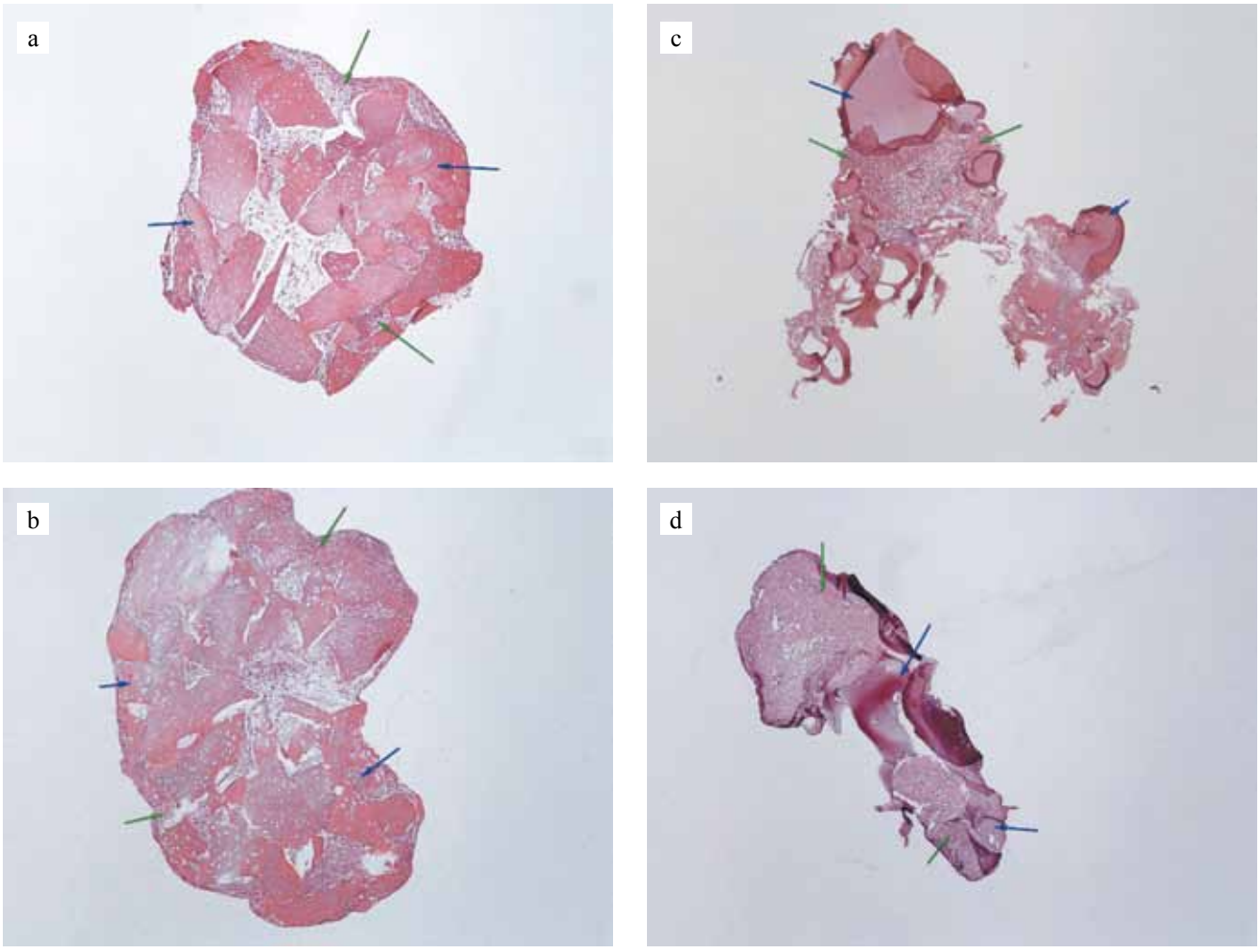


Fig. 4. Histological picture of CEC formation dynamics. H&E staining. 40× magnification. a, b – DPAC-based CECs, c, d – BMCH-based CECs. a, c – 14 days of cultivation in chondrogenic differentiation medium, b, d – 42 days of cultivation in chondrogenic differentiation medium. Blue arrows – cell carrier, green arrows – cells with the obtained ECM

CECs, as mitochondrial dehydrogenases, cytochromes and dehydrogenases located in the cytoplasm take part in reduction of resazurin to resorufin [17]. The difference in the cell count when observed for 14 days (Fig. 2), indicates a greater ability of DC to stimulate the growth of hAMSCs compared to BMCH. Note that for a full in vitro comparative assessment of the prospects of using cell carriers in cartilage tissue engineering, it is necessary to investigate their ability to maintain chondrogenic differentiation of hAMSCs.

Fig. 3. shows the results of SEM morphology of CECs consisting of hAMSCs and DCs or BMCHs after 14 days of culture.

At 14 days of cultivation in chondrogenic culture medium, hAMSCs formed cell layers on both DC and BMCH surfaces (Fig. 3, a, b). In some cells, the use of lanthanide contrasting allowed visualization of intracellular structures due to accumulation of lanthanides in calcium- and phosphorus-rich areas (including $Ca^{2+}$-channels of various membranes, cell contact proteins). We have been able to identify nuclei and plasma membrane edges in some cells. Interestingly, the cytoplasm of the cells included numerous granules whose contents were similar in brightness to the matrix used. These intracellular vesicles may be related to both secretion of components of the extracellular cartilage matrix and resorption of the carrier. Both described processes were observed in histological studies of cartilage CEC specimens. Note that, summarizing this with the observed spread-eagled shape of cells, it can be concluded that physiological metabolic processes were actively taking place in the cells cultured on the surface of the tissue-specific carrier and the collagen-containing hydrogel.

As can be seen in Fig. 4, there was active fibroblast-like cell growth on the DC surface with formation of multilayer layers. The cells and their derived ECM bound numerous DC microparticles, forming large conglomerates.

In CEC samples including BMCH, a heterogeneous cell population forming stratified patches was observed.

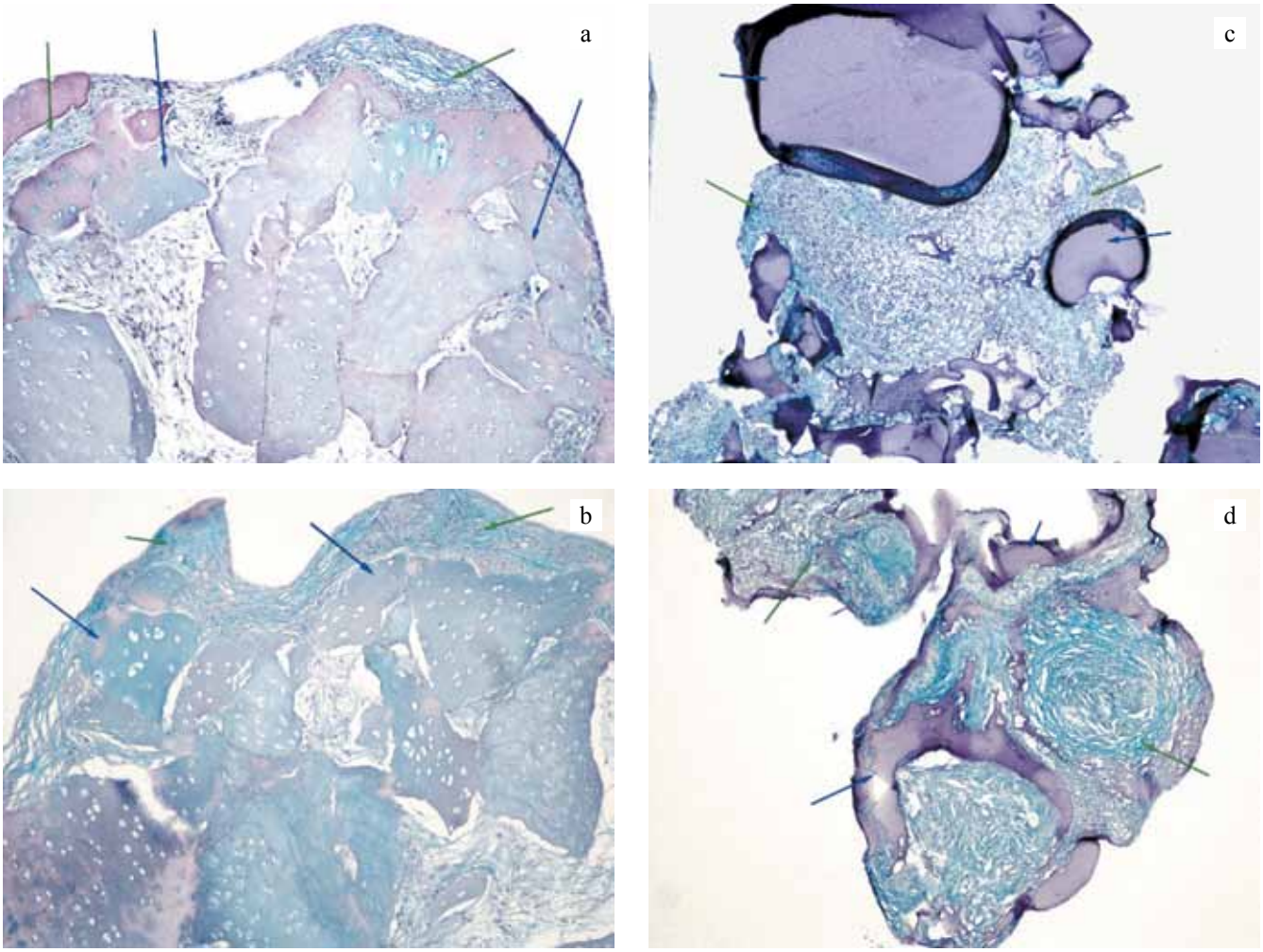


Fig. 5. Histological picture of cartilage CEC formation dynamics. Alcian blue stain for GAGs. 100× magnification. a, b – DPAC-based CEC, c, d – BMCH-based CECs. a, c – 14 days of cultivation in chondrogenic differentiation medium, b, d – 42 days of cultivation in chondrogenic differentiation medium. Blue arrows – cell carrier, green arrows – cells with the obtained ECM

The cells sprouted into the BMCH thickness, which was accompanied by its resorption. Areas of cell layer destruction were also visualized. The sample contained areas not stained with hematoxylin – cell detritus.

The amount of cell detritus in both DC- and BMCH-based CECs increased over time. Note that cells were more evenly distributed on the surface of the DC matrix, while for BMCH, cell adhesion and proliferation were observed only in isolated areas. It should be noted that for both matrices, the cell mass volume did not visually differ. However, on the DC surface, cells with the ECM that were synthesized by them form thin strands, which leads to formation of aggregates of micro-dispersed particles. On the surface of the hydrogel matrix, hAMSCs are unevenly distributed across the surface in the form of large clusters, which can lead to nutrient deficiencies in the cell layer volume.

In cell-derived ECM in CECs including both types of matrices, local positive staining for GAGs and collagen in multilayer areas was observed at 14 days of cultivation in differentiation medium (Fig. 5, a, b and Fig. 6, a, b). Uniform ECM staining for GAGs was observed in CECs containing BMCH and in CECs with DC at 42 days (Fig. 5, b, d). By day 42, there was a significant increase in collagen content in the samples (Fig. 6, b, e).

Results obtained show that hAMSCs are capable of forming cartilage CECs when cultured in chondrogenic differentiation medium on collagen-containing matrices. It can be assumed that chondrogenesis of hAMSCs in CECs with ECM-derived collagen-containing mimetics was influenced by macromolecular components of ECM. Macromolecules such as hyaluronic acid, chondroitin sulphate and type II collagen have been extensively studied as cartilage repair agents. It is thought that the reason for their stimulating effect on chondrogenesis lies in their interaction with cells. For example, introduction of hyaluronic acid into a chondrocyte culture enhanced ECM synthesis. As a possible mechanism, it is suggested that the chondrocyte surface receptor CD44 first binds to the hyaluronic acid molecule that stimulates it and then interacts with the cytoplasmic domain of the TGF β1 receptor, ultimately regulating the genes responsible

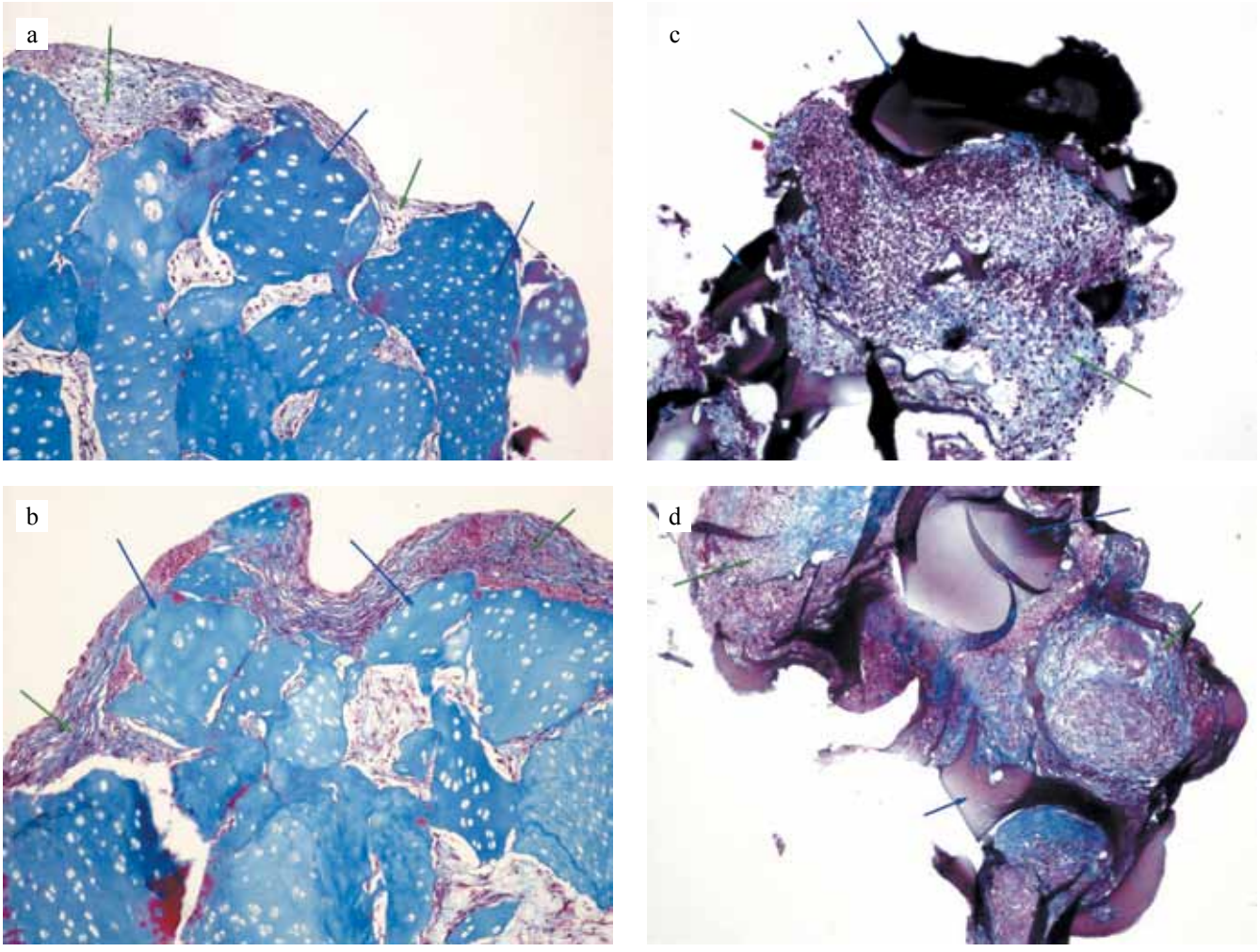


Fig. 6. Histological picture of CEC formation dynamics. H&E staining. 100× magnification. a, b – DPAC-based CEC, c, d – BMCH-based CECs. a, c – 14 days of cultivation in chondrogenic differentiation medium, b, d – 42 days of cultivation in chondrogenic differentiation medium. Blue arrows – cell carrier, green arrows – cells with the obtained ECM

for TGF β1 signaling and improving ECM synthesis [18, 19]. In addition, it has been shown that collagen type II, which is the main protein component of hyaline cartilage ECM from which DC was derived, can promote preservation of chondrocyte morphology and synthesis of more GAGs than type I collagen [20]. Type II collagen has also been shown to enhance chondrogenic differentiation of MSCs when added to agarose matrices [21]. Note that in CEC with DC, the cells were more evenly distributed, whereas in the CEC with BMCH, the cells adhered and proliferated only in certain areas of the matrix surface. This is probably due to preservation of cell adhesion sites on the surface of DC microparticles due to a gentler decellularization procedure compared to the acetic acid hydrolysis to which farm animal tissues are subjected during BMCH fabrication.

## CONCLUSION

The established ability of decellularized cartilage (obtained using the developed protocol) to form cell-engineered constructs with hAMSCs with uniform cell distribution and the cell-derived specific extracellular matrix containing collagen and glycosaminoglycans indicates its potential for regeneration of damaged cartilage. Biopolymer microheterogeneous collagen-containing hydrogel and tissue-specific decellularized cartilage showed they can support chondrogenic differentiation of hAMSCs. The high ability of the obtained decellularized cartilage matrix to support adhesion, proliferation and chondrogenic differentiation of hAMSCs in comparison to the clinically applied equivalent indicates that it has prospects for application in tissue engineering.

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*The authors declare no conflict of interest.*

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# ENDOTHELIAL CELL MONOLAYER FORMATION ON A SMALL-DIAMETER VASCULAR GRAFT SURFACE UNDER PULSATILE FLOW CONDITIONS

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**Objective:** to create a cell-populated small-diameter vascular graft (SDVG) using autologous endothelial cells and extracellular matrix proteins, and to evaluate the efficiency of endothelial cell monolayer formation during shear stress preconditioning in a SDVG. **Materials and methods.** PHBV/PCL tubular scaffolds of vascular grafts were made by electrospinning from a mixture of polyhydroxybutyrate-valerate (PHBV) copolymer and polycaprolactone (PCL) and modified with fibrin. To populate the graft, an endothelial cell culture was isolated from the blood of patients with coronary heart disease. Phenotyping of endothelial colony-forming cell (ECFC) culture was performed by flow cytometry and immunofluorescence microscopy. Cell proliferative and angiogenic activity were also studied. Cell-populated vascular scaffolds were cultured in a pulsatile flow setup with a final shear stress of 2.85 dyne/cm$^2$. The effect of pulsatile flow on monolayer formation was assessed by immunofluorescence, scanning electron microscopy, atomic force microscopy, and whole-transcriptome RNA sequencing. **Results.** Under the influence of pulsatile flow, endothelial cells that were seeded into the tubular scaffold showed an increase in the expression level of endothelial profile proteins, focal adhesion and cytoskeleton. In contrast to endothelial cell culture on a vascular graft surface under static conditions, when cultured under pulsatile flow with 2.85 dyne/cm2 shear stress, endothelial lining cells have an increased ability to adhere and are oriented along the pulsatile flow path. Whole-transcriptome RNA sequencing showed that induced shear stress increased expression levels of differentially expressed genes encoding proteins that ensure vascular development, endothelial integrity, and endothelial metabolism. A protocol for fabrication of a personalized cell-populated biodegradable SDVG under pulsatile flow conditions was developed. **Conclusion.** The use of autologous fibrin and ECFC culture, as well as shear stress preconditioning, allow to obtain a personalized cell-populated SDVG with continuous functional endothelial monolayer adapted to the flow.

*Keywords: tissue engineering, autologous endothelial cells, pulsatile flow, personalized vascular graft.*

## INTRODUCTION

Creation of biodegradable small-diameter vascular grafts capable of being resorbed after implantation and replaced by the patient's own new vessels, frees the patient from the need for replacement. Therefore, it is a priority in tissue vascular engineering [1, 2]. Researchers are looking for an ideal culture that would have a high proliferative potential, full functional activity, and reproducibility.

It is believed that in vitro endothelialization of vascular grafts or early stimulated in situ endothelialization of engineered constructs can be very effective in preventing thrombosis in tissue-engineered vascular grafts.

It is believed that in vitro endothelialization of vascular grafts or early stimulated in situ endothelialization of engineered constructs can be very effective in preventing thrombosis of tissue-engineered vascular grafts [3–5].

Vascular endothelial cells are constantly subjected to shear stress due to frictional force created by blood flow. Through activation of mechanosensors, they recognize changes in local shear stress and cyclic deformation, intracellular signal transduction is modulated, leading to changes in gene expression, cell morphology and structural remodeling [6].

In the straight part of the vessel, shear stress and cyclic stretch have well-defined directions; the response of endothelial cells to targeted mechanical stimuli involves remodeling of the cytoskeleton structure to minimize intracellular stress. These adaptive changes help maintain homeostasis and they have an atheroprotective effect. In the vascular network of complex geometry, multidirectional mechanical stimuli arise, which can contribute to atherogenesis in these areas [7].

In vitro endothelialization of grafts is expensive and requires cell preconditioning by shear stress in order to increase cell viability and resistance to flushing by blood flow from the surface of the cell-based graft after its implantation into the vascular bed [8–9]. It is also

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believed that it is impossible to obtain large quantities of autologous endothelial cells from peripheral blood [10–12]. Therefore, in order to colonize the inner surface of tubular scaffolds with cells, scientists have often resorted to the use of bone marrow-derived mononuclear fraction or adipose-derived stromal vascular fraction, or used induced pluripotent cells in order to observe personalization of biological material, acting as a source of own vascular cells [13–15].

In our previous works, we showed the possibility of obtaining autologous human endothelial colony-forming cells (ECFCs) with high proliferative potential from the mononuclear blood fraction of the majority of patients with coronary heart disease [16].

Adhesion of endothelial cells to the polymer base is impeded by hydrophobicity of the surface, its foreign molecular composition and absence of cell adhesion sites. In order to eliminate these factors, the surface is modified by various extracellular matrix proteins. In tissue engineering, extracellular matrix proteins (collagen, fibronectin, gelatin) from animal sources or allogeneic proteins are usually used as a substrate, which increases the risk of negative immune response after graft implantation into the vascular bed. The use of autologous fibrin, obtained from the patient's peripheral blood as a feeder layer, reduces immunogenicity of protein-coated biodegradable polymeric vascular graft. With autologous fibrin and ECFC culture, one can design a personalized small-diameter vascular graft, while cultivation in the pulsatile flow unit allows to adapt endothelial cells to the active mechanical stimuli existing in natural blood flow.

Our objective is to create a cell-based small-diameter vascular graft using autologous endothelial cells and extracellular matrix proteins and evaluate the efficiency of endothelial monolayer formation under shear stress preconditioning.

## MATERIALS AND METHODS

### Fabrication of biodegradable tubular scaffolds

Biodegradable polymer tubular scaffolds from a mixture of 5% poly(3-hydroxybutyrate-co-3-hydroxyvalerate) solution, (PHBV, Sigma-Aldrich, USA) and 10% poly(ε-caprolactone) solution, (PCL, Sigma-Aldrich, USA) dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (Sigma-Aldrich, USA) in a 1:2 ratio were made by electrospinning on a Nanon-01A machine (MECC, Japan). 22G-gauge needles were used to make the scaffolds. Needle voltage was 23 kV, polymer solution feed rate was 0.3 mL/h, collector rotation speed was 200 rpm, distance from needle to the winding collector was 15 cm, and the winding collector diameter was 4 mm.

### Fibrin modification of the inner surface of tubular scaffolds

The inner surface of tubular scaffolds was modified with fibrin. Fibrinogen was isolated from peripheral blood of patients with coronary heart disease. Blood sampling was performed after obtaining voluntary informed consent to use biological material for research purposes. The study was approved by the local ethics committee (protocol No. 20 of December 9, 2020). Blood collected in vacuum tubes with sodium citrate was centrifuged at 2000 g for 10 min, and the resulting plasma was cooled to 4 °C. Cold ethanol (70 vol%, 4 °C) was added to the plasma at a 4:1 ratio (plasma/ethanol) with constant stirring. Immediately thereafter, the solution was centrifuged at 600 g at 4 °C for 5 min. The supernatant plasma was decanted and the fibrinogen precipitate was dissolved in 0.9% $NaCl_2$ with HEPES at 37 °C (to 30–40 mg/mL fibrinogen concentration) [17]. Fibrinogen was polymerized using thrombin and $CaCl_2$. Polymer grafts were immersed and impregnated with the resulting fibrinogen solution. Next, a solution of thrombin 500 IU/ml (Thrombin from human plasma, T7009, Sigma-Aldrich, USA) and $CaCl_2$ 40 mmol/L were applied to the surface of the tubular scaffold for fibrin polymerization. After polymerization, the tubular scaffold was immersed in sodium phosphate buffer solution (PBS) with ε-aminocaproic acid 2 mg/ml until subsequent cellular seeding.

### Obtaining ECFCs

A culture of endothelial colony-forming cells of the peripheral blood mononuclear fraction with high proliferative activity and full functionality was used for populating the vascular grafts. Blood was taken from patients with coronary heart disease (a group of patients who, with the further development of the disease, would require prosthetic repair to the extent of coronary artery bypass grafting) after obtaining voluntary informed consent to use biological material for research purposes. ECFC culture was obtained based on culturing blood mononuclear fraction (BMF) in media containing growth factors. The studies were performed at the Research Institute for Complex Issues of Cardiovascular Diseases and were approved by the local ethics committee (protocol No. 20 of December 9, 2020). The cultivation technique, immunophenotyping and study of the functional properties of the resulting culture are presented earlier in a paper by our team [16]. A comparative study of the gene expression profile of ECFC culture with mature coronary artery cell culture was conducted [18].

### Phenotyping culture by flow cytometry

For staining, $1 \times 10^5$ cells of the resulting culture washed with PBS were selected. We used a combination of monoclonal antibodies conjugated with various

dyes: with fluorescein isothiocyanate (FITC) – CD3 (BC, A07746), CD34 (BC, IM1870U), vWF (abcam, ab8822); with phycoerythrin (PE) – CD309 (BD, 560494), CD14 (BC, A07764); with allophycocyanin (APC) – CD133 (MACS, 130-090-826), CD31 (BL, 303115); with phycoerythrin-cyanine 7 (PC7) – CD146 (BL, 361008); with Pacific Blue 450 (PB 450) – HLA DR (BL, 307633); with Krome Orange (KrOr) – CD45 (BC, A96416).

Sample preparation was performed according to the protocols of the manufacturers for two panels: 1) CD3, CD14, HLADR, CD45; 2) CD34, KDR, CD146, CD133, CD31, CD45. Cell culture with cobblestone morphology was additionally stained with vWF, CD146. A culture stained with antibodies of the corresponding isotypic control, CD146 – PC7 Mouse IgG1 (BL, 400127), CD31, was used as a control, CD133 – APC Mouse IgG1 (BL, 400121), CD309 – PE Mouse IgG1 (BD, 550617), CD3, CD3, CD34 – FITC Mouse IgG1 (BC, A07795), CD14 – PE Mouse IgG2α (BC, 559319), CD45 – KrOr Mouse IgG1 (BC, A96415), HLA DR – PB Mouse IgG2α (BL, 400235). When intracellular vWF protein was stained, cells were fixed and permeabilized using the IntraPrep kit (BC, A07803, USA). Samples were then resuspended in PBS and analyzed on a CytoFlex laser flow cytometer (BC, USA) using the CytExpert software. The instrument was set up for each panel using control samples stained with the corresponding isotypes; further analysis of all samples was performed using the same instrument settings.

## Cell seeding of a biodegradable tubular scaffold

ECFC suspension was injected into PHBV/PCL scaffolds at 700,000/ml concentration. For the first 8 hours after seeding, the vascular grafts were inverted every 30 minutes to evenly distribute the cells over the surface. The culture medium was replaced after 24 hours. The vascular grafts were cultivated under static conditions for a total of 2 days. After that, the vascular prostheses were connected to the pulsatile flow unit (Harvard Apparatus, USA) at preset settings, providing voltage of 1.27 dyne/cm$^2$ for 24 hours. Then, the voltage was gradually increased to 2.85 dyne/cm$^2$. The operating mode of culturing in the pulsatile flow unit included the following parameters: ejection rate 20 beats/min, ejection volume 0.7 mL, and a final shear voltage of 2.85 dyne/cm$^2$. Culturing was continued in this mode for 5 days under sterile conditions in a $CO_2$ incubator at 37 °C. For the control, similar vascular grafts were cultured under static conditions.

## Scanning electron microscopy

The inner surface of tubular scaffolds was examined by scanning electron microscopy at various stages (before and after fibrin modification of PHBV/PCL scaffolds, after ECFC seeding) on a Hitachi S-3400N microscope (Hitachi, Japan) under high vacuum conditions. Sample preparation consisted of fixation in glutaraldehyde (Sigma, USA) for one day followed by freezing and lyophilization in Freezone 2.5 machine (Labonco, USA) at –40 °C and pressure <0.133 mbar. After that, the samples were mounted on special tables and the conductive gold-palladium coating with a 7 nm thickness was formed on their surface by ion sputtering in the EM CE200 machine (Leika Mikro-systeme GmbH, Austria).

## Immunofluorescence study

After culturing, the endothelial layer formed on the inner surface of the vascular prosthesis was subjected to immunofluorescent staining. The vascular prosthesis was fixed in 4% paraformaldehyde solution for 10 minutes, then cut lengthwise and fixed open on slides with the inner surface upward. Cells on the inner surface were stained for endothelial cell markers: CD31, VEGFR2 (CD309), CD144, vWF, adhesion marker F-actin, and Talin. Intracellular markers (vWF, F-actin) were permeabilized with 0.01% Triton X-100 solution before staining. F-actin was stained with phalloidin conjugated with Alexa Fluor 568 fluorescent dye (Alexa Fluor™ 568 Phalloidin, A12380, Thermo Fisher, USA) according to the manufacturer's instructions. Primary antibodies used for the remaining markers were Mouse anti-CD31 (ab119339), Rabbit anti-VEGFR2 (ab2349), Rabbit anti-CD144 (ab33168), Sheep anti-vWF – FITC (ab8822), Rabbit anti-Talin (ab71333) and secondary antibodies: Donkey anti-Mouse IgG Highly Cross-Adsorbed – AF555 (A-31570), Donkey anti-Rabbit IgG Highly Cross-Adsorbed – AF488 (A-21206) (Thermo Fisher, USA). Incubation with primary antibodies was performed at 4 °C for 16 hours, with secondary antibodies at room temperature for one hour. A 1% bovine serum albumin solution was used to block nonspecific binding. The samples were counterstained with DAPI (Sigma, USA). Prepared glasses were embedded in ProLong (Life technologies, United States) under glass. The preparations were analyzed using an LSM 700 laser scanning microscope (Zeiss, Germany).

## Assessment of cell adhesion and viability

Adhesion and viability of endothelial cells on the surface of vascular grafts were assessed by fluorescence microscopy. Samples were washed in PBS, serially injected with Hoechst 33342 (10 μg/mL, 14533, Sigma Aldrich, USA), followed with ethidium bromide (30 μg/mL, 46067, Sigma Aldrich, USA), and incubated for 10 minutes and 1 minute, respectively. Samples were analyzed on an LSM 700 laser scanning microscope (Zeiss, Germany). Cell adhesion was assessed by counting cell nuclei stained with Hoechst 33342 in the field of view. At least 20 randomly selected fields of view

were analyzed at ×200 magnification. The results were recalculated and presented as units/mm$^2$.

The relative number of dead cells was calculated using the formula: absolute number of dead cells × 100% / absolute number of all adherent cells. The relative number of live cells was determined by subtracting the proportion of dead cells from 100% of adherent cells.

### Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 software. Data are presented as medians (Me) and quartiles (25% and 75%). Statistical significance of differences between the two independent groups was assessed using the nonparametric Mann–Whitney U test. Differences were considered significant at a significance level of $p < 0.05$.

### Whole transcriptome sequencing

An ECFC culture of 10 million cells was used for whole transcriptome sequencing (RNA-seq). Cells were lysed with Trizol (15596018, Invitrogen, USA) followed by isolation of total RNA using the Purelink RNA Micro Scale Kit (12183016, Invitrogen, USA) with accompanying DNAase treatment (DNASE70, Sigma-Aldrich, USA). RNA quality was monitored using the RNA 6000 Pico Kit (5067-1513, Agilent, USA) on a Bioanalyzer 2100 (Agilent, USA) using the RNA integrity index (RIN). Amount of isolated RNA was estimated using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and a Qubit 4 fluorometer (Invitrogen, USA). For 1 μg of isolated RNA, rRNA was depleted using using the RiboCop rRNA Depletion Kit V1.2 (037.96, Lexogen, Austria) with further formation of DNA libraries (SENSE Total RNA-Seq Library Prep Kit, 042.96, Lexogen, Austria). The quality of obtained DNA libraries was analyzed using the High Sensitivity DNA Kit (5067-4626, Agilent, USA) on a Bioanalyzer 2100 (Agilent, USA). DNA libraries were analyzed quantitatively by quantitative polymerase chain reaction (RT-qPCR) on a CFX96 Touch amplifier (Bio-Rad, USA). Next, DNA libraries were mixed in an equimolar manner and sequenced on a HiSeq 2000 platform (Illumina) with a 2 × 125 nucleotide paired-end read length.

The resulting reads were filtered by quality (QV >20) and length (>20), and adaptor sequences were removed using the TrimGalore v.0.4.4 software. After filtering, the average number of reads exceeded 10 million. They were mapped to the human genome (hg38) with Ensembl annotation (v.38.93) using the CLC GW 11.0 program (Qiagen) with the following parameters: Similarity fraction = 0.8, Length fraction = 0.8, Mismatch cost = 2, Insertion cost = 3, Deletion cost = 3; the resulting files were obtained in *.bam* format. To assess differential gene expression, we used multivariate statistical analysis in the CLC GW 11.0 software based on the negative binomial model used in the EdgeR and DESeq2 programs.

The RNA integrity index (RIN) isolated from the ECFC culture was at least 8, indicating that it was of high quality and could be used for RNA-seq (RIN ≥7 is recommended). The amount of total RNA obtained in the samples was at least 29 μg, which was more than enough for rRNA depletion (≥1 μg is recommended).

### Statistical analysis of whole transcriptome sequencing results

Statistical analysis was performed using GraphPad Prism 8 software (GraphPad Software). The data are presented as median, 25th and 75th percentiles, and minimum and maximum. The groups were compared by Mann–Whitney U test. When analyzing differences between cell cultures, statistically significant differentially expressed genes (DEGs) were determined by the frequency of change ≥2 and a p value <0.05 corrected for the mean false discovery rate (FDR). Gene Ontology (GO) gene set enrichment analysis was performed in the categories of molecular functions, biological processes, and cellular components using the Gene Set Test in CLC GW. When comparing cell groups, categories with $p < 0.05$ (FDR) support and a ratio of DEGs to the total number of genes greater than 50% were considered.

## RESULTS

### Characteristics of autologous endothelial colony-forming cells

The phenotype of the resulting ECFC culture corresponded to that of mature endothelial cells: CD31+CD309+vWF+CD146+, no expression of progenitor markers CD34, CD133 (Fig. 1) [16]. The culture had high proliferative activity. Full-fledged functional activity, including angiogenic activity, was detected [16].

### Surface structure of PHBV/PCL scaffolds before and after fibrin modification

The unmodified PHBV/PCL scaffolds exhibited a homogeneous highly porous structure with chaotic filament interlacing. The average fiber diameter was 2.8 [1.9; 3.3] μm, and the pore size was 36.1 [30.2; 38.7] μm. Modification of the polymer scaffold surface with fibrin significantly changed its architectonics (Fig. 2). It was found that during polymerization, fibrin flattened the surface of the polymer scaffold, forming a uniform fine-pored coating on their finest branched fibrils with a fiber diameter of 125 [94.0; 152.0] nm and a pore size of 273 [176.0; 333.0] nm.

On the inner surface of the PHBV/PCL/fibrin cell-populated vascular grafts, well-disseminated endothelial cells were found, indicating high-quality adhesion. Meanwhile, a highly irregular distribution of ECFCs

was found on unmodified PHBV/PCL scaffolds. In the absence of a feeder layer carrying cell adhesion sites, there was no sufficiently dense cell attachment.

## Regime of cultivation of cell-populated vascular grafts under pulsatile flow conditions

The optimal pulsatile flow regime for formation and preservation of endothelial lining on the surface of PHBV/PCL/fibrin vascular grafts was selected.

It was found that increasing the endothelial cell culture time on the graft surface does not lead to increased seeding efficiency. Apparently, this is due to the fact that mature endothelial cells have moderate proliferative activity. After 2 days of culturing endothelial cells in static conditions, most of the graft inner surface was colonized. After 7 days in static conditions, moderate proliferation occurred with the formation of an almost continuous cell layer. When cells were cultured on the prosthetic graft surface in static conditions for more than 7 days (14 and 21 days), a part of cells died with disruption of the cell monolayer.

Under pulsatile flow conditions, we determined the following optimal mode of culturing cell-based vascular graft: 2 days of preliminary culturing in static conditions, 5 days of subsequent culturing in the pulsatile flow unit at the following pulsatile flow parameters: ejection volume 0.7 ml; ejection frequency 20 beats/min; shear stress 2.85 dyn/cm$^2$. The shear stress was gradually increased to the specified value after pre-culturing the cells in static conditions for two days.

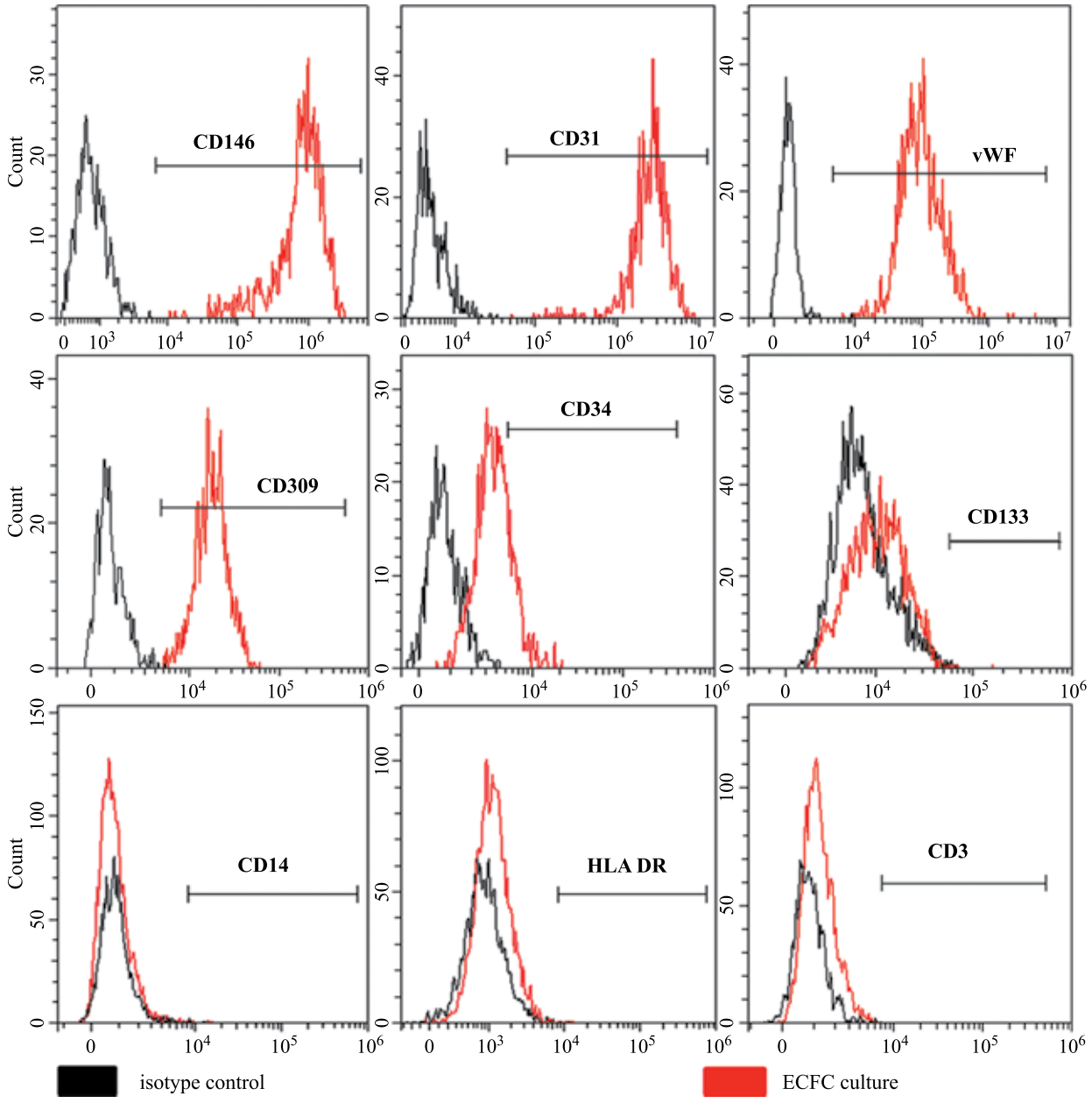


Fig. 1. ECFC culture phenotype. Flow cytometry [16]

## Results of cell seeding of PHBV/PCL/fibrin tubular scaffolds

The viability of endothelial colony-forming cells cultured on the inner surface of PHBV/PCL/fibrin biodegradable scaffolds was fully preserved regardless of the culture conditions. There were also no statistically significant differences in the density of cell populations per unit area (Fig. 3). Results obtained confirm that the chosen culturing regimen had no damaging effect on the endothelial lining, and the fibrin feeder layer provided sufficient adhesive surface characteristics for ECFCs.

Based on the results of staining with specific antibodies, we analyzed the effect of pulsatile flow intensity of 2.85 dyne/cm$^2$ on the phenotype of endothelial colony-forming cells, intercellular contacts, functional activity, cytoskeleton organization, and cell surface adhesion. It was found that ECFCs, which constituted a monolayer on the inner surface of PHBV/PCL/fibrin tubular scaffolds, regardless of the culturing conditions, were characterized by a high level of expression of specific endothelial markers: CD31, CD309, CD144, vWF (Fig. 4). However, under dynamic culturing conditions, these markers were higher, except for CD144 (VE-cadherin). Meanwhile, response to pulsatile flow was reflected in cytoskeletal rearrangements. The average fluorescence intensity of the structural protein F-actin was significantly higher under dynamic culture conditions, ($p < 0.01$). Under the influence of shear stress, chaotic orientation of F-actin microfilaments changed to a preferential orientation of cells in the flow direction, reflecting cell adaptation to shear stress effects. Also adaptive to flow was an increase in expression of the point adhesion protein Talin, ($p < 0.05$). Talin binds integrin receptors and actin cytoskeleton, is a mechanosensitive protein and transmits the mechanical signal of the environment to the cell, allowing it to adapt to the altered conditions.

A change in the morphology of endothelial cells cultured under pulsatile flow conditions was revealed. Under this operating regimen, the cells were stretched along the fluid flow, in close contact with neighboring cells, which was not observed under static conditions.

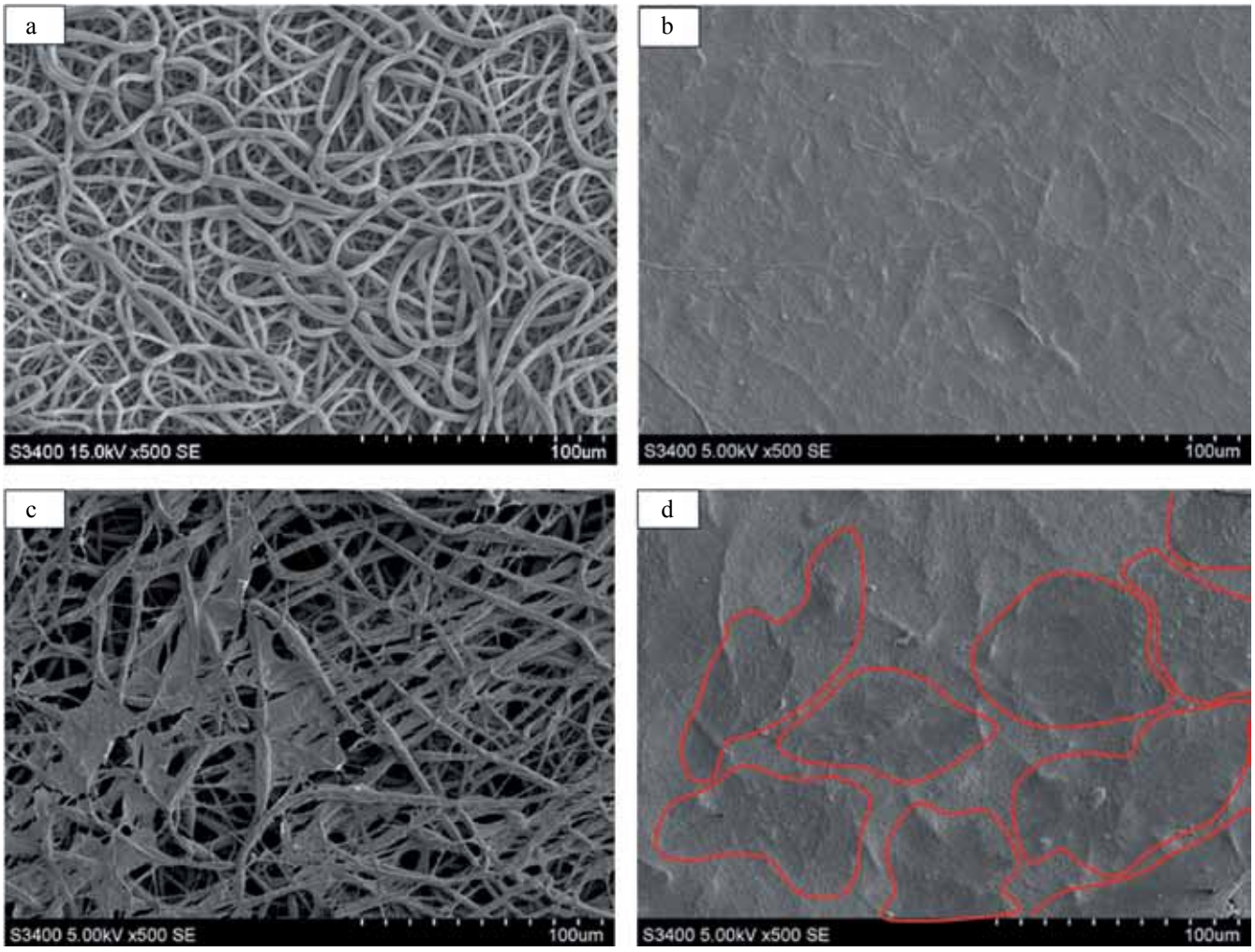


Fig. 2. Scanning electron microscopy of the inner surface of biodegradable vascular prostheses: a – PHBV/PCL; b – PHBV/PCL/fibrin, c – ECFC-populated PHBV/PCL; d – ECFC-populated PHBV/PCL/fibrin. The red line outlines the cell contour. 500× magnification

Endothelial homeostasis, maintained by the correct gene expression profile, affects the long-term patency of the vascular graft. Whole transcriptome sequencing (RNA-Seq) was performed to determine whether shear stress preconditioning has an effect on ECFC gene expression profile.

Sequencing of DNA libraries resulted in paired-end reads of 125 nucleotides long, with total reads ranging from 1–5 billion base pairs and coverage of 9.5–42.7 million reads. After filtering the reads by quality and length, as well as removing adapters, their number remained virtually unchanged. Mapping the library reads to the human genome showed that at least 98.2% of the reads in all samples corresponded to the human genome. Most of the reads (82.2–90.4) were for exons, i.e., the protein-coding part of genes.

Differences between ECFC transcriptomes cultured on the inner surface of PHBV/PCL/fibrin under static and pulsatile flow conditions were found. A total of 185 significant DEGs were identified in ECFC culture. DEGs were annotated using terms suggested in the Gene Ontology database to further disclose their molecular characteristics. DEGs were divided into 125 categories, which

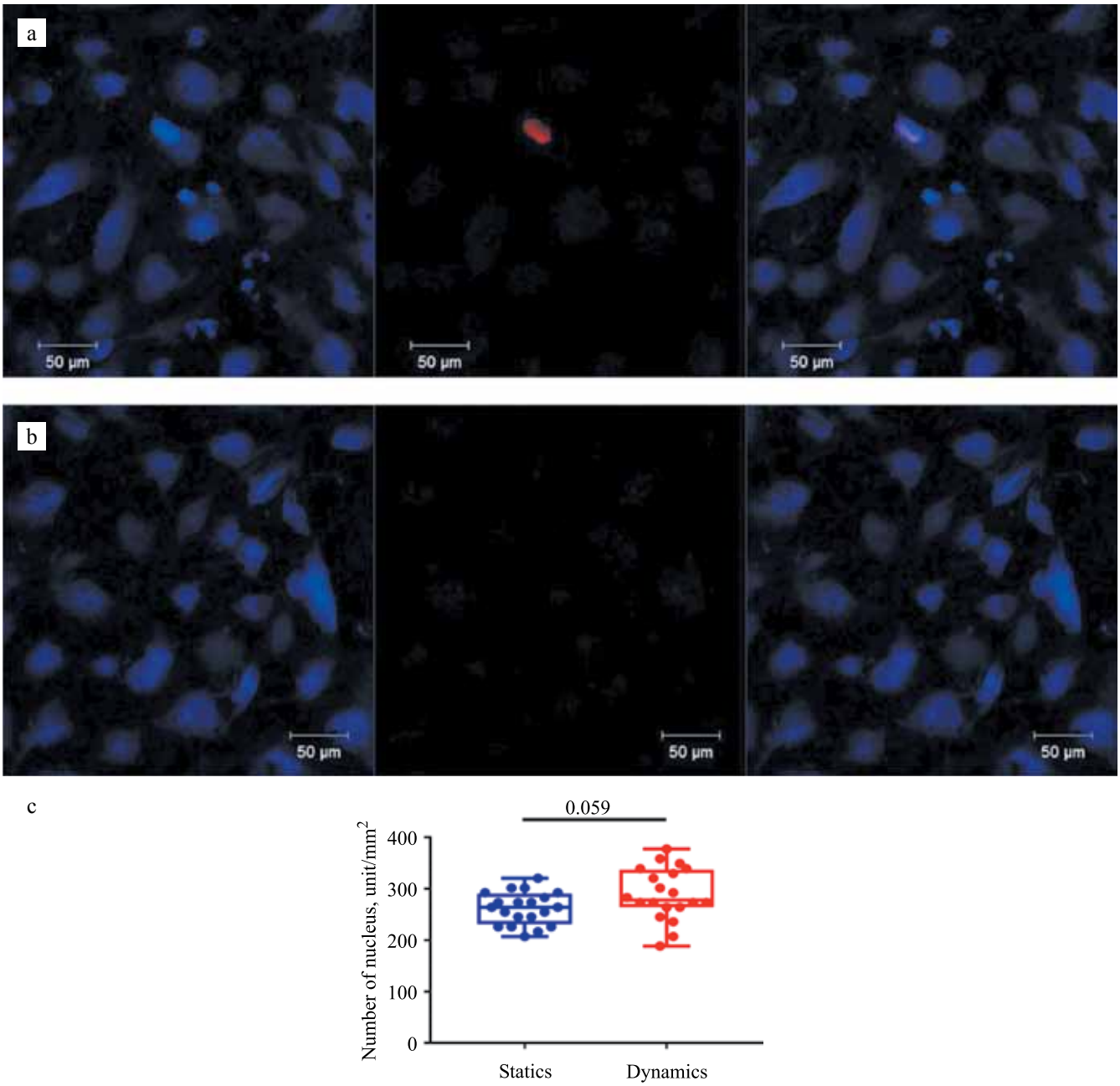


Fig. 3. Viability of human ECFC when cultured for 7 days on the inner surface of PHBV/PCL/fibrin biodegradable vascular prostheses: a – under static conditions; b – under pulsatile flow conditions; c – quantitative analysis of cell population density, $p < 0.05$. Combined staining with Hoechst 33342 fluorescent (blue) and ethidium bromide (red). Confocal laser microscopy, 200× magnification

can be roughly divided into 2 broad groups: endothelial metabolism (62) and processes involved in endothelial biology (63).

A comparative analysis of the differential expression systems associated with the endothelial gene phenotype showed that exposure to pulsatile flow activates metabolism in ECFCs. The Gene Ontology tool was used to detect these differences, which had an advantage when analyzing DEGs in the "endothelial metabolism" category, and determined that 23 DEGs were characterized by increased expression in statics, whereas 39 were characterized by increased expression in dynamics.

Further enrichment of endothelial phenotype-related gene systems by Gene Ontology category enlargement (especially by manual annotation of genes differentially expressed in ECFCs) showed that, in response to pulsatile flow, there is increased expression of gene encoding:

Table 1

**Results of quantitative analysis of immunofluorescence staining of ECFCs cultured under static conditions ("Static") and under pulsatile flow conditions with 2.85 dyne/cm$^2$ shear stress ("Dynamic")**

| | Statics<br>Me (25%; 75%) | Dynamics<br>Me (25%; 75%) |
|---|---|---|
| Mean fluorescence intensity of CD31, AU | 50.5 [40.8; 55.3] | 56.0 [53.5; 62.3] |
| Dyed area of CD309, % | 2.0 [1.8; 2.3] | 3.1 [2.7; 4.3] |
| Mean fluorescence intensity of CD144, AU | 34.5 [31.3; 37.6] | 22.0 [19.6; 23.2] |
| Mean fluorescence intensity of vWF, AU | 5.5 [4.0; 7.3] | 17.5 [13.5; 23.3] |
| Dyed area of Talin, % | 1.7 [1.6; 1.9] | 2.7 [2.1; 3.3] |
| Mean fluorescence intensity of F-actin, AU | 59.7 [52.2; 64.8] | 66.5 [62.7; 71.6] |

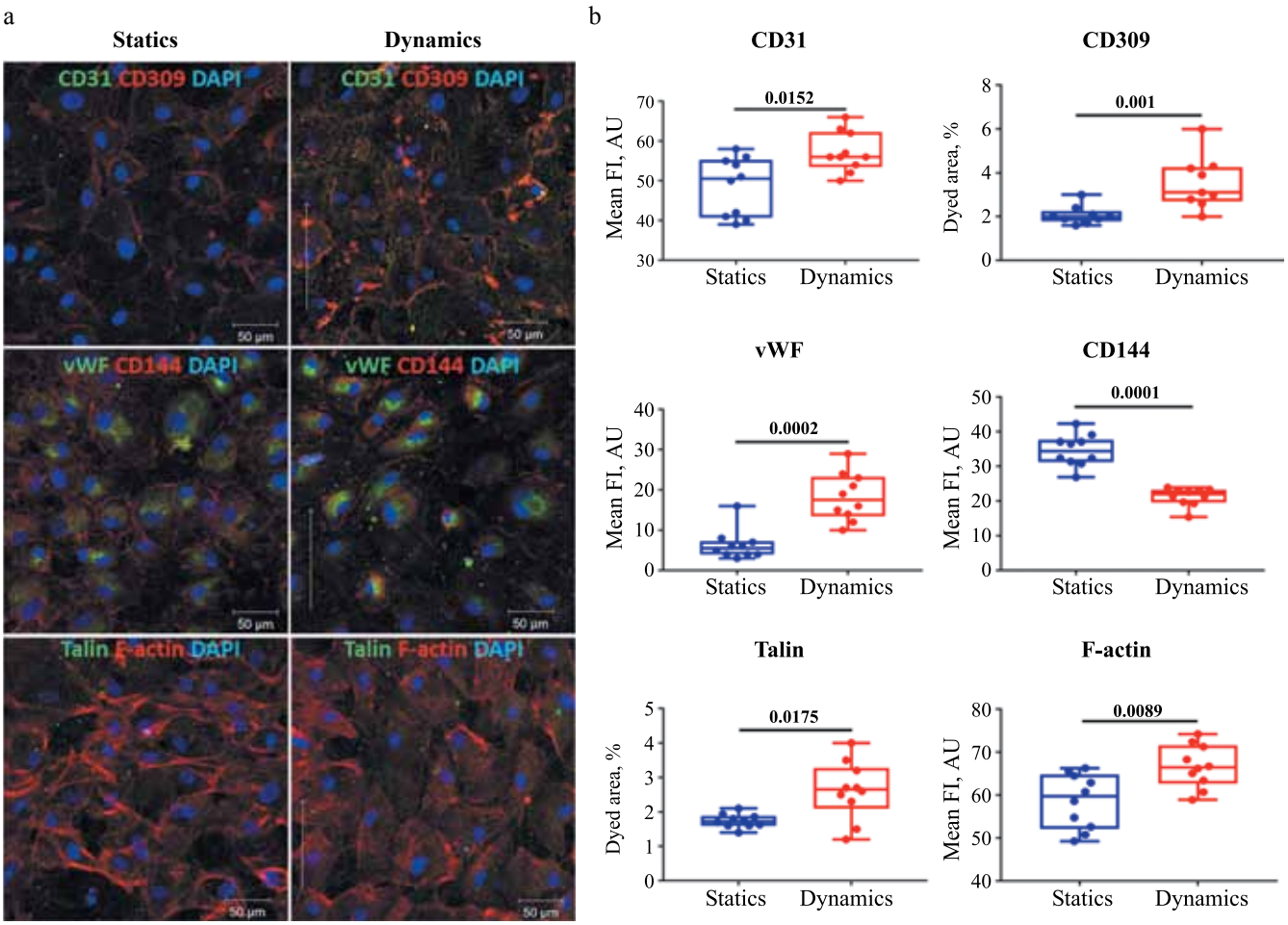


Fig. 4. Combined immunofluorescence staining of ECFCs cultured under static conditions ("Static") and under pulsatile flow conditions with 2.85 dyn/cm$^2$ shear stress ("Dynamic"): CD31/CD309/DAPI; CD144/vWF/DAPI; F-actin/Talin/DAPI. a – representative photographs, scanning confocal microscopy, 200× magnification; b – quantitative analysis. FI is fluorescence intensity

1) proteins responsible for blood vessel development (3 DEGs in pulsatile flow conditions (dynamic) versus 2 DEGs in static conditions);
2) proteins responsible for regulating endothelial integrity (9 DEGs in dynamic versus 6 DEGs in static conditions);
3) proteins stimulating angiogenesis (48 DEGs in dynamic versus 33 DEGs in static conditions);
4) proteins determining endothelial cell proliferation (17 DEGs in dynamic versus 12 DEGs in static conditions);
5) proteins promoting endothelial cell migration (21 DEGs in dynamic versus 15 DEGs in static conditions);
6) proteins responsible for inflammation regulation (20 DEGs in dynamic versus 16 DEGs in static conditions) (Fig. 5).

It was the manual annotation that allowed to establish the differences in genes differentially expressed in ECFCs in response to pulsatile flow in all the gene systems cited.

## DISCUSSION

The native endothelial layer forms a continuous, selectively permeable, thrombosis-resistant barrier between the circulating blood and the vascular wall. It is the endothelium that is mostly subjected to shear stress exerted by blood flow. We have attempted to develop a

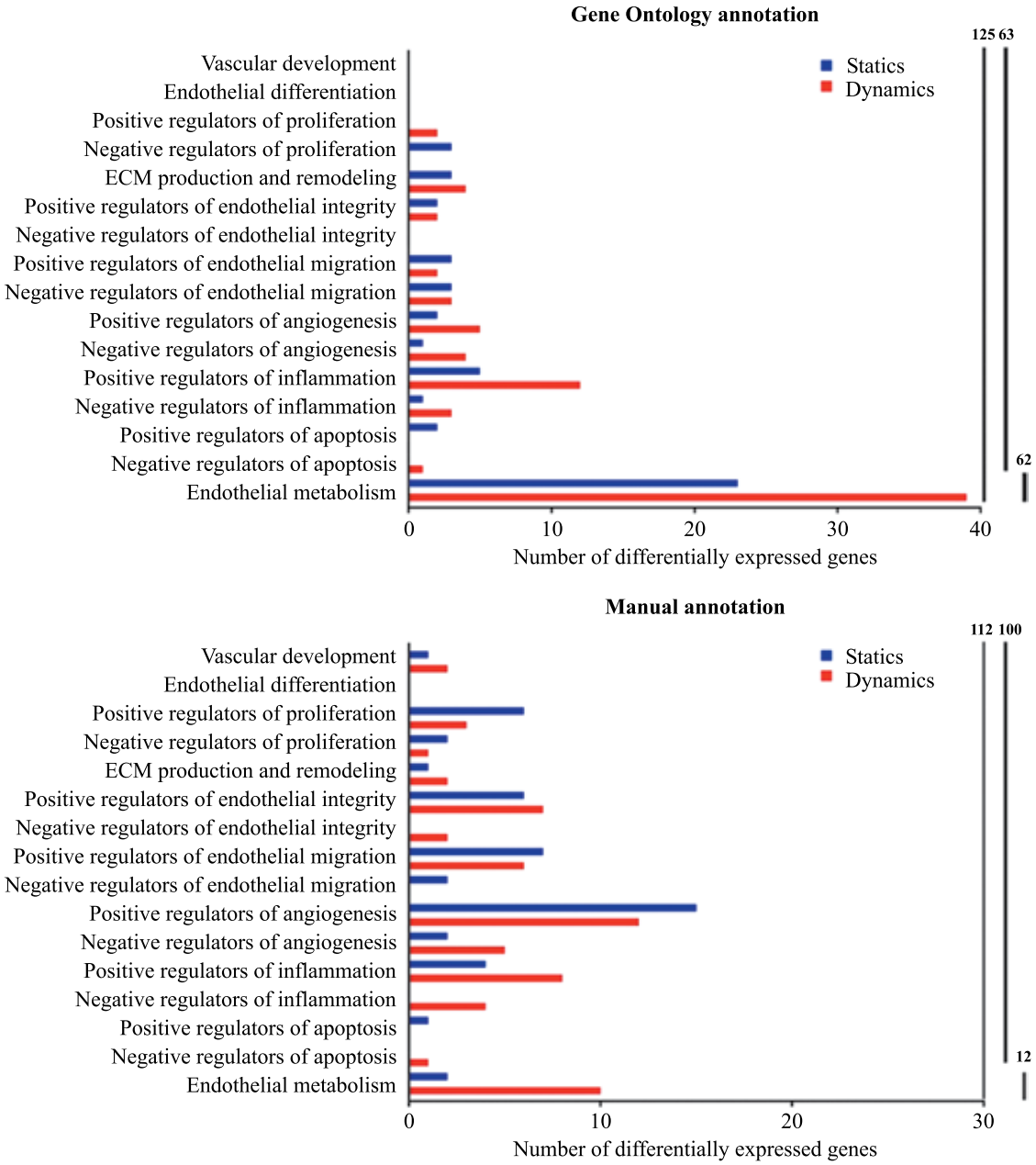


Fig. 5. Qualitative and quantitative comparison of the profile of DEGs in ECFC under static conditions and when exposed to pulsatile flow with 2.85 dyne/cm$^2$ shear stress using bioinformatic tool Gene Ontology and manual annotation. ECM – extracellular matrix

cell-based small-diameter vascular graft under pulsatile flow conditions. The main goal was to obtain a wash-resistant functional endothelial layer on the inner surface of the graft. The efficiency of the selected protocol was evaluated by the formation of endothelial monolayer, its integrity and viability, and the effect of shear stress on endothelial cell phenotype.

Under pulsatile flow, the cells were aligned relative to each other, formed a monolayer and had intercellular interactions. Some of these shear responses, such as cell alignment, were found to be mediated by PECAM-1, VE-cadherin and VEGFR2 receptors [19]. These receptors, which provide adhesion and form a monolayer, carry out mechanotransduction. Laminar shear stress promotes cell elongation and orientation along the vessel wall, activates mechanosensors and intracellular signaling pathways, and induces endothelial gene and protein expression [20]. Thus, shear stress plays a leading role in vascular homeostasis due to its atheroprotective, anticoagulant and anti-inflammatory functions [20–22].

Tondreau et al. obtained complete endothelialization of a decellularized tissue-engineered vascular scaffold produced by self-assembly of the extracellular matrix from fibroblasts. The endothelial monolayer was reconstituted using Human umbilical vein endothelial cell (HUVEC) culture and under flow conditions at a rate of 40 ml/min and a shear stress of 0.65 dyne/m$^2$ for 1 week [23].

In addition, a gradual increase in the applied fluid current from 5 dyne/cm$^2$ to 10 dyne/cm$^2$ was found to promote cell adhesion at the level of 99.31 ± 4.97% after 32 hours of culturing with a nearly complete HUVEC monolayer [24].

Yazdani et al. succeeded in obtaining effective endothelialization at high shear stress. Scaffolds obtained from decellularized porcine carotid arteries (4–5 mm) were populated with autologous endothelial cells and preconditioned for 9 days. Three regimens were tested in the study: low steady shear stress LSS (1.7 dyn/cm$^2$), high steady shear stress HSS (13.2 dyn/cm$^2$), and cyclic high shear stress CSS (13.2 dyn/cm$^2$, 60 bpm). Preconditioning of grafts under HSS and CSS conditions resulted in monolayer formation and stable adhesion as well as cell orientation in the flow direction. In the arteriovenous shunting model, preconditioned grafts under HSS and SSS conditions remained intact, cell morphology was preserved, there was a statistically significant decrease in attachment of blood elements, especially thrombocytes, compared to other conditions. Western blotting demonstrated increased expression of eNOS protein and prostaglandin-I synthase for cells conditioned with cyclic high shear stress compared to cells conditioned with only high shear stress [25].

In a 2016 study by the Melchiorri AJ team, polyglycolic acid (PGA) polymer scaffolds populated with endothelial progenitor cells (EPCs) were subjected to a low shear stress of 0.6 dyne/cm$^2$, similar to the venous system. The researchers found increased proliferation, infiltration, and differentiation of EPCs under dynamic loading. In dynamically cultured grafts, there was increased expression of functional endothelial markers – vWF and VEGF – in comparison to statically cultured EPCs by PCR results [26].

Under normal physiological conditions, endothelial integrity maintains the dynamic balance between mechanical shear stress and biological responses [27]. Shear stresses resulting from various flow patterns initiate various signaling events in the endothelium, including mechanosensitivity, intracellular stress transmission, conversion of mechanical force into biochemical signals, and feedback mechanisms [28–29]. Focal adhesion proteins are a dynamic mechanosensitive multiprotein complex that binds integrin receptors of the extracellular matrix to intracellular actin. This complex is represented by many different proteins (vinculin, zyxin, talin, paxillin), which perform scaffold, adaptor, and regulatory functions [30–31]. The presence of focal adhesion proteins reflects the cell's response to the external environment and allows adhesion of the cellular matrix to adapt to the

Table 2

**Characterization of DNA libraries prepared from ECFC RNA cultured under static and pulsatile flow conditions and their sequencing results**

| Sample ID | DNA-library average, nucleotides | Coverage, million reads | Percent of reads mapped to hg38 | Percent of reads mapped to exons |
|---|---|---|---|---|
| | ECFC *Statics* | | | |
| S1 | 356 | 15.7 | 98.2 | 84.9 |
| S2 | 354 | 14.5 | 98.4 | 84.5 |
| S3 | 389 | 42.7 | 98.2 | 82.2 |
| S4 | 360 | 9.5 | 98.2 | 84.1 |
| | ECFC *Dynamics* | | | |
| D1 | 346 | 15.1 | 98.2 | 88.8 |
| D2 | 351 | 15.5 | 98.3 | 89.8 |
| D3 | 353 | 15.3 | 98.3 | 90.4 |

composition and mechanical properties of the extracellular matrix [32–34].

Culturing ECFCs on the surface of PHBV/PCL/fibrin scaffolds under pulsatile flow conditions changed the orientation of cells in the flow direction, resulted in increased expression of specific endothelial markers CD31, CD309 and vWF, and stimulated the expression of structural protein F-actin and focal adhesion protein Talin. In addition, endothelial cells exhibited increased expression of genes encoding proteins that:

– ensure blood vessel development;
– are responsible for the regulation of endothelial integrity;
– stimulate angiogenesis;
– determine endothelial cell proliferation;
– promote endothelial cell migration;
– responsible for the regulation of inflammation.

## CONCLUSION

Personalized biodegradable, cell-based, small-diameter vascular graft can be created when autologous fibrin is used as a feeder layer, and an autologous ECFC culture is used for cell colonization. Endothelial lining cells formed on the internal surface of the graft under shear stress conditions possess increased synthetic activity, adhesion ability, and are oriented along the pulsatile flow path. Shear stress also affects cell culture transcriptome, enhancing endothelial ability to migrate, proliferate, and maintain integrity.

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*The authors declare no conflict of interest.*

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# EXPRESSION OF TISSUE INHIBITORS OF METALLOPROTEINASES TYPE 1 AND TYPE 2 IN THE LEAFLETS OF EXPLANTED BIOPROSTHETIC HEART VALVES: A NEW PATHOGENETIC PARALLEL BETWEEN STRUCTURAL VALVE DEGENERATION AND CALCIFIC AORTIC STENOSIS

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**Objective:** to study cellular and lipid infiltration, as well as the expression of tissue inhibitors of metalloproteinases (TIMP) types 1 and 2 in biological prosthetic heart valves (BPHVs) explanted due to dysfunction. **Material and Methods.** We examined 17 leaflets from 6 BPHVs, dissected from the aortic and mitral positions during valve replacement. For microscopic analysis, fragments of the BPHV leaflets were frozen and serial sections were made using a cryotome. In order to study cellular infiltration and the degree of degenerative changes in the prosthetic biomaterial, the sections were stained with Gill's hematoxylin and eosin; Oil Red O stain was used to assess lipid deposition. Immunohistochemistry was used for cell typing and detection of TIMP-1/-2. The stained samples were analyzed by light microscopy. **Results.** Cellular and lipid infiltration of xenogeneic tissues was detected in all BPHV flaps studied. Recipient cells coexpressed pan-leukocyte and macrophage markers PTPRC/CD45 and CD68. Positive staining for TIMP-1/-2 co-localized with cell clusters but was absent in acellular sections. **Conclusion.** Cells infiltrating xenogeneic BPHV tissues express TIMP-1/-2. This suggests that BPHV immune rejection pathophysiology is partially similar to that of calcific aortic stenosis.

*Keywords: bioprosthetic heart valves, structural valve degeneration, calcific aortic stenosis, cellular infiltration, lipids, tissue inhibitors of metalloproteinases.*

## INTRODUCTION

Despite advances in medical technologies, there are still no effective methods of conservative treatment of severe valve pathologies; so affected heart valves are replaced by mechanical or biological prostheses [1]. Bioprosthetic heart valves (BHVs) are made of chemically stabilized animal tissues [2]. They compare favorably with their mechanical counterparts with low thrombogenicity, but their biological component is subject to structural degeneration [3, 4]. The latter is manifested by dissociation, fragmentation and calcification of the fibrous component of the extracellular matrix (ECM) of the flail apparatus, becoming the cause of BHV regurgitation or stenosis [3, 4]. Despite modern achievements in biomaterial chemical modification methods and improvement in BHV design, this problem remains unresolved; up to half of prosthetic valves require replacement as early as 15 years after implantation in patients [1].

Interestingly, the results of modern studies indicate that a number of pathophysiological features of structural degeneration of BHV biomaterial and calcific aortic stenosis (CAS) are similar. Thus, degenerative changes arising in the affected native aortic valve (AV), associated with fibrosis and calcification, are caused by lipid deposition and macrophage infiltration of the leaflets [5]. Recent studies demonstrate that during degeneration of BHV biomaterial, lipids and immune cells also accumulate in the tissue, which is accompanied by production of matrix metalloproteinases (MMPs) [6, 7]. It is assumed that these processes can contribute significantly to degradation of ECM prosthetic biomaterial [8]. In this regard, in-depth study of pathophysiological patterns of BHV degeneration is of significant interest, as it potentially opens the way to development of drug therapy aimed at slowing down degeneration of both native heart valves and their substitutes.

One of the factors regulating ECM remodeling of native AVs includes tissue inhibitors of metalloproteinases (TIMPs) types 1 and 2, overexpressed in CAS [9, 10]. TIMP-1/-2 are multifunctional proteins that, in addition to inhibiting MMPs, act like signaling molecules involved in regulation of apoptosis, cell proliferation and differentiation [11, 12]. Production of TIMP-1/-2 cells, as well as MMPs, indicates their ECM remodeling activity. To date, there have been no published data on TIMP expression in BHV tissues.

The objective of this work is to study cellular and lipid infiltration of leaflets explanted due to BHV dys-

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function, and to detect TIMP-1/-2 expression in the implant tissues.

## Material and methods

For this study, we used epoxy-treated BHVs manufactured by NeoCor (Russia), excised from aortic or mitral positions in 6 patients during valve repair operations in 2019–2020. The mean age of recipients with primary valve replacement was 54.5 ± 10.5 years. The studied BHVs lasted for an average of 12 ± 8 years. The study was approved by the local ethics committee of the Research Institute for Complex Issues of Cardiovascular Diseases. BHVs were included in the study subject to the patients' signed voluntary informed consent. Endocarditis as a cause of dysfunction was a criterion for excluding BHVs from this study.

After macroscopic description of BHVs obtained during reoperations, samples were prepared for microstructural study of the material. From each BHVs, the central part of 1–3 flaps was taken from the base to the free edge, as well as areas with pronounced degenerative changes, which were frozen at –140 °C. Then, using a Microm HM 525 cryotome (Thermo Scientific, Germany), serial 6 ± 1 μm-thick sections were prepared and placed on slides, 4–6 pieces at a time. A total of 17 specimens from 6 explanted BHVs were studied, including xenoaortic bioprosthetic valves KemCor (n = 2) and PeriCor (n = 2), as well as the pericardial models UniLine (n = 1) and TiAra (n = 1).

In order to study the intensity of infiltration by recipient cells and determine the degree of degenerative changes in the prosthetic biomaterial, the sections were stained with Gill's hematoxylin and eosin in accordance with a protocol optimized for circulatory system tissues [13]. The presence of lipids in the structure of BHV biomaterial was determined by staining the sections with Oil Red O (Abcam, UK). For this purpose, the sections were fixed in 4% paraformaldehyde (Sigma-Aldrich, USA), then washed with phosphate-buffered saline (PBS) (Sigma-Aldrich, USA) and incubated in isopropanol for 5 minutes. Then they were stained with Oil Red O for 15 minutes, washed with 60% isopropanol, and contrasted with Mayer's modified hematoxylin (Abcam, UK). Finally, the sections were washed in tap water and then in double-distilled water and covered with Mowiol (Sigma-Aldrich, USA).

For cell typing and TIMP-1/-2 detection, we used manual immunohistochemical staining using antibodies against PTPRC/CD45, CD68, TIMP-1, and TIMP-2 (Abcam PLC, UK). Before staining, sections were fixed for 10 minutes at room temperature with 4% paraformaldehyde followed by three washes (5 minutes each) in PBS (pH 7.4) on a shaker (Polymax 1040, Heidolph, 25 rpm). Immunohistochemical reaction was performed using NovoLink Polimer Detection System kit (Leica Microsystems Inc., USA) according to the manufacturer's modified protocol. First, endogenous peroxidase was blocked with 4% hydrogen peroxide solution (Peroxidase Block) for 5 minutes. The sections were then washed twice in PBS and blocked for 60 minutes with 0.4% casein saline with adjuvant reagents (Protein Block) for nonspecific antibody binding. Primary antibodies were diluted according to the manufacturer's protocol in 1% bovine serum albumin saline in proportions of 1 to 1000, 1 to 2000, and 1 to 3000 for antibodies against CD68, TIMP-1/2, and PTPRC/CD45, respectively. The sections were incubated with antibodies in the dark at +4 °C overnight, then washed three times in PBS. When stained for CD68 and TIMP-2, the sections were additionally treated for 30 minutes with anti-mouse rabbit antibodies (Post Primary), followed by three washes in PBS. Next, the sections were incubated for 30 minutes with anti-rabbit antibodies (Novolink Polymer), and then again washed three times in PBS and treated for 2 minutes with 0.087% diaminobenzidine solution. Then, the sections were washed with double-distilled water and placed in hematoxylin (from the kit) for 10 minutes. After that, we blued the sections in running water (5 minutes), dehydrated them in three shifts of 95% ethanol (5 minutes each), and cleared them in three shifts of xylene (5 minutes each), concluding with Vitrogel (BioVitrum, Russia) under a coverslip. Intact epoxy-treated xenogeneic tissues were used as controls: porcine aortic valve leaflets and bovine pericardium purchased from Neocor (Kemerovo, Russia). Also, one slice was isolated on each glass with negative control of primary and secondary antibodies.

Samples with histological and immunohistochemical stains were analyzed using an AxioImager.A1 light microscope (Zeiss, Germany). The images were processed using AxioVision software (Zeiss, Germany).

## RESULTS

### Macroscopic description of explanted BHVss

BHVs included in this study showed signs of primary tissue failure in the form of leaflet flail ruptures in the commissural area, perforations as well as signs of calcification (Fig. 1). Pannus fixation of the cusps along the frame struts on the outflow side was noted, which contributed to some limitation in their mobility. Pannus was noted in 4 out of 6 BHVs, while calcification was observed in varying degrees for all the studied implants.

### Histological examination of flaps of explanted BHVs

At the microstructural level, degenerative changes in the biomaterial were in the form of loosening and fragmentation of collagenous fibers. Infiltration of prosthetic biomaterial by recipient cells was detected in all examined samples (Fig. 2). Cellular infiltrates were localized predominantly on the surface or in the loosened pre-surface layers of the xenogeneic ECM near the base

of the flaps. Cell penetration deep into the biomaterial was noted only for xenoaortic prostheses, and the most massive cellular infiltrates were recorded near perforations and large calcifications, where pronounced ECM debridement was observed. The biomaterial was affected to a greater extent by cellular infiltration on the outflow side.

Lipid infiltration in varying degrees was noted in all samples studied. Lipid localization did not depend on the presence of cells in the ECM. Samples of intact epoxy-treated xenogeneic tissues showed no positive staining with Oil Red O dye.

## Immunohistochemical analysis of flaps of explanted BHVs

Immunohistochemical staining of sections demonstrated that most cells in the infiltrates coexpressed the panleukocyte and macrophage markers PTPRC/CD45

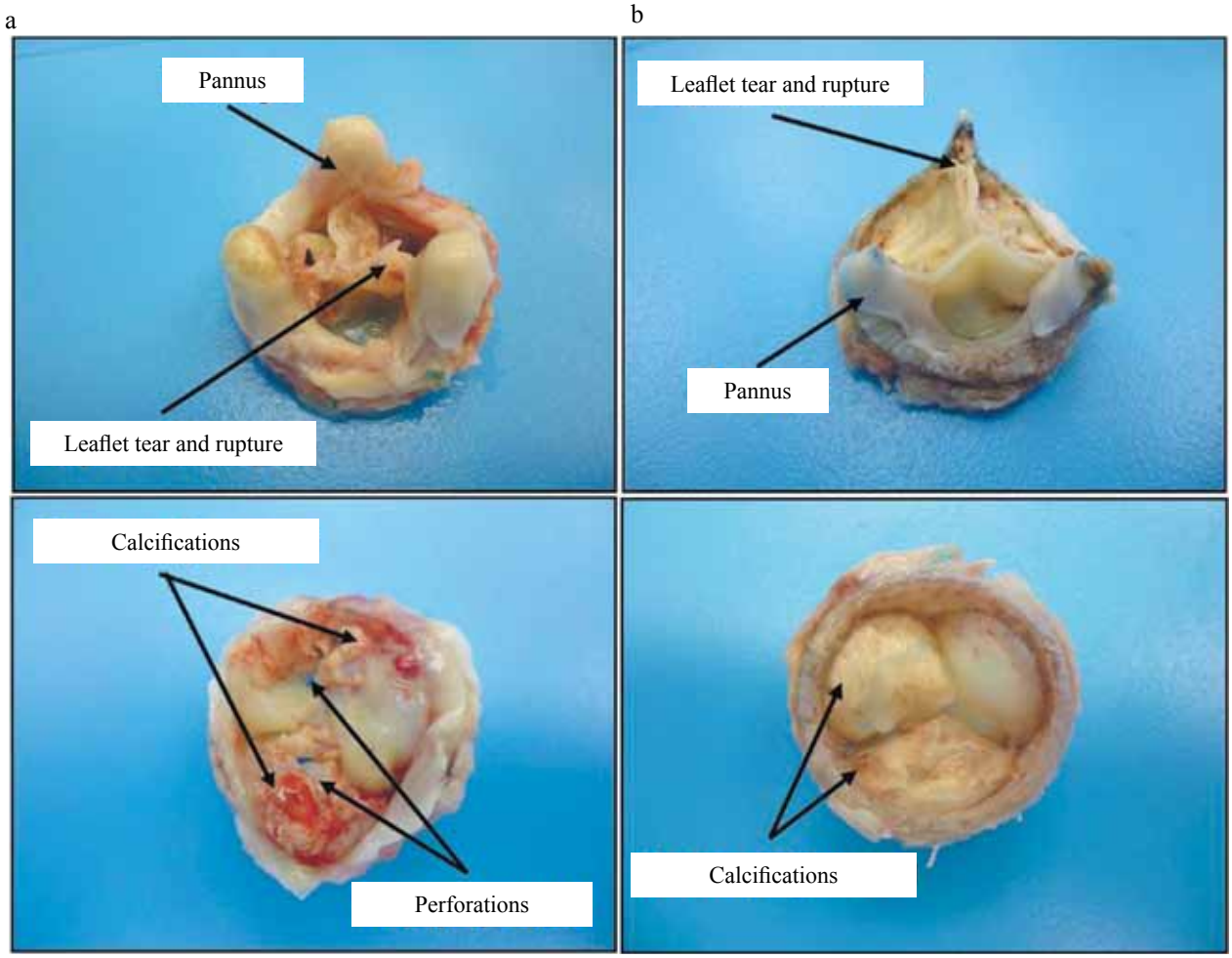


Fig. 1. Heart valve bioprostheses removed due to dysfunction: a – xenoaortic prosthesis (PeriCor); b – xenopericardial prosthesis (UniLine); Upper and lower rows – view from the outflow and inflow side, respectively

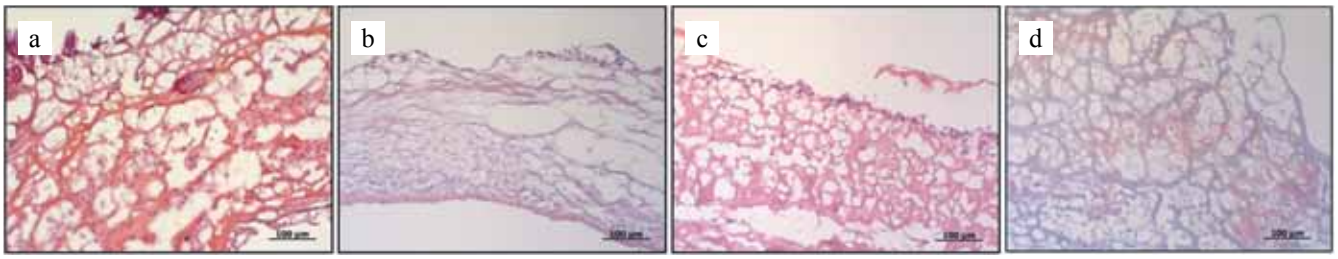


Fig. 2. Cellular and lipid infiltration of heart valve bioprosthesis leaflets: a – large cellular infiltrate near the calcific (Gill's hematoxylin and eosin staining); b and c – cellular infiltration of the surface layers of xenogeneic tissue of the xenoaortic and xenopericardial prosthesis respectively (Gill's hematoxylin and eosin); d – lipid spots in the thickness of the prosthetic leaflets (Oil Red O stain)

and CD68, allowing them to be identified as macrophages (Fig. 3). It was also possible to identify these cells as a source of TIMP-1/-2. Positive staining for these molecular agents was noted in all samples studied in co-localization with cellular infiltrates (Fig. 4). No staining for TIMP-1/-2 was observed in acellular ECM.

No positive staining for PTPRC/CD45, CD68, or TIMP-1/-2 was observed in all controls.

## DISCUSSION

Evidence obtained is consistent with the results of other original studies that investigated the cellular and

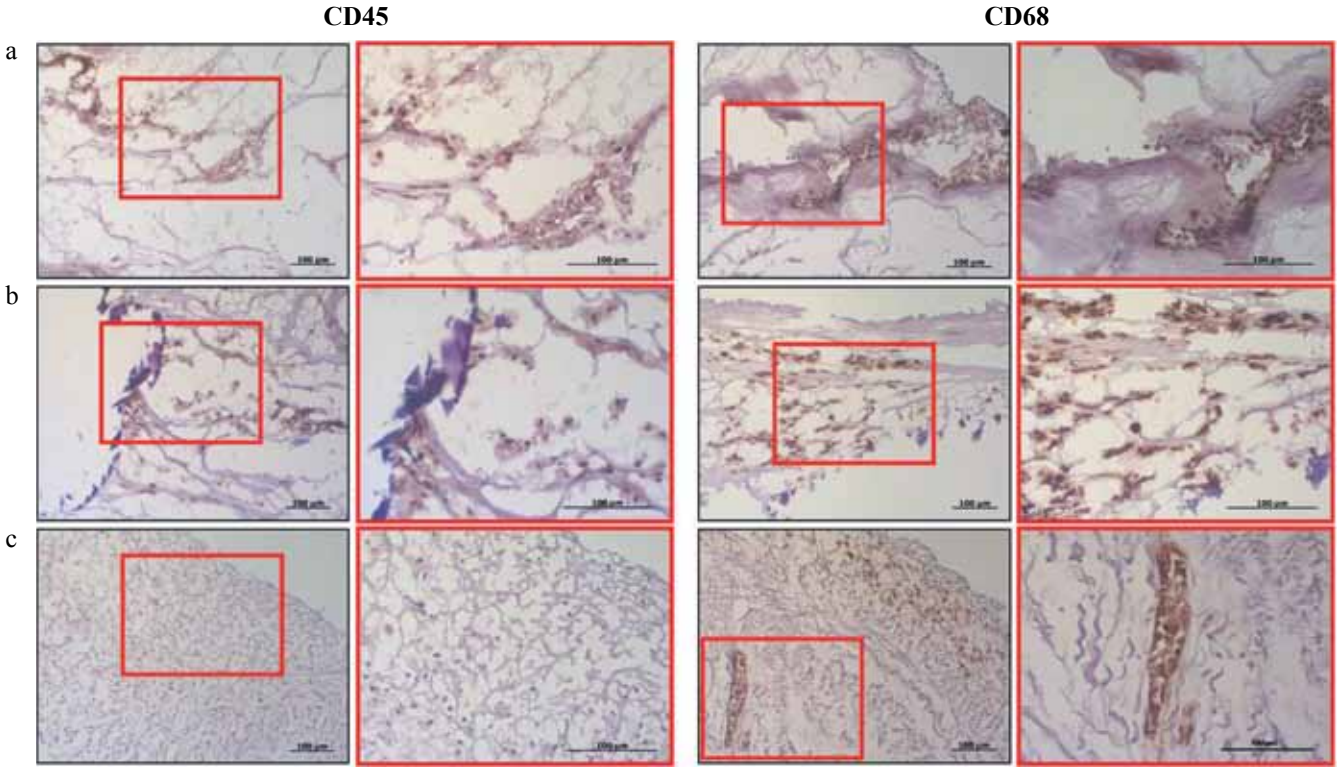


Fig. 3. Results of immunohistochemical staining of cardiac valve bioprosthesis leaflets for PTPRC/CD45 and CD68: a – clusters of immune cells in the loosened subsurface layers of xenogeneic prosthesis leaflets; b – large inflammatory infiltrates near calcium deposits in xenogeneic prosthesis leaflets; c – diffuse cellular infiltration of the surface layers of xenogeneic prosthesis leaflets. Pay attention to the penetration of macrophages deep into the xenopericardium due to the pseudovessels

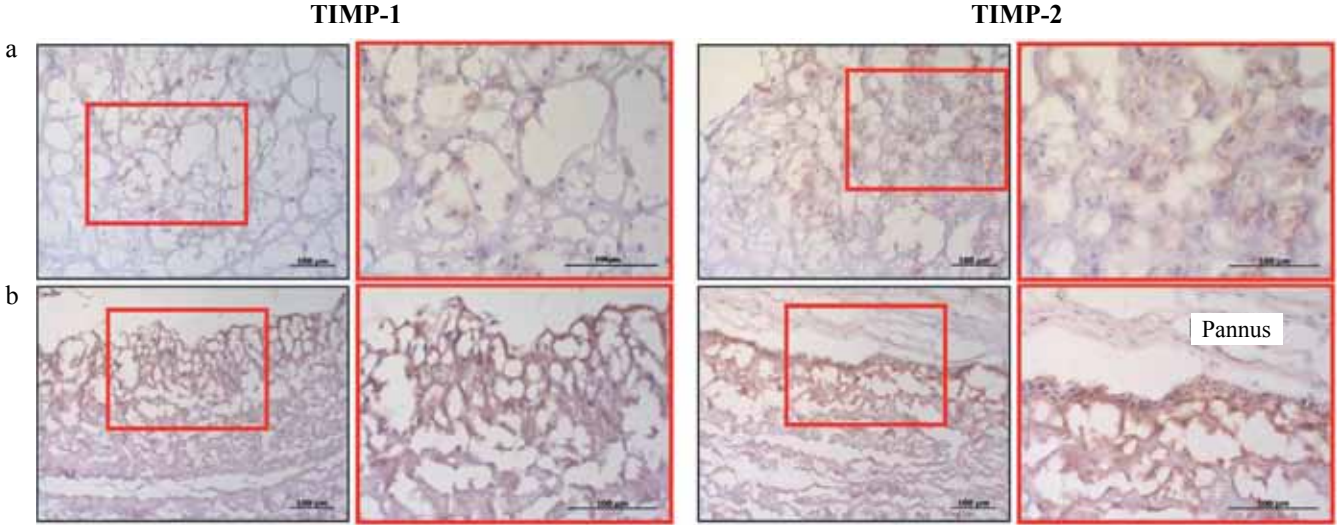


Fig. 4. The results of immunohistochemical staining of heart valve bioprosthesis leaflets for TIMP-1/-2: a – staining of infiltrates in the thickness of xenoaortic prosthesis leaflets; b – staining of infiltrates located in the surface layers of the xenopericardial prosthesis biomaterial

lipid infiltration of BHV xenogeneic tissues [6, 7]. It also confirms the conclusions made by us earlier [14]. Staining of most cells for PTPRC/CD45 and CD68 markers indicates the predominance of macrophages in the composition of cell infiltrates formed in the BHV flaps. It is important to note that macrophages dominate regardless of the timing of implant functioning. This indicates a chronic type of the inflammatory response arising in response to BHV implantation. Interestingly, inflammation in the native AV leaflets, which is the driving force of CAS, is also chronic in nature [5].

Lipid deposition in the flaps of BHVs of foreign and domestic models has been previously studied, although the exact contribution to the structural destruction of the prosthetic biomaterial has not yet been established [6, 15]. Potentially, lipid accumulation can promote activation of oxidative processes in the flaps. It has been demonstrated that the biological component of BHVs is highly susceptible to degradation through oxidation-dependent mechanisms [16]. In addition, lipid uptake by macrophages stimulates the latter to degenerate into foam cells, which are found in the tissues of BHVs [15]. Foam cells secrete a wide range of bioactive compounds, including chemokines, mitogens and growth factors, so they can stimulate fibroblast migration, proliferation and synthesis of connective tissue. This process is intrinsic to the pathogenesis of CAS, where it is responsible for fibrosis and sclerosing of AV leaflets [5]. Apparently, it is also partly responsible for pannus formation on the surface of BHVs.

We found for the first time that cells infiltrating BHV flaps express TIMP-1/-2 cells, but the clinical significance of this phenomenon is still unknown. Experiments on knockout mice show that overexpression of TIMP-1/-2 has an antiatherogenic effect by suppressing the activity of MMPs and reducing the inflammatory response in the vessels [17]. It is suggested that TIMP-1/-2 may play a similar role in native AV lesions, with increased expression of these factors being a reflex mechanism protecting ECMs against proteolytic degradation [10]. TIMP-1/-2 expression in BHV tissues can have both positive and negative significance. We previously found that macrophages infiltrating BHVs produce MMPs [14]. Thus, TIMP-1/-2 can protect the prosthetic biomaterial from proteolysis. Meanwhile, decreased activity of proteolytic enzymes may promote proliferation of connective tissue over the BHV flaps. Further research is needed to test the hypotheses presented.

## CONCLUSION

The xenogeneic biomaterial of BHVs is subject to lipid accumulation as well as infiltration by immune cells, which in turn express TIMP-1/-2. These observations suggest that there is a partial similarity in the pathophysiology of structural degeneration of BHV biomaterial with that of CAS. The direct role of TIMP-1/-2 in the development of BHV dysfunction is currently unknown and requires to be studied.

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*The authors declare no conflict of interest.*

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# THE EFFECT OF EXOGENOUS PEROXIREDOXIN 6 ON THE MORPHOFUNCTIONAL STATE OF ISOLATED RAT KIDNEY

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**Objective:** to investigate the role of peroxiredoxin 6 (PRX6) in preserving the morphofunctional state of ischemic isolated kidney during perfusion. **Materials and methods.** The model of an isolated perfused rat kidney was used. Ischemia time was 5 and 20 minutes, perfusion was 50 minutes. To evaluate the effectiveness of PRX6 at different ischemia times, we used the conventional criteria of kidney function and histological methods. **Results.** During short warm ischemia times, exogenous PRX6 improves the morphofunctional state of an isolated kidney during perfusion. During this period, the main criteria for functioning of the isolated ischemic kidney reach acceptable values, renal parenchyma is without severe damage. By the end of perfusion, there was an increase in urine flow rate, glomerular filtration rate, fractional glucose reabsorption, urine urea concentration and proportion of primary urine from 1.5 to 2 times compared with the control lesion. At 20-minute ischemia, the isolated kidney can be recognized as non-viable according to the functioning criteria; the positive effect of PRX6 is leveled. **Conclusion.** The use of recombinant peroxiredoxin 6 for preserving the morphofunctional state of isolated kidneys can be an effective approach in preventing ischemia–reperfusion injury.

*Keywords: isolated kidney, ischemia, perfusion, peroxiredoxin.*

## INTRODUCTION

The popular use of an isolated kidney as a subject of research is down to the convenience of its use for studying renal secretory function, drug metabolism, screening of clinically relevant drug interactions and many aspects of renal metabolism without systemic influences such as blood pressure, hormones or nerve innervation [1–3]. An isolated kidney is an ex vivo model that consists of a whole kidney isolated from the vasculature. Two modifications of the model can be distinguished. Most researchers use the modification described by Nishitsutsuji-Uwo et al. – kidney perfusion through the superior mesenteric artery without ischemia period [4, 5]. The second modification is kidney perfusion retrogradely, through the abdominal aorta without an ischemia period [3, 6]. To interpret the results, it is important that the isolated kidney functions throughout the perfusion period. A number of criteria are used to assess the functioning [1]. In this work, we used a retrograde perfusion model of the kidney; the principal point was the presence of ischemia period of the isolated kidney.

Ischemia and subsequent organ reperfusion activate pathological processes; they trigger an avalanche-like growth of reactive oxygen species and development of oxidative stress, thereby leading to structural and functional tissue damage [7, 8]. Ischemia-reperfusion injury (IRI) is the main cause of primary graft dysfunction and reduced graft viability [9]. In this case, to reduce the concentration of reactive oxygen species and reduce the lesion of isolated organs, the use of antioxidant drugs can be the main therapy direction. In this regard, the use of peroxiredoxin antioxidant enzymes can be a promising solution due to their wide distribution in the body and multifunctionality [10–13]. Among the family members, peroxiredoxin 6 (Prx6) has been the subject of the largest number of studies indicating its protective role in free-radical pathologies [8, 14–16], including in the protection of isolated organs from IRI [17, 18]. Given the protective role of Prx6, the possibility of its application for preserving the morphofunctional state of isolated ischemic kidney should be studied.

The **objective** is to study the role of peroxiredoxin in preserving the morphofunctional state of ischemic isolated kidneys during perfusion.

## MATERIALS AND METHODS

Male Wistar rats weighing 230 g were used in the experiments. The animals were kept in a vivarium at the Institute of Cell Biophysics (ICB), Pushchino, Moscow. Experiments with the laboratory animals were conducted in accordance with the provisions of the 1986 European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. The main document regulating the conduct of this study is the ICB Guidelines for Working with Laboratory Animals, No. 57. dated December 30, 2011.

Recombinant Prx6 was obtained at the Laboratory of Reception Mechanisms, Institute of Cell Biophysics, according to the previously described method [19].

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We used the isolated perfused kidney (IPK) model proposed by J. Czogalla el al. with some modifications [3]. The rats were anesthetized by intravenous injection of 0.5 ml of 3.5% (3 mg/kg) Zoletil 100. Heparin (5000 U/ml) was used to prevent blood clotting. Ischemia was initiated by decapitation. Two time periods of ischemia were chosen: 5 and 20 minutes. The perfusion time was 50 minutes. DMEM culture medium (GIBCO, Invitrogen) with 4.5 g/L glucose content was used as the perfusion solution. The medium included urea (5 mM/liter), creatinine (80 μmM/liter), 6% BSA, and 0.6% BSA. Before perfusion, the solution was oxygenated with an $O_2/CO_2$ mixture (95%/5%), pH 7.4. Solution temperature was subnormothermic (30–35 °C). The isolated kidney perfusion algorithm is shown in Fig. 1.

*Ischemia period.* With the onset of ischemia, within 5 minutes, the right kidney was isolated from the common vascular system by consecutive application of ligatures on the vessels and catheterization of the renal artery through the abdominal aorta. Additionally, the inferior vena cava was dissected and then catheterized. The right ureter was isolated, catheterized and placed in a urine collection tank [3, 20]. After 5 minutes of ischemia, blood was removed from the vascular bed of the isolated kidney by flushing it with a perfusion buffer for 5 minutes. To determine the effects of exogenous peroxiredoxin 6, 0.2 mg/ml Prx6 was added to the perfusion buffer and perfused in the isolated kidney during blood removal from the vascular bed. Perfusion rate at this stage was 3 ml/min. The exogenous Prx6 concentration in the perfusion solution was chosen based on previously obtained data on effective reduction of IRI of the small intestine and kidneys using Prx6 [8, 14].

*Perfusion period.* At the end of ischemia, the isolated kidney was perfused for 50 minutes with a perfusion buffer. There was no Prx6 in the perfusion buffer at this stage. The first 20 minutes of perfusion was the stabilization period. Throughout the perfusion period, urine was collected from the ureter every 10 minutes. At the end of perfusion, the renal tissue was fixed for further examination. All rats were divided into 10 groups (N = 5) (Table 1).

To assess the effectiveness of Prx6 as a means of preserving the morphofunctional characteristics of the isolated kidney throughout the perfusion period, several generally accepted functioning criteria were analyzed [1]. The magnitude of these criteria was determined every 10 minutes in the studied urine samples.

– Perfusion pressure, mmHg. (PP) was maintained at 90–100 mmHg and regulated by changing perfusion flow rate (PFR). PFR was considered satisfactory if it corresponded to normal renal blood flow rate – 4–5.3 ml/min·g of the kidney [21].
– Urine flow rate (UFR), μl/min, was calculated by dividing the collected volume of urine from the ureter by the time. The first urine collection was initiated 20 minutes after perfusion had started, since the first 15 minutes of urination was irregular. UFR values below 30 μl/min are unsatisfactory and indicate the absence of ultrafiltration processes [1].
– Glomerular filtration rate (GFR), ml/min·g, was determined by creatinine clearance using the equation [5]:

$$GFR = \frac{M_{Cr} \times UFR}{P_{Cr}},$$

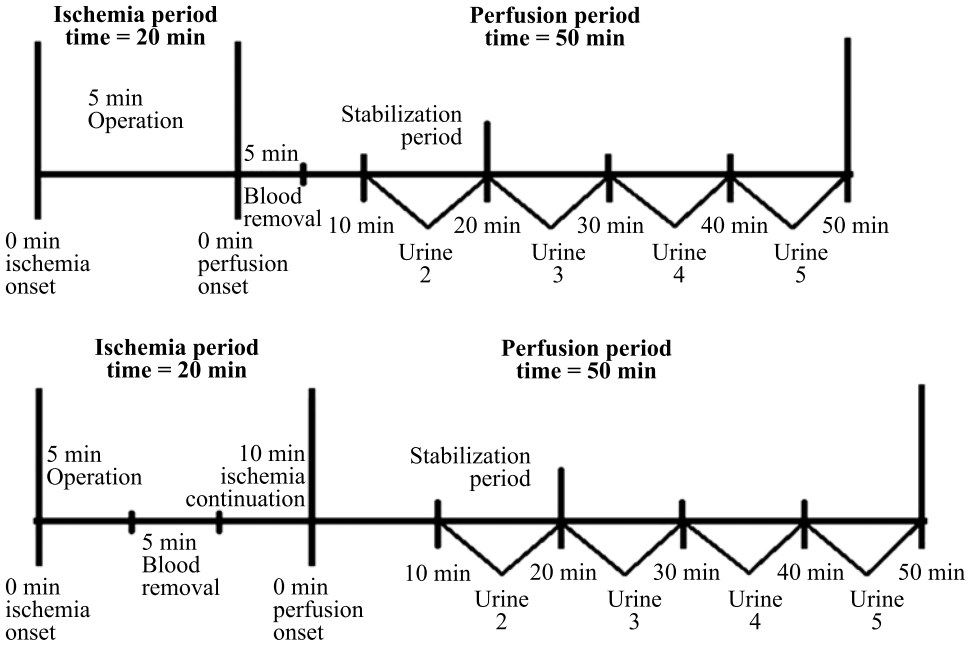


Fig. 1. Isolated kidney perfusion algorithm: а – ischemia time 5 min; б – ischemia time 20 min. Prx6 (0,2 mg/ml) was added to perfusion buffer at the stage blood removal. There is no Prx6 in the perfusion buffer during the perfusion period

$M_{Cr}$ is urine creatinine level (mg/ml), UFR is urine flow rate (ml/min), and $P_{Cr}$ is perfusate creatinine concentration (mg/ml). The minimum allowable value for GFR is >0.5 mL/min [6]. Creatinine levels in the investigated urine and perfusate samples were determined on Reflotron Plus device (Roche Diagnostics, Switzerland) every 10 minutes.

– Reabsorbed glucose fraction (RGF), %, is expressed as the fraction of glucose from ultrafiltrate reabsorbed by the kidneys [1, 5]:

$$RGF = 1 - \frac{UFR \times M_{Gl}}{GFR \times P_{Gl}},$$

$M_{Gl}$ is urine glucose level, $P_{Gl}$ is perfusate glucose level, GFR is glomerular filtration rate. Glucose levels in tested urine samples were determined on Accu-Chek glucometer (Roche, Germany) every 10 minutes. RGF reflects the functionality of proximal channels and should be at least 90% in IPK model [1, 2].

– Terminal urine percentage (TUP), %, is expressed as a ratio of terminal urine amount in the sample to GFR. This criterion reflects the % of ultrafiltrate that is excreted as terminal urine [22].

– The urine urea level (UUL), μmol/10 min, was measured as urea concentration in urine sample multiplied by the amount of secondary urine in the sample. Urea concentration in the tested urine samples was determined on a Reflotron Plus device (Roche Diagnostics, Switzerland) every 10 minutes. Urea concentration in perfusate was 5 mM.

Renal tissue was subjected to histological examination, including H&E staining of paraffin sections of renal tissue (VITROSTAIN Biovitrum, Russia). Slice thickness was 3 microns. Microscopic analysis of sections was performed on a Leica DM 6000 microscope with a Leica DFC 490 digital camera.

Statistical analysis and plotting were performed using SigmaPlot 11.0 software (Systat Software Inc.). Results were expressed as mean value ± standard deviation. A P value <0.05 was considered statistically significant.

## RESULTS

### Assessment of the functional state of isolated ischemic kidney

Groups 1–4 were subjected to a 5-minute ischemia and 50-minute perfusion. The design of the experiment is shown in Fig. 1.

No urine formation was observed when 6% BSA was injected into the perfusion buffer (group 1), although PP and PFR were consistent with normal values throughout the perfusion period. The absence of urine made it impossible to assess the functional criteria under study. In group 2 (0.6% BSA), by the end of perfusion, of all criteria, only PFR and UFR corresponded to minimally permissible values (5.7 ± 0.5 ml/min·g and 49 ± 2.5 μL/min, respectively).

The dynamics of the functioning criteria of the isolated ischemic kidney during perfusion in groups 3–4 is shown in Fig. 2. PP and PFR were constant during perfusion in both groups and had acceptable values. Prx6 had no effect on the dynamics of these criteria (Fig. 2, a). For UFR, there was an increase during perfusion and criterion values were above the minimum acceptable values throughout the perfusion period in both groups. With Prx6, there was a significant 1.5-fold increase in UFR by the end of perfusion relative to group 3 (115 ± 23 μL/min and 178 ± 28 μL/min, respectively) (Fig. 2, b, Table 2). In group 3, GFR did not change during the perfusion period and did not reach minimum permissible values (0.2 ± 0.04 mL/min·g) by the end of perfusion (Fig. 2, b); RGF, although increasing during perfusion, also did not reach minimum tolerated values at 71% by the end of perfusion (Fig. 2, d, Table 2). Using Prx6 brings these criteria to values above the minimum. The data revealed a statistically significant difference between groups 3 and 4 in these criteria by the end of per-

Table 1

**Experimental groups**

| Experimental group | Ischemia time (min) | Additive/stage of adding additive to the perfusion buffer |
|---|---|---|
| 1 | 5 | 6% bovine serum albumin (BSA) / perfusion period |
| 2 | | 0.6% BSA / perfusion period |
| 3 | | No additive |
| 4 | | Prx6 / blood removal stage |
| 5 | 20 | 6% BSA / perfusion period |
| 6 | | 0.6% BSA / perfusion period |
| 7 | | No additive |
| 8 | | Prx6 / blood removal stage |
| 9 | 0 | No perfusion |
| 10 | 90 | No perfusion |

*Note.* BSA was in the perfusate at the perfusion stage. Prx6 was in perfusate at the blood removal stage. Group 9 – kidneys of intact animals, group 10 – kidneys without perfusion period.

fusion (Table 2). TUP is higher in group 3, here 63% of the primary ultrafiltrate was excreted as terminal urine; in contrast, in the Prx6 group, this figure was 1.5-fold lower ($p < 0.05$) (Fig. 2, g). Both groups showed an increase in UUL toward the end of perfusion; however, this criterion was significantly (2-fold) higher in the Prx6 group (7.3 ± 1.8 μmol/10 min and 13 ± 1.7 μmol/10 min, respectively) (Fig. 2, e). Table 2 summarizes the values of the renal function criteria in groups 1–4 at the end of perfusion.

Groups 5–8 were subjected to a 20-minute ischemia period, with a 50-minute perfusion period. The design of the experiment is shown in Fig. 1.

When 6% BSA was injected into the perfusion buffer (group 5), no urine formation was observed, PFR did not meet the minimum acceptable values. Absence of urine made it impossible to assess the function parameters under study. In group 6 (0.6% BSA), by the end of perfusion, of all the criteria, only UFR corresponded to

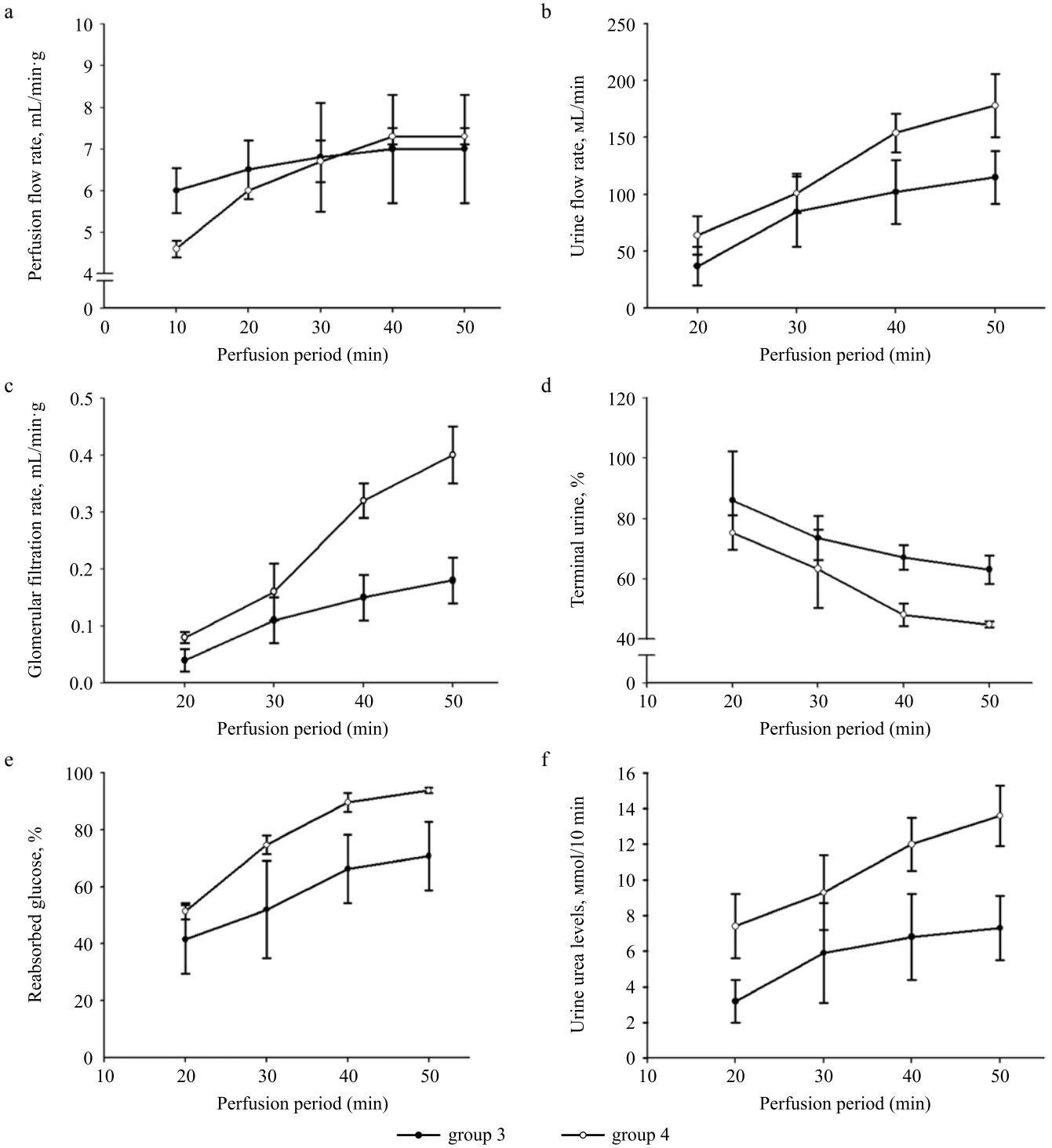


Fig. 2. The dynamic of the criteria of the functioning of an isolated ischemic kidney during perfusion (ischemia 5 minutes / perfusion 50 minutes). The first 20 minutes of perfusion is the stabilization period

the minimum acceptable values (44 ± 4.6 μl/min). There was increased pressure in both groups, with reduced perfusion flow rate.

The dynamics of the functioning criteria of the isolated ischemic kidney during perfusion in groups 7–8 is shown in Fig. 3. Perfusion pressure and perfusion flow rate were constant during perfusion in both groups and had acceptable values. Prx6 had no effect on the dynamics of these parameters (Fig. 3, a). In both groups, UFR was above the minimum acceptable values throughout the perfusion period. With Prx6, there was no significant increase in UFR by the end of perfusion relative to group 3 (137 ± 40 μL/min and 146 ± 41 μL/min, respectively) (Fig. 3, b, Table 2). The GFR and RGF values were below the minimum tolerated values throughout the perfusion period in both groups. The use of Prx6 did not take GFR and RGF above the minimum values even by the end of perfusion (0.16 ± 0.05 mL/min and 44%, respectively) (Fig. 3, c, E). In groups 7–8, TUP and UUL did not change significantly during perfusion. By the end of perfusion, the values of these criteria did not differ significantly between the groups (Fig. 3, d, f, Table 2). Table 2 shows the values of renal function criteria in groups 7–8 at the end of perfusion.

Table 2 shows the values of the kidney function criteria in the experimental groups at the last 10-minute perfusion interval.

## Assessment of the morphological state of the isolated ischemic kidney

Fig. 4 shows the morphology of renal tissue from different experimental groups. The kidneys of intact animals had distinguishable sections of the nephron in the cortical layer: renal corpuscle and renal convoluted tubules: proximal (canal cells have a pronounced brush border) and distal (Fig. 4, a). An hour and a half of ischemia and absence of perfusion led to development of foci of necrobiosis and necrosis in the renal parenchyma, desquamative changes in the ducts, and focal capillary-venous full-blooded cortical substance with erythrostasis (Fig. 4, b). During short periods of ischemia in the isolated kidney after perfusion, the general morphofunctional structure of the nephron was preserved (Fig. 4, c, d). For groups 3–4, a general morphological pattern was observed: hydropic dystrophy in the juxtamedullary zone, foci of canal lesions, and absence of cellular debris. When Prx6 was used, there was decreased lesion of the tubular epithelium; the proximal tubular nephrocytes had a pronounced brush border. The distal tubules were unchanged (Fig. 4, d). With an increase in the isolated kidney ischemia time to 20 minutes in groups 7–8, a similar morphological picture was observed by the end of perfusion, particularly disorder in the morphofunctional structure of the nephron. The presence of granular masses in capsule lumen and focal spasm of capillary loops were noted. Foci of hydropic dystrophy of canal epithelium appeared, absence of brush border of proximal canal nephrocytes, necrosis of individual epitheliocytes and cell groups. There was a depletion in the tubules due to decreased epithelial height and lumen dilatation. Homogeneous masses and desquamated epithelial cells were noted in the lumen of canals. The lesion was zoned and pronounced in the cortical layer. The degree of lesion decreased towards the lateral margin. The use of Prx6 did not cause a change in the morphological state of the renal tissue and did not reduce the lesion.

## DISCUSSION

The IPK model is recognized for the study of renal function [1, 23], and is also of practical importance for assessment of preservation methods [22, 24]. In the IPK model, three interrelated renal transport processes – filtration, secretion and reabsorption – are close to their in vivo functioning criteria; in addition, the glomeruli and proximal tubule functions are preserved. The disadvantages of the model include impaired renal hemodynamics

Table 2

**Functioning criteria at the end of the perfusion period**

| Criteria | | | PFR, ml/min·g | UFR, μl/min | GFR ml/min | RGF, % | UUL, μmol/10 min | TUR, % |
|---|---|---|---|---|---|---|---|---|
| Min values | | | 4–5.3 | >30 | >0.5 | >90 | | |
| 5 min ischemia | No. 1 | 6% BSA | 7.5 ± 2.8 | – | – | – | – | – |
| | No. 2 | 0.6% BSA | 5.7 ± 0.5 | 49 ± 2.5 | 0.01 ± 0.01 | 50 | 4.2 ± 0.5 | 61 ± 0.5 |
| | No. 3 | No additive | 6.9 ± 1.3 | 115 ± 23 | 0.18 ± 0.04 | 71 ± 12 | 7 ± 1.8 | 64 ± 4.7 |
| | No. 4 | Prx 6 | 7.3 ± 0.2 | 178 ± 28 | 0.4 ± 0.05 | 94 ± 1 | 13 ± 1.7 | 45 ± 1 |
| 20 min ischemia | No. 5 | 6% BSA | 2 ± 0.7 | – | – | – | – | – |
| | No. 6 | 0.6% BSA | 3.4 ± 1 | 44 ± 4.6 | 0.05 ± 0.01 | 21 ± 6 | 2.8 ± 3.6 | 92 ± 6.5 |
| | No. 7 | No additive | 5.6 ± 1 | 137 ± 40 | 0.16 ± 0.05 | 46 ± 13 | 9.6 ± 3 | 85 ± 3.2 |
| | No. 8 | Prx 6 | 5.1 ± 0.3 | 146 ± 41 | 0.16 ± 0.04 | 44 ± 13 | 9.6 ± 1 | 91 ± 2.3 |

due to high perfusion flow rate and impaired functions of the distal tubules [1].

In this study, we used a model of retrogradely perfused mouse isolated kidney [3]. Perfusion was performed with DMEM synthetic medium. In our IPK model, the principal point was having a thermal ischemia period of 5 and 20 minutes. The period of 5 minutes was chosen as the minimal period of blood flow interruption. The 20-minute thermal ischemia period is described as the most optimal for subsequent recovery of isolated kidney function during transplantation [26]. Ischemia is known to trigger a number of pathological reactions that lead to disruption in the functions of vital cell structures. Subsequent reperfusion leads to progression of pathological processes. Studies in recent years have repeatedly pointed to the key role of oxidative stress in the pathogenesis of renal IRI. The main participants in the chain of events leading to oxidative stress are reactive oxygen and nitrogen species: there is a sharp increase in the generation of these free radicals in kidney cells [7]. In this

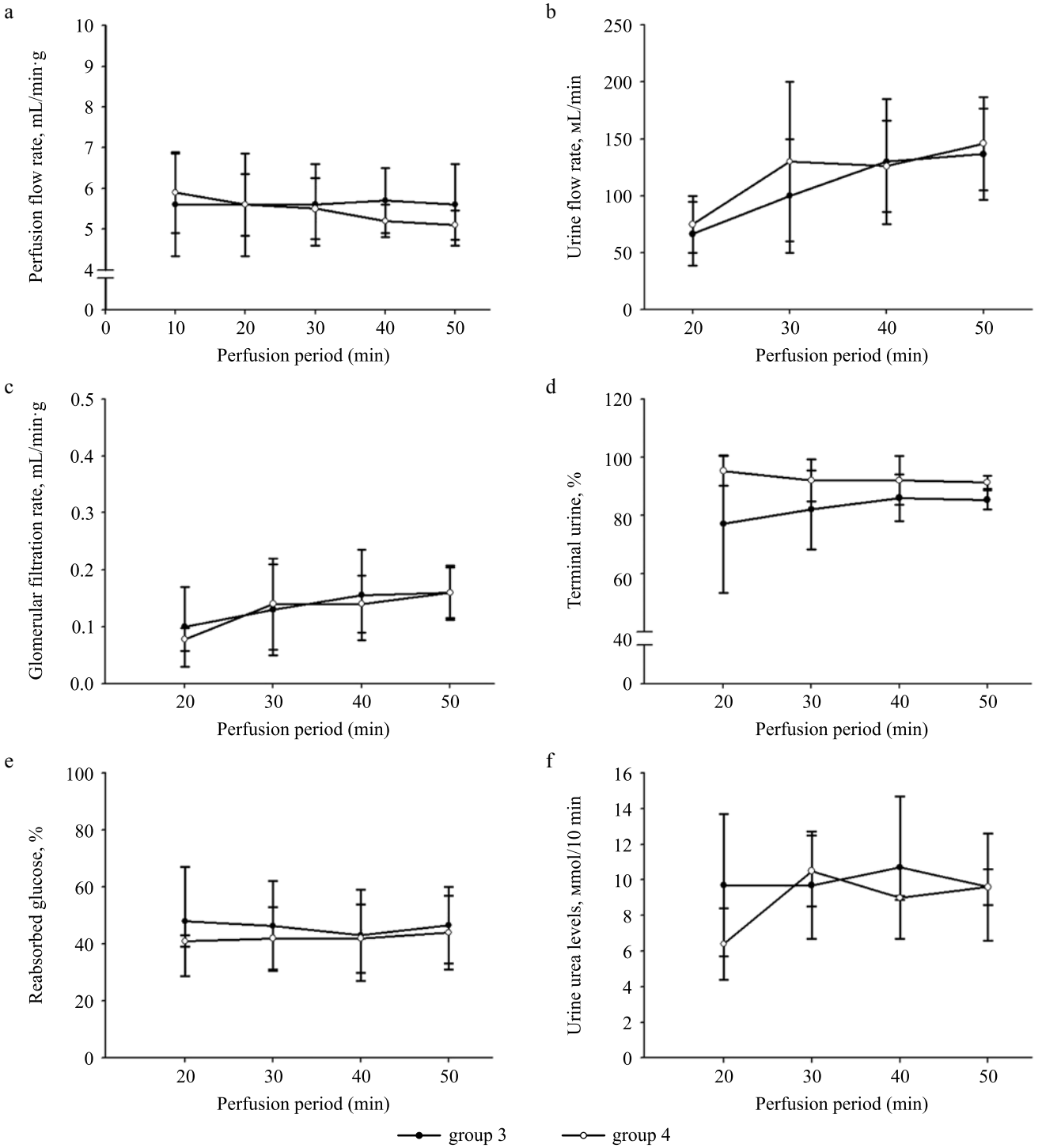


Fig. 3. The dynamic of the criteria of the functioning of an isolated ischemic kidney during perfusion (ischemia 5 minutes / perfusion 50 minutes). The first 20 minutes of perfusion is the stabilization period

work, exogenous antioxidant enzyme Prx6 was used to protect the isolated organ from the damaging effects of IRI and to improve the morphofunctional characteristics of the isolated ischemic kidney. As part of the perfusion solution, Prx6 was injected locally into the isolated kidney at the onset of ischemia. Thus, Prx6 was in the kidney tissue throughout the ischemia period and at the beginning of perfusion. It has been previously shown that when an isolated kidney is perfused with exogenous Prx6, the protein is distributed through the vessels of the renal glomeruli and the vessels accompanying the thin tubule [20]. Besides, the renal tissue has its own pool

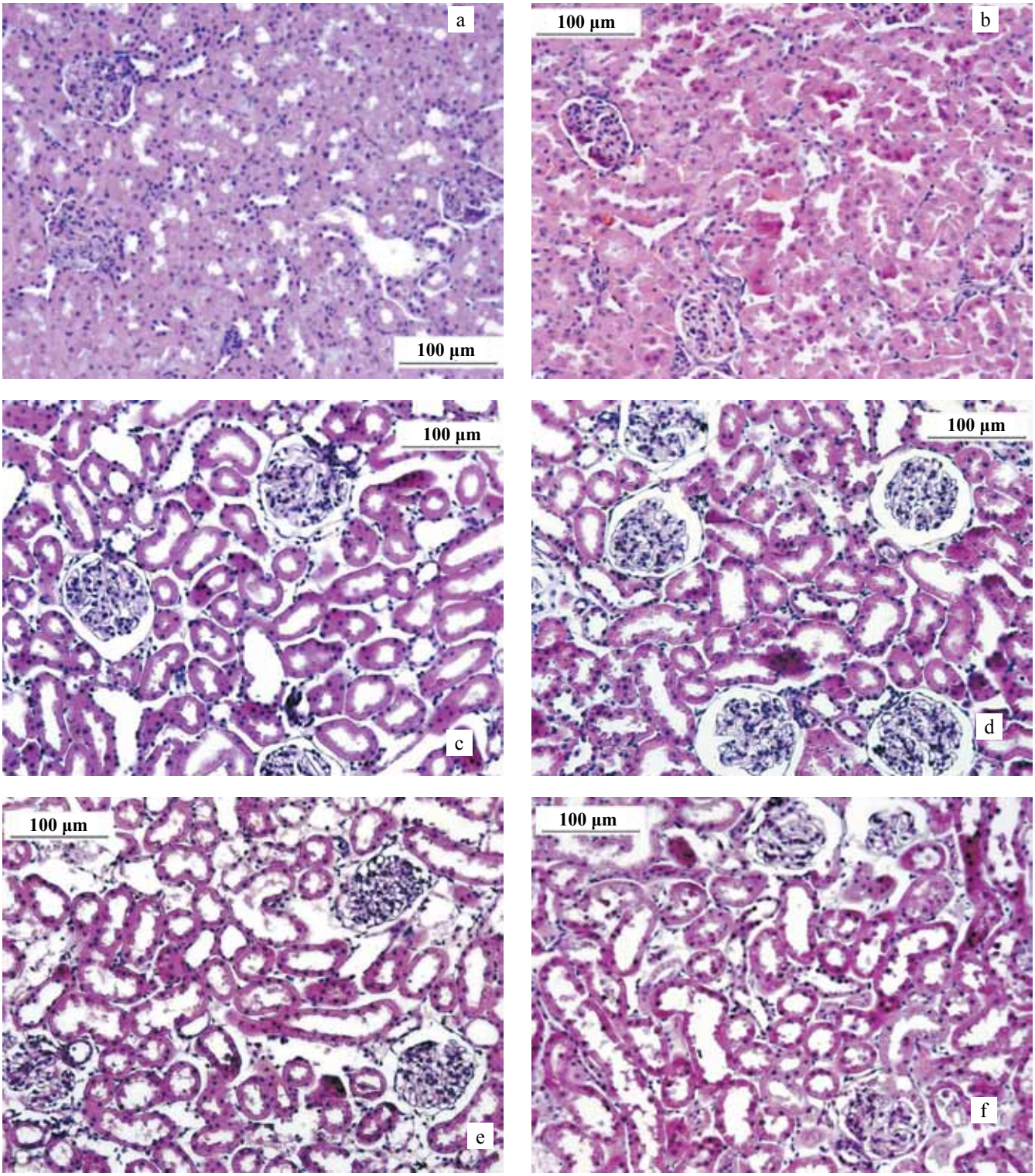


Fig. 4. Morphology of renal tissue: а – native control; б – kidney, no perfusion, ischemia 90 minutes; в – group 3 (ischemia period 5 min / perfusion period 50 min, no Prx6); г – group 4 (ischemia period 5 min / perfusion period 50 min, + Prx6); д – group 7 (ischemia period 20 min / perfusion period 50 min, no Prx6); е – group 8 (ischemia period 20 min / perfusion period 50 min, + Prx6). Eosin-hematoxylin, ×200

of Prx6 in the tubular segments of the nephron, and it is at these sites that increased Prx6 expression is observed during IRI [27]. To assess the effectiveness of Prx6 as a means to preserve the morphofunctional characteristics of the isolated ischemic kidney, several generally accepted functioning criteria were analyzed: PP, PFR, UFR, GFR, RGF, TUP [1, 22] and UUL.

PP was maintained at 90–110 mmHg by changing the PFR from 2 to 7.5 ml/g/min·g in different groups (Table 2). By the end of perfusion, maximal PFR was observed at 5-minute ischemia amidst a 6% BSA background; minimal PFR was observed at 20-minute ischemia on a 6% BSA background. Despite high PFR in group 1 (6% BSA), there was no urine formation. It is noted that adding high BSA doses to the buffer decreases GFR; on the contrary, isolated kidneys perfused with synthetic medium without BSA addition tend to increase GFR and RGF [22]. Macroscopically, there was an about 1.5-fold increase in the size of isolated kidneys during perfusion. This is a natural process, as there are factors contributing to fluid accumulation in the tubules – lack of nerve control of afferent arterioles and blockage of lymphatic vessels [4].

Monitoring of the main criteria of isolated kidney functioning began after hemodynamic equilibrium was established, when PFR and PP held constant values (stabilization period (20 minutes)).

At 5-minute ischemia, the isolated kidney function criteria in all groups had values above the minimum (Table 2). For group 2 (0.6% BSA) and 3 (No additive), these parameters can include PP, PFR, and UFR. For GFR and RGF, the values are below the minimum acceptable values. Histological evaluation of renal tissue in these groups revealed foci of internal canal lesions. Damage of proximal canals already in the early stages of ischemia was due to their high level of metabolic activity [25]. The use of Prx6 extends the list of criteria of isolated kidney functioning with admissible values: PP, PFR, UFR, GFR and RGF. When Prx6 was used, there was increased UFR, GFR, and RGF and decreased TUP, compared with group 3. This pattern indicates improved filtration, secretion and reabsorption of ultrafiltrate when using Prx6 during perfusion of ischemic isolated kidneys. Any value above 90% using Prx6 indicates preservation of the functionality of the tubular structures of isolated ischemic kidney by the end of perfusion. This is confirmed by histological analysis results. A decrease in the lesions of tubular structures using Prx6 is also confirmed by the almost 2-fold increase in the amount of urea in terminal urine, compared to group 3. Since the main sites responsible for urea transport are the proximal tubule and the thin segment of the ascending part of the loop of Henle [25], we cannot say whether these very tubular structures are preserved. Increase in UUL levels may not be directly related to preservation of tubular structures; however, morphological evidence obtained suggests that this very process significantly contributes to the urea transport process. Thus, by functional criteria, exogenous Prx6 significantly improves the functional state of the isolated kidney during perfusion after short thermal ischemia periods.

Evaluation of the isolated kidney after 20 minutes of ischemia showed that with increasing duration of ischemia, among the functioning criteria, only UFR has acceptable values. The other criteria have values below the minimum (Table 3). The use of Prx6 does not raise these criteria to higher values. Within the time group, absence of BSA in the perfusate increases GFR and RGF, but does not take them to the required level. Low RGF indicates impaired glucose reabsorption processes in proximal channels [28]. A similar situation is observed with UUL, there are increased levels in the absence of BSA in the solution. Thus, after 20 minutes of ischemia, the kidney can be considered non-viable. Lack of kidney function is most likely due to the fact that ischemia duration is of fundamental importance for the survival of the isolated organ. In the ischemic kidney, with the beginning of perfusion, hypo- and hyperperfusion foci appear. Against the background of acute tubular necrosis, there is spasm of arterioles supplying the glomerulus, which can lead to intraorgan blood flow disruption and no-reflow [26]. The presence of tubule lesions was confirmed by histological analysis. Previously, it was shown that increasing ischemia duration to 45 minutes leads to critical parenchymal injury and decrease in UFR to less than 30 μl/min. The use of Prx6 at these times leads to decreased lesion of the isolated kidney, compared to the control lesion. However, despite the protective effect of Prx6 at the critical stages of ischemia, renal parenchyma injury remains high [20].

Thus, the use of synthetic media in the IPK model is appropriate only in the early stages of ischemia. During this period, some criteria of isolated kidney functioning reach acceptable values. Renal tissue structure is preserved, but there are foci of dystrophic changes in the juxtamedullary zone and proximal canals. With increasing ischemia duration, renal tissue damage intensifies, tubular necrosis develops, which manifests as a decrease in functionality criteria and cessation of functioning of the isolated ischemic organ. The use of exogenous Prx6 positively influences the morphofunctional state of isolated kidneys only in the early stages of ischemia. In this case, the criterion values are higher than the minimum allowable, which may indicate the functioning of the isolated ischemic kidney for a certain period when it is perfused with a synthetic medium. Morphologically, the morphostructural integrity of nephrons was noted in the early stages of ischemia.

Prx6 is a polyfunctional antioxidant enzyme with peroxidase activity, low phospholipase activity, and participates in many processes in the cell [10–13]. The fact that Prx6 protects the isolated kidney at short ische-

mia periods indicates that in this case the peroxidase activity of Prx6 makes the main contribution to organ protection. The experience of using recombinant Prx6 for protection of isolated organs has been reported. The protective effect of Prx6 has been shown in a perfusion model of isolated rat heart. It was noted that Prx6 normalizes heart rate, maintains myocardial contractile activity, and prevents lipid peroxidation [17]. Exogenous Prx6 has shown its protective effect for preconditioning of rat heart transplant. Prx6 has been shown to reduce the severity of cardiac IRI and promote normalization of its morphofunctional state during heterotopic transplantation [18].

## CONCLUSION

In a model of an isolated perfused kidney, exogenous recombinant Prx6, locally injected before ischemia, was shown to have a renoprotective effect. When interpreting obtained evidence, a fundamental factor was taken into account: response of the isolated kidney differs from the natural one due to suppression of humoral regulation and absence of innervation [4]. In this regard, the effect of Prx6 in the isolated kidney may probably differ from its protective effects in the kidney in vivo [8].

Based on the data obtained, it is reasonable to suggest that the use of exogenous recombinant Prx6 may be an effective approach to preserving the isolated kidney during transplantation, since the inclusion of Prx6 in the composition of known perfusion solutions as a powerful antioxidant agent will help to reduce free-radical oxidation processes. In this regard, further experimental studies are required.

*We used equipment provided by the Optical Microscopy and Spectrophotometry Department at Pushchino Scientific Center for Biological Research, Moscow, to obtain the microphotographs presented in this paper.*

*The authors declare no conflict of interest.*

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# XENOGENEIC LYMPHOCYTIC RNA STIMULATES SKELETAL MUSCLE REGENERATION

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**Objective:** to find evidence of the existence of distant lymphocytic RNA control of physiological myogenesis as a way to control the muscle tissue regeneration process. **Materials and methods.** The study was conducted on male Wistar rats, n=33. In the first part of the experiment, 12 rats were subjected to regular 40-day physical activity (swimming), half of them were intraperitoneally injected 4 times with total RNA isolated from pig spleen lymphocytes at 30 days of age; 6 rats made up the intact control group. In histological preparations of different skeletal muscle groups, the width and cross-sectional area of muscle fibers, the area of nuclei, and the number of myocytes and myosatellite cells were evaluated. In the second part of the experiment, 15 intact rats were injected with the studied xenogeneic RNA and the amounts of ribonucleic acids in peripheral blood lymphocytes, spleen lymphocytes, and skeletal muscles were determined 2 hours and 24 hours after injection. **Results.** After the 40-day physical activity, the width of the fibers and the area of myocyte nuclei in the skeletal muscles increased; the absolute number of myosatellite cells and the area of their nuclei did not change. After administration of xenogeneic RNA in the trained rats, in addition to an increase in the thickness and cross-sectional area of muscle fibers, the absolute number of myosatellite cells in *m. biceps femoris*, in *m. triceps brachii*, and in *m. pectoralis major* increased 1.4-fold, 1.3-fold, and 1.4-fold, respectively; the area of myosatellite nuclei increased on average by 7%. In intact rats, two hours after xenogeneic RNA injection, the amount of RNA in skeletal muscles remained unchanged, it increased by 19% in spleen lymphocytes, and by 16% in peripheral blood lymphocytes. At 24 hours, the RNA amount in the lymphocytes remained significantly higher than the control values, while in the muscle tissue, it didn't differ from the control. **Conclusion.** Xenogeneic lymphocytic RNA stimulates physiological myogenesis by activating myosatellite cell proliferation.

*Key words: myogenesis, lymphocytes, RNA, regeneration.*

Lymphoid cells are actively involved in regulation of regenerative processes [1, 2], thus providing body reactivity or resistance in a changing external or internal environment. This includes tissue adaptive responses to increased stress. Restoration of skeletal muscle integrity after acute injury and its physiological regeneration with moderately pronounced contractile activity occurs due to proliferation and differentiation of myosatellite cells, committed progenitor cells located between the basal membrane and sarcolemma of the muscle fiber. Hormones, growth factors, and neurotransmitters, many of which are synthesized and secreted by lymphoid cells, are involved in regulation of these processes. In the last 10 years, evidence has emerged that after acute muscle injury, T helpers of different phenotypes emerge in muscle tissue already at the initial stages of repair morphogenesis, whose presence significantly accelerates myosatellite emergence from dormancy and is accompanied by formation of complete muscle fibers both in vivo and in vitro conditions [3, 4]. However, the authors noted that severity of regenerative response does not correspond to quantitative composition of the T cells that have come into the muscle tissue. They suggested that T lymphocyte regulation of myogenesis probably occurs not by contact but remotely through soluble bioactive substances produced by T cells.

The lymphoid system is part of the neuroimmunoendocrine regulatory circuit; it is able to inform body tissues about modulation of its own function, as well as about changes occurring in the organ 'served' by it [5]. The intermediaries in this intercellular signaling system, besides hormones, cytokines, and growth factors, are RNA molecules (extremely diverse in their functional properties) capable of copying and transporting information, regulating gene expression, and catalyzing chemical bond formation. There is evidence that total RNA isolated from morphogenetically active cells has a more pronounced pro-regenerative effect than the cells themselves [6]. Earlier we have proved that lymphocytic total RNA has no allogeneic and xenogeneic limitations; this is particularly so with total RNA isolated from human peripheral blood lymphocytes, stimulated bone marrow

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erythropoiesis in rats both in vivo and in vitro. The aim of the present study was to find evidence for the existence of remote lymphocytic RNA control of physiological myogenesis as a way to control muscle tissue regeneration.

## MATERIALS AND METHODS

We used 33 male Wistar rats weighing 260–310 g. Experiment was performed in compliance with the principles of humanity set out in the European Community directive (86/609/EEC) and the Declaration of Helsinki, in accordance with Order No. 199H of the Russian Ministry of Health dated April 1, 2016 "On approval of rules of good laboratory practice". The animals were kept in standard cages (n = 6) with free access to water and food, at +24 ± 2 °C air temperature in a vivarium, in accordance with the SP 2.2.1.3218 rules. Daily ration of the animals consisted of specialized pelleted feed corresponding to the content of nutrients, vitamins and minerals of international standards and GOST R 50258-92 (Complete Feed for Laboratory Animals). All painful manipulations with the animals and their euthanasia by cervical dislocation were performed under ether anesthesia in a room separate from the vivarium.

The first part of the experiment used 18 animals, which were divided into 3 groups of 6 animals each. Group 1 consisted of intact rats, group 2 was the control rats participating in the training process for 6 weeks, and group 3 included rats participating in the training process for 6 weeks and receiving total RNA injections. The training process was a swimming load in a 200 L tank, the water layer was 0.5 m thick, and the water temperature was +22–23 °C. The rats were placed in the tank 3 times a week, and duration of exercise was increased every week by 5 minutes (from 30 to 55 minutes) [7]. The lymphocytes, the sources of morphogenetically active xenogeneic RNA, were isolated by suspending the spleen of a 30-day old piglet in a glass homogenizer, then filtering the suspension through a capron filter and centrifuging the cells 3 times in sterile 0.9% NaCl solution. Total RNA was isolated by guanidine thiocyanate-phenol-chloroform extraction. The concentration of isolated RNA was determined spectrophotometrically by the optical density of the preparation at 260 nm wavelength. The resulting total RNA was lyophilized and stored in sterile vials at +5 °C. Prior to injection, lyophilised RNA was dissolved in sterile 0.9% NaCl solution. RNA was injected intraperitoneally into rats through sterilizing filtration (sterile syringe nozzles with 0.22 μm pore diameter). A total of 4 injections were given to each group 3 rat once a week. The RNA dose at each injection was 30 μg/100 g body weight, and the volume of the injected solution was 0.5 ml. To assess the morphofunctional state of the skeletal muscle tissue, the preparations were fixed with 10% neutral formalin. After standard histological tracing, preparation of paraffin blocks and slicing, the preparations were stained with hematoxylin and eosin. The stained preparations were studied using a LEICA DMRXA microscope (Germany), a digital video camera LEICA DFC 290 (Germany), connected to a personal computer, obtaining images of micropreparations in *TIFF files in the RGB colour space. Licensed version of ImageScope M image analysis software (Russia) was used for morphometric studies. Myosatellite and myocyte counts were measured at 400× magnification (40× lens; 10× eyepiece). The area of cell nuclei was determined using the "manual selection" function at 1000× magnification.

In the second part of the experiment, 15 animals were divided into 3 groups. Five intact rats constituted the control group and were intraperitoneally injected with 0.5 ml of 0.9% NaCl solution. 10 rats were intraperitoneally injected with 30 μg/100 g weight of xenogeneic total RNA dissolved in 0.5 ml of 0.9% NaCl solution. Then five individuals were then withdrawn from the experiment 2 hours after RNA administration, the remaining 5 individuals were withdrawn after 24 hours. In all 15 rats, total RNA was isolated from peripheral blood lymphocytes (isolated by centrifugation in ficoll-verogran solution with density gradient 1.119 g/cm$^3$, PanEco, Russia), spleen lymphocytes and pectoralis major tissue by guanidine thiocyanate-phenol-chloroform extraction and its amount was measured spectrophotometrically by optical density of the preparation at 260 nm wavelength.

Statistical data processing was performed using a licensed software package: Excel 2020 and PAST version 4.03. Mann–Whitney non-parametric method was used to assess the significance of differences between the groups. Data are presented as arithmetic mean and its error (M ± m). Differences $p \leq 0.05$ were considered statistically significant.

## RESULTS

The dynamic characteristics of swimming movements in land rodents are such that the greatest paddling efforts are provided by the biceps femoris, pectoralis major and triceps brachii muscles. Histomorphological examination of these muscles revealed that regular moderate-intensity physical activity leads to hypertrophy of muscle fibres but is not accompanied by regenerative hyperplasia. After a 40-day training process, muscle fibre width and myocyte nuclear area increased in all skeletal muscles studied (Table). At the same time, the number of mature muscle cells decreased significantly in the biceps femoris, despite pronounced contractile activity and tension during swimming. These results are consistent with those obtained by other researchers, who found partial fragmentation and focal globular necrosis of myocytes in the muscle tissue of rodents subjected to regular swimming exercise [8]. The cellular form of muscle tissue regeneration under regular moderate-intensity physical activity was absent: absolute number of myosatellite cells and the size of their nuclei in all the studied muscles did not differ from those of intact animals.

Injection of xenogeneic total RNA was accompanied by both a significant increase in indicators characterizing the development of a hypertrophic muscle tissue regeneration, and appearance of signs of cellular hyperplasia in the muscle tissue experiencing regular physical activity (Table). In experimental rats injected with RNA, the cross-sectional area of muscle fibres increased 1.2-fold on average, and the thickness of the fibres increased by 8–10%. Visually, the fibres were more densely and compactly arranged (Fig. 1). In the biceps femoris, RNA injection allowed the number of actively functioning mature muscle cells to remain at the physiological level (myocyte count in group 3 rats did not differ from that registered in intact animals).

Obviously, under the influence of exogenous lymphocytic RNA, the mature myocyte pool started replenishing due to proliferation of myosatellite cells: in all the studied striated muscles, the count of these committed progenitor cells and their nuclei sizes increased as compared to those of the control group rats that received no lymphocytic RNA. Myosatellite cells were easily visualized in histological preparations (Fig. 1): they had rounded large light nuclei with evenly distributed chromatin in the form of large clumps and small dust-like inclusions.

In healthy young rodents living under laboratory conditions, the proportion of myosatellite cells in striated muscles does not exceed 25%. After regular forced swimming, the proportion of young proliferating muscle cells in the fore and hind limb muscles in trained animals was significantly higher in percentage terms than in intact rats (Fig. 2), although the absolute count of myosatellite cells in the skeletal muscles did not change (Table). This relative increase in the proportion of myogenic progenitor cells in the trained animals was associated with decrease proportion of mature myocytes resulting from the regular damaging effects of physical exercise. Against the background of xenogeneic lymphocytic RNA administration, the proportion of muscle progenitor cells increased more markedly, and its increase was observed not only in the limb muscles, but also in the pectoralis major. An increase in the percentage of myosatellite cells in trained animals receiving lymphocytic RNA was coupled with an absolute increase in the number of these cells. Thus, compared with group 2, in group 3 rats, the myosatellite cell count in biceps femoris increased 1.4-fold, in triceps brachii – 1.25-fold, in the pectoralis major – 1.43-fold (Table). The increased proliferative activity of myosatellite cells in group 3 rats was also evidenced by a marked increase in the size of the nuclei of these cells.

The radar chart demonstrating the severity of signs of hypertrophic and hyperplastic regenerative processes in specific skeletal muscles (Fig. 3) after administration of lymphocytic RNA shows that both types of regenerati-

Table

**Effect of moderate-intensity physical activity and xenogeneic lymphocytic RNA on physiological myogenesis**

| Indicators | Intact rats (group 1), n = 6 | Physical activity (swimming) | |
|---|---|---|---|
| | | Control (group 2), n = 6 | RNA injection (group 3), n = 6 |
| ***M. biceps femoris*** | | | |
| Muscle fiber width (μm) | 26.1 ± 1.4 | 31.1 ± 0.7* | 34.6 ± 2.5*▲ |
| Muscle fiber cross-sectional area (μm$^2$) | 1450.0 ± 58.6 | 1514.3 ± 41.7 | 2009.0 ± 127.9*▲ |
| Myosatellite cell count (cells/mm$^2$) | 275.9 ± 22.7 | 302.2 ± 14.9 | 423.1 ± 28.6*▲ |
| Area of myosatellite nuclei (μm$^2$) | 30.7 ± 0.6 | 31.0 ± 0.9 | 32.2 ± 1.7 |
| Myocyte count (cells/mm$^2$) | 1058.4 ± 46.5 | 877.2 ± 36.9* | 1001.2 ± 64.4 |
| Area of myocyte nuclei (μm$^2$) | 14.7 ± 0.9 | 20.9 ± 0.8* | 19.6 ± 1.1* |
| ***M. pectoralis major*** | | | |
| Muscle fiber width (μm) | 25.0 ± 1.0 | 30.4 ± 0.9* | 32.6 ± 1.9*▲ |
| Muscle fiber cross-sectional area (μm$^2$) | 1404.6 ± 77.2 | 1470 ± 27.5 | 1786.2 ± 92.3*▲ |
| Myosatellite cell count (cells/mm$^2$) | 265.8 ± 14.9 | 280.7 ± 11.3 | 399.3 ± 23.2*▲ |
| Area of myosatellite nuclei (μm$^2$) | 29.9 ± 0.9 | 30.5 ± 1.3 | 32.0 ± 1.6*▲ |
| Myocyte count (cells/mm$^2$) | 791.4 ± 40.5 | 785.5 ± 32.2 | 965.4 ± 51.3*▲ |
| Area of myocyte nuclei (μm$^2$) | 14.2 ± 0.6 | 17.7 ± 0.7* | 17.3 ± 0.5* |
| ***M. triceps brachii*** | | | |
| Muscle fiber width (μm) | 26.7 ± 0.6 | 30.8 ± 0.8* | 33.5 ± 2.0*▲ |
| Muscle fiber cross-sectional area (μm$^2$) | 1632.7 ± 54.1 | 1745.8 ± 82.9 | 1815.0 ± 65.9 |
| Myosatellite cell count (cells/mm$^2$) | 292.6 ± 20.3 | 327.8 ± 13.7 | 411.1 ± 31.6*▲ |
| Area of myosatellite nuclei (μm$^2$) | 31.11 ± 1.05 | 32.24 ± 0.83 | 33.99 ± 1.47*▲ |
| Myocyte count (cells/mm$^2$) | 910.6 ± 33.4 | 923.7 ± 33.4 | 977.4 ± 50.1 |
| Area of myocyte nuclei (μm$^2$) | 17.1 ± 0.6 | 20.8 ± 0.6* | 17.4 ± 0.7▲ |

*Notes.* * – differences between groups 2, 3 and group 1 ($p < 0.05$); ▲ – differences between group 3 and group 2 ($p < 0.05$).

ve activity are almost equally evident in the hind limb muscles. In the forelimb muscles, regenerative response to RNA injection during exercise was weaker than in the other muscles studied, but hyperplastic processes associated with activation of satellite cell proliferation predominated. The greatest response to the lymphoid RNA signal was registered in the pectoralis major: under the influence of xenogeneic lymphocytic RNA, muscle fibers increased their size by 21%, and the myosatellite cell count in these muscles increased by 42%. Probably, this phenomenon was associated with both the initial relatively higher proportion of myosatellite cells in *m. pectoralis major* compared to the limb muscles (Fig. 2) and with increased load on the pectoral muscles when swimming.

When studying the in vivo properties of any exogenous bioactive substances, the question always arises as to how exactly these substances perform their regulatory functions. It is currently unknown which cells in the body are the target for exogenous morphogenetically active lymphocyte RNA, i.e. whether it acts directly on the tissue or transmits an information signal to recipient T lymphocytes, which, in turn, activate or inhibit cell proliferation and differentiation. Within the existing concept of lymphoid RNA regulation of repair processes [5, 9], we suggest that morphogenetically active lymphocytic RNA acts on cells of other, non-lymphoid organs indirectly, through T lymphocytes committed to a particular tissue or organ. Therefore, in the second part of the experiment, we evaluated the dynamics of total RNA content in peripheral blood lymphocytes, spleen lymphocytes and muscle tissue of intact animals after administration of xenogeneic lymphocytic RNA (Fig. 4). Even a day the introduction of exogenous RNA into the animals, the content of total RNA in the striated muscle tissue did not change, i.e. exogenous lymphocytic RNA did not penetrate into myocytes. In contrast to muscle cells, peripheral blood lymphocytes and spleen lymphocytes

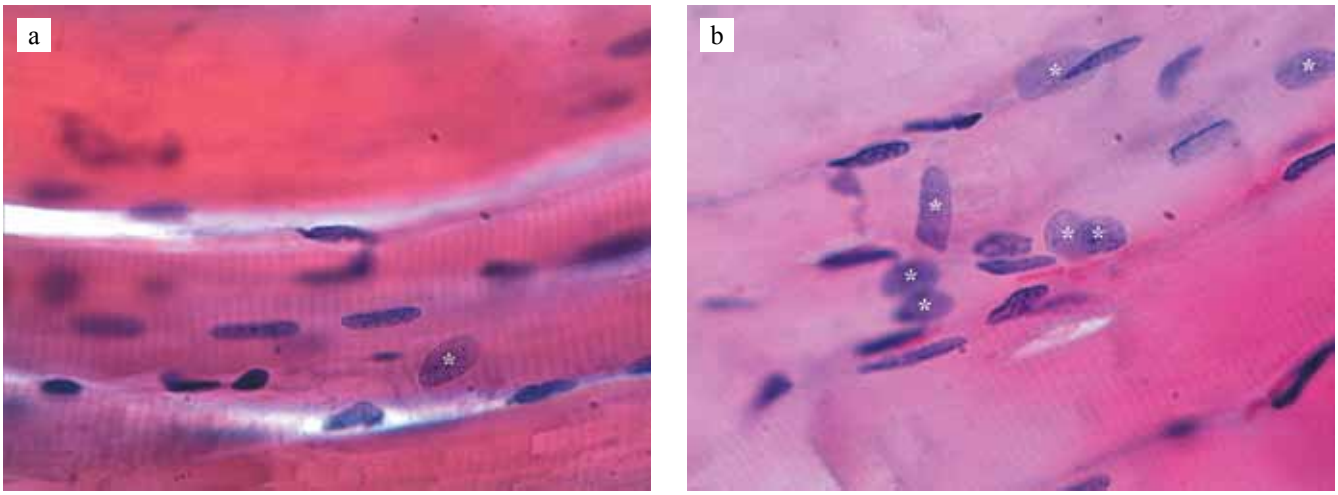


Fig. 1. Myosatellite cells (nuclei*) in the transverse striated muscle fiber of the *m. biceps femoris*: (a) intact muscle (group 1), (b) muscle after injection of lymphocytic RNA against the background of regular load of moderate intensity (group 3). H&E staining, 1000× magnification, oil immersion

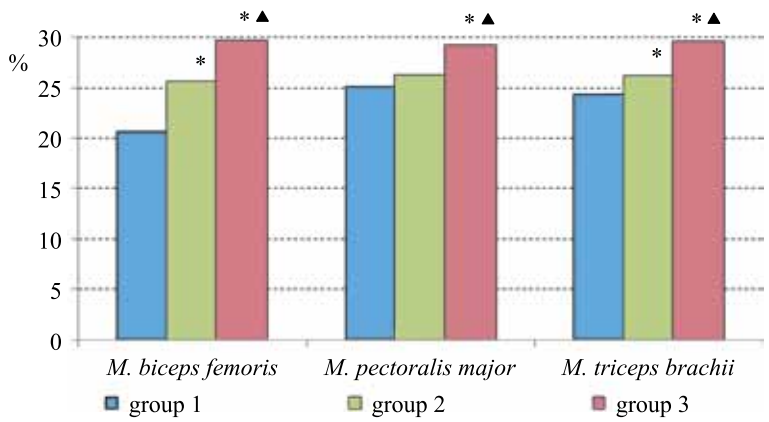


Fig. 2. Percentage of myosatellite cells in the muscles of intact rats (group 1), in the muscles of rats after regular exercise (group 2), in the muscles of rats after regular physical activity and injection of xenogeneic lymphocytic RNA (group 3). * – differences between groups 2, 3 and group 1 ($p < 0.05$), ▲ – differences between groups 2 and 3 ($p < 0.05$)

appeared to actively absorb exogenous RNA for at least 2 hours after injection. By 24 hours after lymphocyte RNA injection, RNA content in peripheral blood and spleen lymphocytes decreased, but remained significantly higher than the control level.

## DISCUSSION

The fact that allogeneic and xenogeneic RNA can rapidly penetrate into lymphoid cells has long been known: 3% of RNA, isolated from the peripheral blood lymphocytes of healthy subjects or patients with chronic lymphoblastic leukemia, was found inside allogeneic lymphocytes and lymphoid cells of mice spleen after 3 minutes of incubation, and after 15 minutes, 8% of exogenous RNA was already inside these cells [10]. A slight decrease in specific radioactivity of the cells after repeated washing and treatment with pancreatic ribonuclease indicated that exogenous RNA was indeed absorbed by lymphocytes instead of being adsorbed on their membrane. Homologous total RNA penetrated into lymphoid cells of the spleen just as quickly, stimulating their synthetic activity [11]. Current reports suggest that the main players in lymphoid regulation of myogenesis are T helper cells, namely regulatory T cells (Treg), which are found in large numbers around regenerating muscle fibers [12, 13]. It is assumed that this is a special population of Treg that proliferates not in the lymph nodes and spleen, but at the muscle tissue damage site. A special feature of these "muscle-type" Treg cells is their ability to synthesize growth factor amphiregulin. Since amphiregulin-producing T cells have been found among T lymphocytes in the spleen, it is possible that

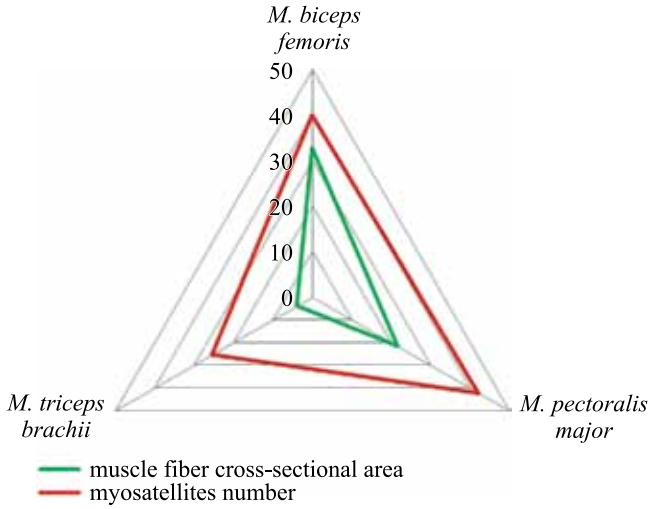


Fig. 3 Severity of hypertrophy and hyperplasia in different skeletal muscles after injection of xenogeneic lymphocytic RNA under conditions of regular physical activity (as a percentage of control values)

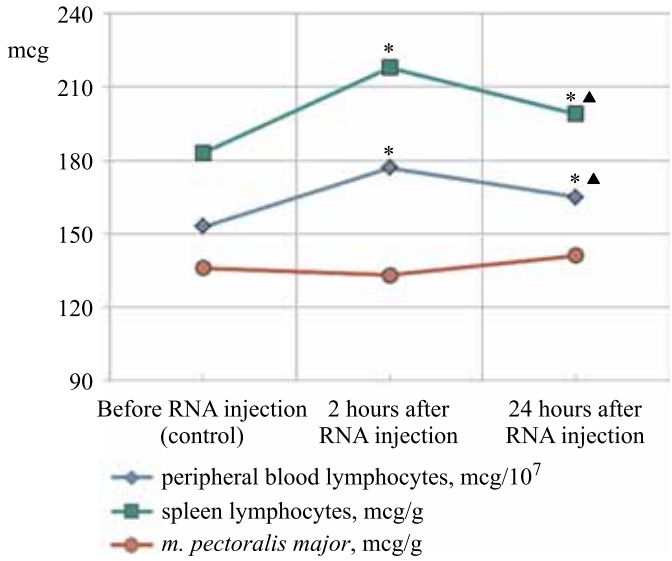


Fig. 4 Dynamics of total RNA content in tissues of intact rats after injection of xenogeneic lymphocytic RNA. * – differences from control ($p < 0.05$), ▲ – differences between the indicators obtained 2 hours and 24 hours after RNA injection ($p < 0.05$)

these already "trained" T cells migrate from the spleen to the injured muscle. To date, it has been proven that Tregs "serving" a particular muscle through amphiregulin directly enhance proliferation and differentiation of myosatellite cells in vivo and in vitro, while the regenerative potential of myosatellite cells is significantly reduced in Treg-deprived mice [12]. In addition, memory T cells, which start expressing specific myoregulatory proteins when repeated muscle damage occurs, have been found in laboratory rodents [14].

The results we arrived at provide evidence that the signal for enhanced proliferation of a tissue-specific clone of regulatory T lymphocytes located in muscles can be transmitted remotely via RNA molecules. First, we used animal lymphocytes during the active histogenesis period (weight of piglets increases 8-fold during the first month of life) as a source of morphogenetically active RNA.

Secondly, injection of xenogeneic morphogenetically active lymphocytic RNA into experimental rats not only promoted sarcoplasmic and myofibrillar hypertrophy of muscle fibers, but also stimulated cell regeneration, accompanied by increased myosatellite cell count in the striated muscle tissue. Thirdly, after RNA injection in the muscle tissue itself, the amount of ribonucleic acids did not change, but significantly increased in both spleen and peripheral blood lymphocytes. Most likely, microRNA molecules, which are not degraded by plasma and tissue RNAases, mediate this remote RNA signaling from lymphoid cells to skeletal muscle.

Both regenerative functions of different miRNAs and miRNA spectra secreted by different types of T lymphocytes are being actively studied worldwide, and there is every reason to believe that the RNA mechanisms of regenerative lymphocyte information transmission will be deciphered in the near future. The first step on this path has already been taken. In 2011, the microRNA-223 family was discovered in T lymphocytes [15], and in 2020, microRNA-223-3p was shown to be activated at an early stage of skeletal muscle regeneration after damage, and knockout of the gene encoding this microRNA synthesis leads to increased inflammation, inhibition of regeneration and development of interstitial fibrosis in the striated muscle tissue [16].

It was also found that microRNA-27 intensively expressed in T-lymphocytes [17] suppresses myostatin synthesis, a protein inhibiting myosatellite proliferation [18]. Therefore, increased microRNA-27 expression in T cells in muscle tissue injury may be one of the mechanisms by which T lymphocytes stimulate myogenesis.

The efficacy of specifically xenogeneic lymphocytic RNA suggests that T-lymphocytic control of the effector link of the motor system is a universal mechanism of regulation of the functional state of muscle tissue in all mammals, because the ability to move in space is a vital function necessary for realization of the motivation to find food or a mate. Further study on lymphocyte regulation of myogenesis is a top priority for medicine, since creation of xenogeneic RNA preparations suitable for use in regenerative medicine may become one of the advanced methods of subcellular therapy in muscular dystrophies, as well as other diseases associated with skeletal muscle conditions.

*The authors declare no conflict of interest.*

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# SERTOLI CELLS: IMMUNOMODULATORY PROPERTIES, METHODS OF ISOLATION AND CULTURE

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Due to complications caused by the inevitable use of immunosuppressive drugs in organ and cell transplantation, the use of natural mechanisms of immunological tolerance identified in animal and human organisms arouses interest. It has long been known that there are certain areas in them, including the testis, where immune reactions are virtually impossible. Our review focuses on the role of Sertoli cells that provide testicular immune privilege. Methods of isolation and cultivation of Sertoli cells are described and their potentials in biology and medicine are discussed.

*Keywords: testis, Sertoli cells, immune privileged cells, cell culture.*

## INTRODUCTION

Transplantation of donor organs can provide radical help to patients with end-stage chronic diseases in several organs, primarily kidneys, heart and liver. However, shortage of suitable donors significantly limits the use of this treatment. Theoretically, the use of animal organs and tissues as a donor source could provide unlimited supply and meet the need for transplantation treatment, but aggressive immune rejection of xenografts is the main obstacle to their use in clinic [1, 2]. At the same time, drug prolongation of their survival requires doses of immunosuppressive drugs that are highly toxic and therefore unacceptable for use in recipients [3–7]. In this connection, the use of natural mechanisms of immunological tolerance identified in animals and humans is of interest.

It has long been known that there are certain areas of the body where immune reactions are virtually impossible. These include organs or parts of them, such as the testis, brain, anterior chamber of the eye, ovary, pregnant uterus, and placenta [8]. These areas were named immune privileged zones. Placement of both allogeneic and xenogeneic organs (or their fragments), tissues and cells into them does not lead to graft rejection. In particular, it has been repeatedly shown that foreign transplants injected intratesticularly survive longer than those implanted elsewhere [9]. However, the mystery of this phenomenon persists to a certain extent, and the mechanisms providing such immune privilege remain insufficiently clear.

This review focuses on elucidating the factors that provide immune privilege to the testis and the role played by Sertoli cells, which are non-germ cells localized in the seminal epithelium, in ensuring this immune tolerance.

## IMMUNOMODULATING PROPERTIES OF SERTOLI CELLS

Sertoli cells (SCs) were first described back in 1865 by Enrico Sertoli but remained largely unexplored until data on the structure and functional abilities of these unique cells were obtained in 1975 [10].

It was shown that SCs undoubtedly play a key role in spermatogenesis. They are the first to differentiate in the fetal gonads and, in turn, induce development of seminal tubules [11]. The CSs, being somatic cells, support and control germ cells during their development and full spermatogenesis in adult animals [12–16]. In the mature testis, the SCs maintain the necessary structural and functional state of the seminal epithelium, are responsible for formation of blood-testis barrier and secrete biomolecules, such as transferrin and androgen-binding protein, providing nutrition for germ cells and phagocytosis of degenerating germ cells [10, 14].

An extremely important fact is that, in addition to nutrients and growth factors, SCs can secrete various immune defense factors, such as cytokines [17]. Moreover, co-culture with SCs promotes proliferation and preservation of viability of various cells, in particular, neurocytes and islet cells [18–20]. The immunomodulatory properties of SCs were confirmed after their co-culture with human pancreatic islets followed by islet xenotransplantation to animals with experimental diabetes [20, 21].

Assumption of similarity in the morphofunctional properties of SCs and mesenchymal stem cells (MSCs) at the early stage of differentiation should be considered as quite significant in elucidation of immunomodulatory and trophic effects of SCs [22]. It was found that surface markers of SCs and MSCs were almost identical. At the same time, the proliferative activity of SCs as well as the propensity for osteogenic and adipogenic differen-

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tiation was weaker than that of MSCs. Nuclear staining showed that, in comparison to MSCs, chromatin in SCs began to aggregate and was in slightly higher abundance. The β-galactosidase staining showed that SCs were in a slightly aging state. In addition, they secreted cytokines in slightly lower amounts than MSCs.

Basically, spermatozoa is protected against immune detection and destruction through formation of the blood-testis barrier, which, in fact, is an aggregate population of SC connecting complexes. The mechanisms of the formation of immunoprotective function of SCs have not yet been clearly defined. This may be due to the fact that these cells do not express the major histocompatibility complex antigens class I or II and, therefore, cannot be detected by the immune system [23]. One of the putative underlying mechanisms for immune protection is the expression of FasL (CD 95 ligand) [24, 25], by which isolated SCs induce localized immune privilege for co-transplanted cells. This is very similar to the already well-defined mechanism of mammalian immune response suppression. An alternative or additional mechanism beyond the testicular immunoprotective activity of SCs is the pathway of suppression of activated lymphocyte proliferation. A dose-dependent effect of inhibition of spleen lymphocyte proliferation by conditioned medium obtained after SCs incubation has been found. Apparently, this occurs through a corresponding decrease in interleukin 2 (IL-2) secretion by lymphocytes, since addition of exogenous IL-2 could not reverse this effect [23–25].

For a detailed analysis of the reasons providing immune privilege to testes and elucidating the subtle mechanisms of SCs influence on survival of allogeneic and xenogeneic cell transplants, it is necessary to obtain a preparation of viable SCs by means of efficient enzymatic treatment of testicular tissue and/or by selecting an adequate cultivation regime.

## ISOLATION OF SERTOLI CELLS

As a rule, methods of SC isolation from testes are aimed at eliminating from the obtained preparation, connective tissue contaminating its elements and interstitial cells. Most studies on isolation of SCs have been performed on rodents [26–30].

The most common method of isolating SCs purified from contaminants is to use sequential enzymatic treatment of testicular tissue [26, 29–31]. The SC isolation procedure is usually carried out as follows: first the testes are decapsulated, and the protein sheath consisting of fibroblasts, mesenchymal stem cells and extracellular matrix is mechanically removed using forceps and scissors. The decapsulated testes are carefully crushed to release interstitial cells (Leydig cells, peritubular myoid cells, macrophages, endothelial cells, fibroblasts and mesenchymal stem cells), which are then washed out with a series of decantations or low-speed centrifugation. The tissue remaining in the sediment is subjected to two-step incubation in a mixture of enzymes (collagenase, trypsin and hyaluronidase) in various combinations, depending on the specific protocol. The first step involves digestion of the extracellular matrix, which leads to detachment of more interstitial cells from the outer surface of the seminal tubules. After washing off the detached cells, the isolated tubules are further incubated in a second enzyme mixture to shred the tubules into individual SCs and germ cells, which are then incubated at 32–37 °C. Since the germ cells have no tendency to adhere, they are easily washed off with hypotonic saline or during subsequent nutrient medium changes. The main SC isolation stages are presented below (Fig.).

There are modifications of the above-described testicular tissue processing protocol, consisting in crushing the tubules only after the first enzymatic digestion or in using filtration (instead of centrifugation) to separate the cells from the tubule microfragments obtained as a result of crushing [32, 33]. To increase the purity of SC preparation, it has been proposed to separate the isolated cells obtained by enzymatic digestion by centrifugation in density gradients or by placing them for a short period in lectin-coated cups before washing out unattached cells [34]. According to several authors, enzymatic methods can be applied on testicular tissue of both prepubertal and adult rats and mice [30, 35, 36]. However, as has been shown in some published works, germ cell contamination can be high when using age-matched animals, which necessitated increasing the incubation time of the testicular tissue with each enzymatic mixture. In addition, the use of hypotonic shock has been strongly recommended for germ cell removal [30, 37].

Thus, several factors can affect the amount of cell mass secreted and the purity of the resulting SCs preparation, including the age of the donor. In mice, the population of Sertoli cells drops from 50% of the total number of testicular cells immediately after birth to <1% in adults [38, 39].

Although only mature SCs are thought to perform their functions in spermatogenesis in vivo, such as formation of the blood-testis barrier and fluid secretion, it is widely recognized that they do not proliferate and can only be maintained in culture for a limited time [12]. At the same time, some researchers obtained proliferating "sertoli-like" cells from the testes of adult rats and mice, which could be cultured for several weeks [40, 41].

## CULTURE OF SERTOLI CELLS

The use of primary cultures has enabled molecular and genetic studies that have contributed to elucidating the mechanisms by which SCs maintain germ cells and influence the process of spermatogenesis [10, 11]. Obtaining SC cultures and studying their morphophysiological properties during incubation in vitro are fundamental tools for studying the molecular mechanisms that maintain homeostasis and develop pathological processes in the testes. In this case, it is possible to follow the morphophysiological changes occurring in different cells

under the influence of all kinds of specific substances introduced into the culture medium, including hormones, growth factors and other substances with both stimulating and inhibitory effects [12]. However, due to the fact that it is almost impossible to fully simulate the complex interactions between different cell types in the testis under in vitro conditions, results obtained under culture conditions must be interpreted with caution and, if possible, confirmed in in vivo experiments. For example, testosterone plays a crucial role in regulation of spermatogenesis, but SCs do not seem to respond to this androgen under culture conditions, most likely due to reduced expression of androgen receptors [13, 14]. Nevertheless, in vitro systems have been shown to reflect many features of SCs previously observed in vivo, such as dense compound formation, transferrin secretion, germ cell phagocytosis, and response to follicle-stimulating hormone [15]. Besides, most of the properties first observed in vitro have been shown to occur in vivo [42].

Since immature SCs are less differentiated and more prone to proliferation, their adaptive capacity under culture conditions is higher than that of cells obtained from adult rodents. Therefore, most studies are performed on SCs isolated from prepubertal (immature) animals, usually at the postnatal period of 18–22 days in rats and 10–18 days in mice [1, 26, 32, 43, 44]. Meanwhile, there is evidence that immature SCs can behave in vitro in many respects in the same way as their adult counterparts [45]. For example, primary cultures of immature SCs showed comparable kinetics with cultures from mature donors in terms of phagocytosis and expression of connective proteins involved in formation of the blood-testis barrier [46, 47]. Recent studies have shown a reduced content of intracellular lipids and proteins involved in cellular metabolism in SCs isolated from 20-day-old mice as compared to similar parameters determined in SCs obtained from adult mice [48]. Therefore, caution should be exercised when extrapolating the results obtained in studying SCs from immature rodents to the presumed physiological situation in adults.

The basic conditions under which SCs are cultured should be briefly described. Usually, Dulbecco's modified eagle medium with or without fetal bovine serum and with additives such as insulin, transferrin, sodium selenite, and epidermal growth factor are used when incubating SCs [32, 48–50]. Although the use of various additives is intended to mimic the microenvironment that exists in vivo, it is unclear how their absence affects SCs cultures. Interestingly, reducing the amount or having no serum in the culture medium has been suggested to improve the purity of primary SCs cultures [40, 41, 51]. Not using nutrient additives, primarily serum, as if creating conditions for "starvation" of SCs, is aimed at avoiding a significant increase in the number of rapidly proliferating contaminant cells, such as peritubular myoid cells, fibroblasts, endothelial cells and MSCs [40, 52]. In addition, the presence of serum affects phagocytic activity and can inhibit response to certain hormones, such as follicle-stimulating hormone [53, 54]. Meanwhile, it has long been shown that SCs can retain their viability, morphology, and secretory activity in the absence of serum [55]. However, a number of reports in recent years indicate that, if optimal conditions for serum use are observed when obtaining primary SC cultures, negative consequences can be avoided [35, 56, 57]. Therefore, despite the fact that the "starvation" technique is quite successfully used in obtaining cleaner SCs populations, a comparative study of the effect of different conditions of serum application should be performed to further clarify its effectiveness.

## CONCLUSION

Given the unique morphophysiological features of Sertoli cells, development of methods for their isolation and cultivation can be considered very promising. Studies using SC cultures in vitro and in vivo will not

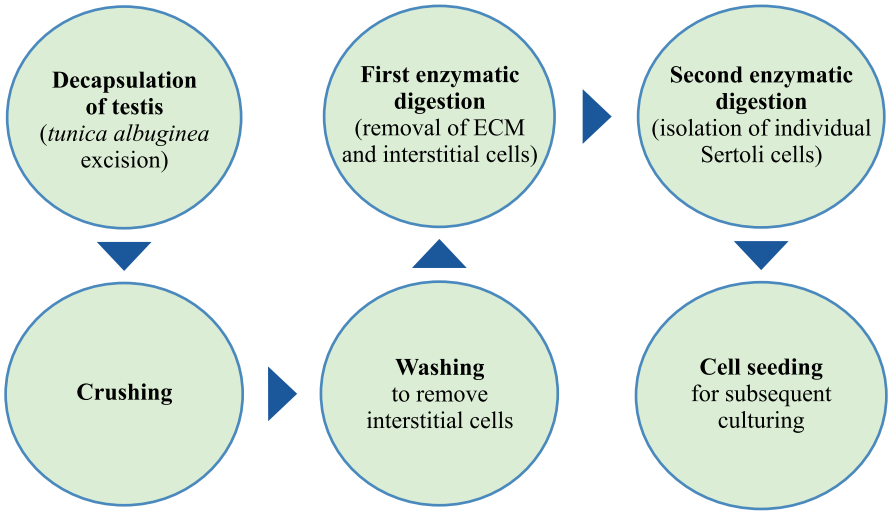


Fig. Main stages of Sertoli cell isolation

only produce new evidence on male genital diseases and develop methods for their correction, but also solve a number of problems in various fields of biology and medicine, primarily in tissue engineering and transplantology. The ability of Sertoli cells to stimulate the growth and survival of a number of cells during co-culture and exert an immunomodulatory effect on them can be used to improve cell transplantation outcomes.

*The authors declare no conflict of interest.*

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# MATURE RAT HEPATOCYTE DEDIFFERENTIATION INTO LONG-LIVED PROLIFERATING HEPATIC PROGENITOR CELLS

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**Objective:** to obtain long-lived proliferating cells with progenitor features by dedifferentiation of mature rat hepatocytes using combinations of small molecules. **Materials and Methods.** Hepatocytes isolated from rat liver by perfusion were cultured in the presence of a cocktail of three small molecules – Wnt signaling pathway activator (CHIR99021), TGF-β inhibitors (A83-01) and ROCK kinase (Y27632). The morphological characteristics and growth features of the culture were assessed using fluorescence and phase-contrast microscopy during cell culture. Cell proliferative activity was analyzed using real-time time-lapse imaging. The expression of surface and intracellular markers was analyzed using flow cytometry and high-resolution fluorescence microscopy. **Results.** Using a cocktail of small molecules, Y-27632, A-83-01, and CHIR99021, long-lived proliferating cells that express progenitor cell markers, such as α-fetoprotein and HNF4α, were obtained from mature rat hepatocytes. The cells had hepatocyte-like morphology and formed discrete clusters of proliferating cells, forming a single cell layer during culturing. Removal of the small molecules from the medium led to expansion of fibroblast-like cells and elimination of potentially progenitor hepatocyte-like cells. **Conclusion.** Proliferating progenitor cells can be obtained by dedifferentiation of mature hepatocytes.

*Keywords: hepatocytes, dedifferentiation, hepatic progenitor cells, small molecules, signaling pathways, Y-27632, A-83-01, CHIR99021.*

## INTRODUCTION

Despite the fact that primary rat hepatocytes were first isolated from the liver more than 50 years ago [1], and the first report on isolation of hepatocytes from the human liver appeared 20 years later [2], issues on long-term culture of these cells and their preservation in functionally active state in vitro still remain unsolved. Meanwhile, it has been reliably proved that in conditions of chronic liver injury, hepatocytes are able to de-differentiate into proliferating bipotent progenitor/stem cells [3]. In this regard, coming up with ways to obtain progenitor cells from mature hepatocytes in vitro is of great importance. This may not only help to better understand the origin of hepatic progenitor cells and reprogramming mechanisms, but also offer an unlimited source of cells for generation of functional hepatocytes, which are widely used in pharmacology, clinical medicine, tissue engineering and disease modeling.

Over the past few years, scientists have managed to achieve certain successes in this direction. For example, protocols for transdifferentiation of mesenchymal stem cells into functionally active hepatocyte-like cells have been developed [4]. Many works have appeared in which hepatocytes were obtained from induced pluripotent stem cells [5], and a number of studies have described approaches to obtaining the so-called induced hepatocytes from various somatic cells [6]. All these methods, of course, have certain scientific interest, but at the same time have a number of limitations in terms of prospects of use in clinical practice. In particular, the use of genetic modifications for cell reprogramming poses a number of problems for the safety of their clinical use. One of the promising current approaches to address safety issues is cell reprogramming using the so-called small molecules. Small molecules are low molecular weight compounds that can include lipids, monosaccharides, secondary messengers, other natural compounds, as well as drugs and other xenobiotics. Small molecules have a number of undeniable advantages over any other way of regulating/altering cell fate: as a rule, they are permeable to cells; they are easier to synthesize and standardize; their cost is low, which allows using this method for mass production of cells with given properties. More importantly, the effects of small molecules can be regulated by varying their concentrations and combinations, thus providing a higher degree of temporal and spatial control over the

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function of the target protein or signaling pathway [7]. In 2017, Katsuda et al. [8] published a paper showing that it is possible to obtain proliferating hepatic bipotent progenitor cells from mature rat hepatocytes using a cocktail of small molecules such as Y-27632, A-83-01 (ROCK kinase and TGF-β inhibitors, respectively) and CHIR99021 (Wnt signaling pathway inhibitor) without any genetic modifications. The obtained cells, which the authors called chemically induced liver progenitors (CLiPs), had stem cell properties, i.e., they proliferated in culture and differentiated into hepatocytes and cholangiocytes. Around the same time, another work was published, which described a method for obtaining hepatic progenitor cells from mature murine hepatocytes by creating in vitro conditions simulating chronic liver injury conditions in vivo, when not only proliferation of mature hepatocytes occurs but also their reversible transformation into progenitor cells of the ducts occurs [9]. In addition to the aforementioned small molecules CHIR99021, A83-01 and Y27632, this medium also contained epidermal growth factor (EGF) and hepatocyte growth factor (HGF), Yap signaling activators – lysophosphatidic acid and sphingosine-1-phosphate. Proliferating duct-like cells (hepPDCs) obtained from hepatocytes in this manner were able to undergo more than 30 passages without obvious morphological changes or karyotype disruption. Gene expression profiles showed that these cells express markers of hepatocytes, cholangiocytes and hepatic progenitor cells, and are also capable of differentiating into mature functionally active hepatocytes [9]. Using a similar culture medium with slight modifications, the authors obtained progenitor cells from mature human hepatocytes after 2 years [10]. Also in 2019, the work of Kim et al. [11], who used a cocktail of two small molecules A83-01 and CHIR99021 in combination with EGF and HGF to obtain human hepatic progenitor cells from mature hepatocytes, which they named human chemically derived hepatic progenitors (hCdHs). The resulting hCdHs cells expressed hepatic progenitor cell markers and underwent about 10 passages in culture, maintaining a normal karyotype and the ability to differentiate into hepatocytes and bile duct epithelial cells in vitro. Gene profiling confirmed reprogramming at the transcriptional level, showing expression of the genes responsible for the progenitor state and suppression of mature hepatocyte genes. In intrasplenic transplantation in several animal models, hCdHs cells effectively repopulated the liver parenchyma [11]. More recently, work was published in which in a similar manner, using a cocktail of two small molecules A83-01, CHIR99021 and HGF, bipotent progenitor cells were derived from mature hepatocytes of nonhuman primates [12].

Taking into account the importance of the problem of obtaining proliferating liver cells in culture for solving the problems of cell biology, the lack of work on this topic in national science, as well as the importance of developing technology for cell mass reproduction and use in regenerative medicine, we aimed our work at obtaining proliferating cells with progenitor features by dedifferentiation of mature rat hepatocytes using combinations of small molecules.

## MATERIALS AND METHODS

Rat liver cells were isolated by two-stage perfusion using a collagenase buffer, followed by washing and seeding on culture plates modified with bovine collagen type I. The first stage of perfusion was performed with 250 ml of Liver Perfusion Medium (LPM) (Gibco™, USA), the first two minutes at 5 ml/min, followed by the remaining volume at 25 ml/min. The second stage of perfusion was performed with Hanks' collagenase buffer (HBSS without $Ca^{2+}$ and $Mg^{2+}$, without phenol red, (Gibco™, USA)) with addition of 0.03% type IV collagenase (PanEco, Russia) and 0.5% bovine serum albumin BSA (Diaem, Russia) in 250 ml/min at 25 ml/min. At the end of perfusion, the liver was placed in L-15 medium cooled to +2…+4 °C (Gibco™, USA) and transported from the operating room to a laminar box. Next, the Gilson capsule was opened with a scalpel, and the contents were crushed in a sterile Petri dish on ice. Then they were passed through a cell sieve with a pore size of 100 μm, washed with Hepatocyte Wash Medium (Gibco™, USA), cooled to +2…+4 °C. The cell suspension was centrifuged at +2 °C at 50 g for 5 minutes. The cell precipitate was resuspended in a fresh portion of medium and the procedure was repeated three times. Next, the precipitate was resuspended in William's E Medium (Gibco™, USA) with the addition of 5% FBS, (HyClone, UK), 1% antimycotic antibiotic (Gibco™, USA) and 200 mM L-glutamine (PanEco, Russia), after which live cell counts were made on a BioRad TS20 cell counter.

In order to determine the working concentrations of small molecules, we performed colorimetric analysis of cell proliferation in an MTT assay. As a cell model of hepatocytes, we used a human line HepG2, which was cultured under standard conditions (37 °C, 5%: $CO_2$) in complete growth medium based on DMEM/F12 (Gibco™, USA) with addition of 10% FBS, penicillin/streptomycin (100X, PanEco, Russia) and 200 mM L-glutamine (PanEco, Russia). For the test, serial dilutions of each inhibitor were added to the wells of a 96-well flat-bottom plate: for A-83-01, the concentration range was 2.5 μM to 0.03 μM, for CHIR99021 from 15 μM to 0.2 μM, for Y-27632 from 50 μM to 0.75 μM. Then, cell suspension (10,000 cells/well) was added to the wells. Each point in one experiment in four replicates. Intact cells cultured under the same conditions but without adding inhibitors were used as the control. The cells were incubated with small molecules for 4 days. After that,

culture medium was drained from the wells, 30 μl/well of MTT solution (5 μg/ml (Sigma, USA)) was added, and incubated in a $CO_2$ incubator for 2–4 hours until formazan crystals precipitated, which were then dissolved in 100 μl/well of DMSO (dimethyl sulfoxide; PanEco, Russia). Optical density (OD) was measured on a Tecan infinite M200 Pro plate reader (Tecan, USA) at 565 nm wavelength. Cell proliferation level was measured as described previously [13], using the formula:

(OD of induced cells minus OD blank) / (OD of control cells minus OD blank) × 100%, where OD blank is the optical density in the wells containing MTT and DMSO solution but no cells. OD of control cells is the optical density in the wells containing intact cells.

For surface modification, a solution of type I bovine collagen in cold (+2…+4 °C) DPBS at the rate of 12.5 μg of collagen per 1 $cm^2$ surface was added to culture vials and Petri dishes, then placed for 1 hour in an incubator at 37 °C. Then, the liquid was drained and the cell suspension was inoculated at a given concentration: $2 \times 10^5$ cells/$cm^2$. After two hours of incubation under standard conditions (37 °C, 5%: $CO_2$), William's E Medium and unattached cells were removed and Hepatozyme-SFM medium (Gibco™, USA) was added with 1% antibiotic antimycotic (Gibco™, USA), 200 mM L-glutamine (PanEco, Russia), a combination of small molecules in the following concentrations: 1.25 μM for A-83-01, 5 μM for CHIR99021, and 12.5 μM Y-27632. Control cells were cultured in the same growth medium without adding the small-molecule cocktail. After 48 hours, the growth medium was replaced with William's E Medium with the same additives. Cells were passaged when 50–70% confluence was achieved according to the standard technique using Versen solution (PanEco, Russia) and TrypLe reagent (Gibco™, USA).

At certain time intervals, cells on Petri dishes were stained with the Live/Dead Assay complex of vital dyes (Invitrogen, USA), which allowed us to evaluate the morphology of adherent cells and distinguish between live and dead cells. The study was performed using a Leica DMi8 Thunder super-resolution microscopic system equipped with a Leica DFC9000 GTC camera (Leica, Germany).

Cell proliferation was assessed using a microscopy system with automatic zeitrapher imaging (IncuCyte ZOOM, USA). The system allows us to evaluate the confluence of cell monolayer in real time with high accuracy and calculate the growth curves of cell culture.

For the analysis, we used passage 1 cells, which were seeded into 25 $cm^2$ culture vials at the rate of $4 \times 10^3$ cells/$cm^2$. Cell growth was studied on 4 types of media:

1. William's E Medium with addition of a small-molecule cocktail;
2. William's E Medium;
3. DMEM/F-12 Medium with addition of a small-molecule cocktail;
4. DMEM/F-12 Medium.

The analysis was performed in real time for 26 days. Growth curves were plotted automatically by the device's software.

For cytofluorometric analysis of expression of surface marker CD29, passage 1 cells cultured with addition of a small-molecule cocktail were removed from the plate, incubated with antibodies to CD29, labeled with FITC (BD Biosciences, USA) for 1 hour. After incubation, the cells were washed twice in DPBS, fixed in CytoFix (BD Biosciences, USA) for 15 minutes, and measured on a BD FACSAria III flow cytometry sorter (BD Biosciences, USA). At least 10,000 events were analyzed. Data was processed using FlowJo_V10 software.

The expression of intracellular markers in cells cultured in the presence of a small-molecule cocktail was analyzed by flow cytometry and fluorescence microscopy. For this purpose, cells were permeabilized in 0.1% Triton X-100 solution (Sigma, USA) for 10 minutes at room temperature. Then they were incubated with primary antibodies against Ki-67, α-fetoprotein (AFP), HNF4α, and cytokeratin 18 (all by SantaCruz Biotechnology, USA) for 1 hour, and then with FITC-labeled secondary anti-species antibodies (all by Sigma, USA) for 40 minutes. Cells stained only with secondary anti-species antibodies were used as controls for nonspecific binding. For fluorescence microscopy, cell nuclei were doped with DAPI (1 μg/mL) (4',6-diamidino-2-phenylindole; Invitrogen, USA) and the preparation was encased in fluorescence protector (Anti-Fade Fluorescence Mounting Medium, Abcam, USA). Fluorescence analysis and flow cytofluorimetry were performed as described above.

## RESULTS AND DISCUSSION

At the first stage of work, we analyzed the effect of small molecules on the proliferation level of HepG2 cells and determined the optimal concentrations of small molecules for further work.

Fig. 1 shows that all the three small molecules in certain concentration ranges induced an increase in the proliferative level of the HepG2 cell line. Thus, the ROCK kinase inhibitor (Y27632) increased the level of cell proliferation in the concentration range from 0.75 to 12.5 μM (Fig. 1, a), the Wnt signaling pathway inhibitor (CHIR99021) stimulated proliferative activity in the range from 0.2 to 7.5 μM (Fig. 1, b), whereas the TGF-β inhibitor (A83-01) increased cell proliferation throughout the entire concentration range of 0.03 to 2.5 μM used (Fig. 1, b). Based on the data obtained and guided by previously published works in which the same small molecules were used to reprogram hepatocytes, the following working concentrations of small molecules were

determined: 12.5 μM for Y-27632, 5 μM for CHIR99021 and 1.25 μM for A-83-01.

Fluorescence and phase-contrast microscopy were used to analyze the effect of the small-molecule cocktail on formation of proliferating cell culture from rat hepatocytes. After isolation, rat hepatocytes were divided into two cultures. In the control culture, cells were cultured without addition of small-molecule cocktail, in the experimental culture, cells were cultured with addition of small-molecule cocktail in the working concentrations determined in preliminary experiments. Fluorescence microscopy of cells stained with the Live/Dead™ complex 24 hours after cell isolation showed that a significant number of viable cells were present in both the control and the experimental variant (Fig. 2).

It is noteworthy that already one day after isolation, the difference in the level of viability of control and experimental cultures was clearly visible. A much higher level of cell death can be observed in the control cells (Fig. 2, a) compared to the experimental cells that were cultured in the presence of the small-molecule cocktail (Fig. 2, b). Morphologically, two types of cells can be distinguished in the experimental culture: hepatocyte-like and oval-shaped cells.

On the fifth day of the experiment, even more significant differences in the experimental and control cell cultures were noted (Fig. 3, a, b). By this time, in the control culture, mainly single strongly expanded cells with signs of plasma and nuclear membrane disruption and increased vacuolization were preserved (Fig. 3, a). Whereas in the experimental culture along with the same mature cells, transitioning into the state of cell death, groups of newly formed cells were visualized (Fig. 3, b).

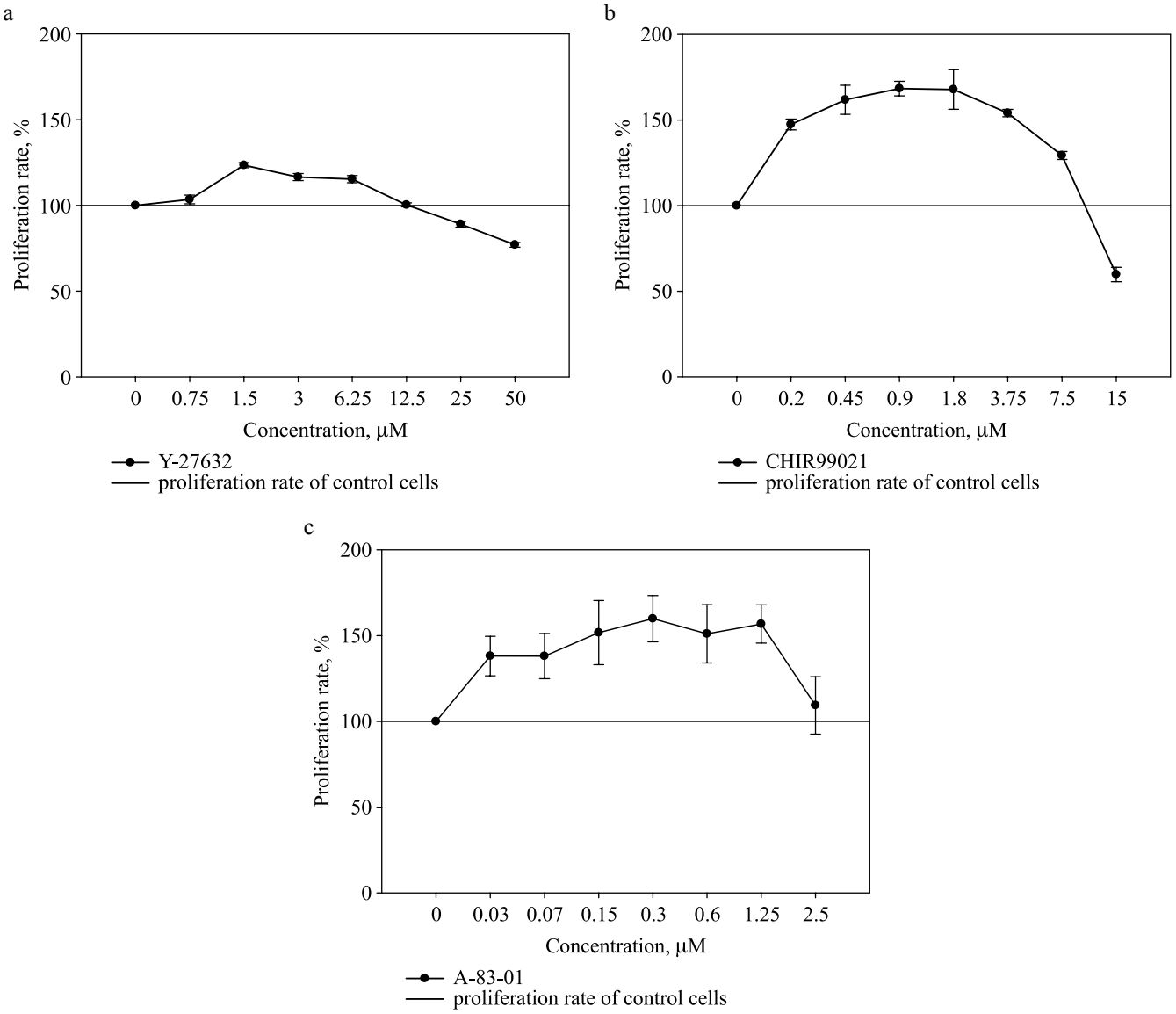


Fig. 1. Dependence of HepG2 cell proliferation level on serial dilutions of small molecules in the culture medium: a – in the presence of Y-27632; b – in the presence of CHIR99021; c – in the presence of A-83-01

By day 7 of the experiment, the process of cell death continued in the control culture (Fig. 3, c). In the experimental culture, proliferation enhancement with the formation of small cell clusters was clearly visualized (Fig. 3, d). On day 10 of the experiment, due to complete cell death in the control culture, only the experimental variant was monitored. During this time in the experimental culture, there was increased count of cells in the formed clusters, compaction of their structure and formation of the extracellular matrix (Fig. 4).

After 15 days of the experiment, there was cell expansion in clusters, which led to their outgrowth, fusion with neighboring clusters, and formation of a cell layer. The level of cell death in the culture was insignificant (Fig. 5).

For all subsequent experiments, cells after the first passage were used, which from the moment of isolation were continuously cultured in the presence of the small-molecule cocktail. To evaluate the effect of different growth media and the small-molecule cocktail on cell growth in culture, proliferation analysis was performed, and growth curves were plotted using a microscopy system with automatic zeitraffer imaging. Analysis of the growth of cell population on different media with and without addition of the small-molecule cocktail showed that the cells that were cultured on media (DMEM/F12 and William's E) without addition of small molecules demonstrated the most rapid growth and uniform expansion (Fig. 6, a and b).

However, visual analysis of images revealed that in both variants of the growth media in the absence of the small-molecule cocktail, growth of the cell population occurred due to expansion of fibroblast-like cells. Characteristic clusters of hepatocyte-like cells in these variants were absent throughout the experiment (26 days).

On the other hand, when cells were cultured in both types of growth media (DMEM/F12 and William's E) in the presence of small-molecule cocktail after the first passage, formation of hepatocyte-like cell clusters took place, the same as we observed in the initial experimental variant after isolation of hepatocytes before passage. Thus, it is shown that for maintenance of growth and proliferation of hepatocyte-like, but not fibroblast-like, cells, not only the initial exposure to the small-molecule cocktail is necessary, but also their constant presence in the growth medium throughout the entire period of cultivation. It is noteworthy that when cultured on a DMEM/F12 medium, cell cluster formation began significantly earlier (48 hours after cell planting), whereas in William's E medium, it was noted only on day 7 of culturing. The number of cell clusters in the field of view also differed. For each cell culture sample, 80 fields of view per 25 $cm^2$ culture vial were analyzed. When cultured on DMEM/F12 medium in the presence of small molecules, cell clusters occurred in 90% of the analyzed fields of view. When growing on William's E medium with addition of small molecules, this figure reached only 50%.

Phase-contrast microscopy of cells at different stages of cell culture growth allowed us to visualize formation of individual clusters. The center of such cluster formation is large, mostly irregularly shaped binuclear cells (Fig. 7).

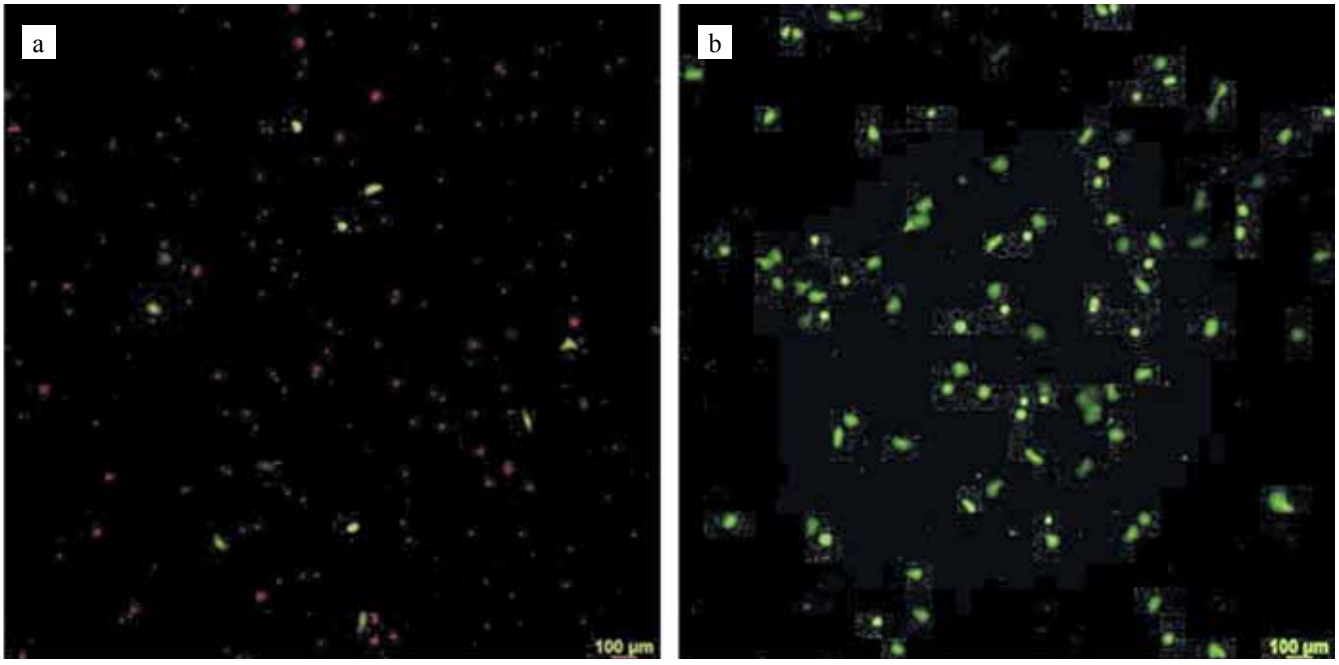


Fig. 2. Fluorescence microscopy of liver cells 24 hours after isolation. Live/Dead™ staining. a – control cells (without addition of the small-molecule cocktail), b – experimental cells (with addition of the small-molecule cocktail). Green color – live cells, red – cells in a state of death. 50× magnification

A significant number of cells containing two nuclei are found in the emerging cell cluster. It is known that one of the characteristic features of the liver is hepatocyte polyploidy, i.e. increased number of the set of chromosomes per cell. Polyploid hepatocytes can be presented in several variants: they can be tetraploid (bicore with a 2n set of chromosomes or unicore with a 4n set of chromosomes) or octaploid (bicore with a 4n set of chromosomes or unicore with an 8n set of chromosomes) [14]. The functional role of hepatocyte polyploidy is still not entirely clear. However, it has been shown that polyploid hepatocytes have increased proliferative capacity, expressing a number of genes associated with the passage of the cell cycle [15].

Further, in the process of culturing, there is active cell division and peripheral growth of the cluster with formation of significant clusters of densely packed cells (Fig. 8, a) at a later stage in close proximity with fibroblast-like cells (Fig. 8, б).

Expression of surface and intracellular markers was analyzed on cells after the first passages cultured with addition of the small-molecule cocktail.

CD29 or integrin β1 belongs to the family of integrin proteins that are involved in cell interactions with extracellular matrix proteins such as collagen, laminin, and fibronectin, as well as in heterodimers, participates in intercellular interactions, and plays a functional role in cell migration, adhesion, and survival. CD29 is widely

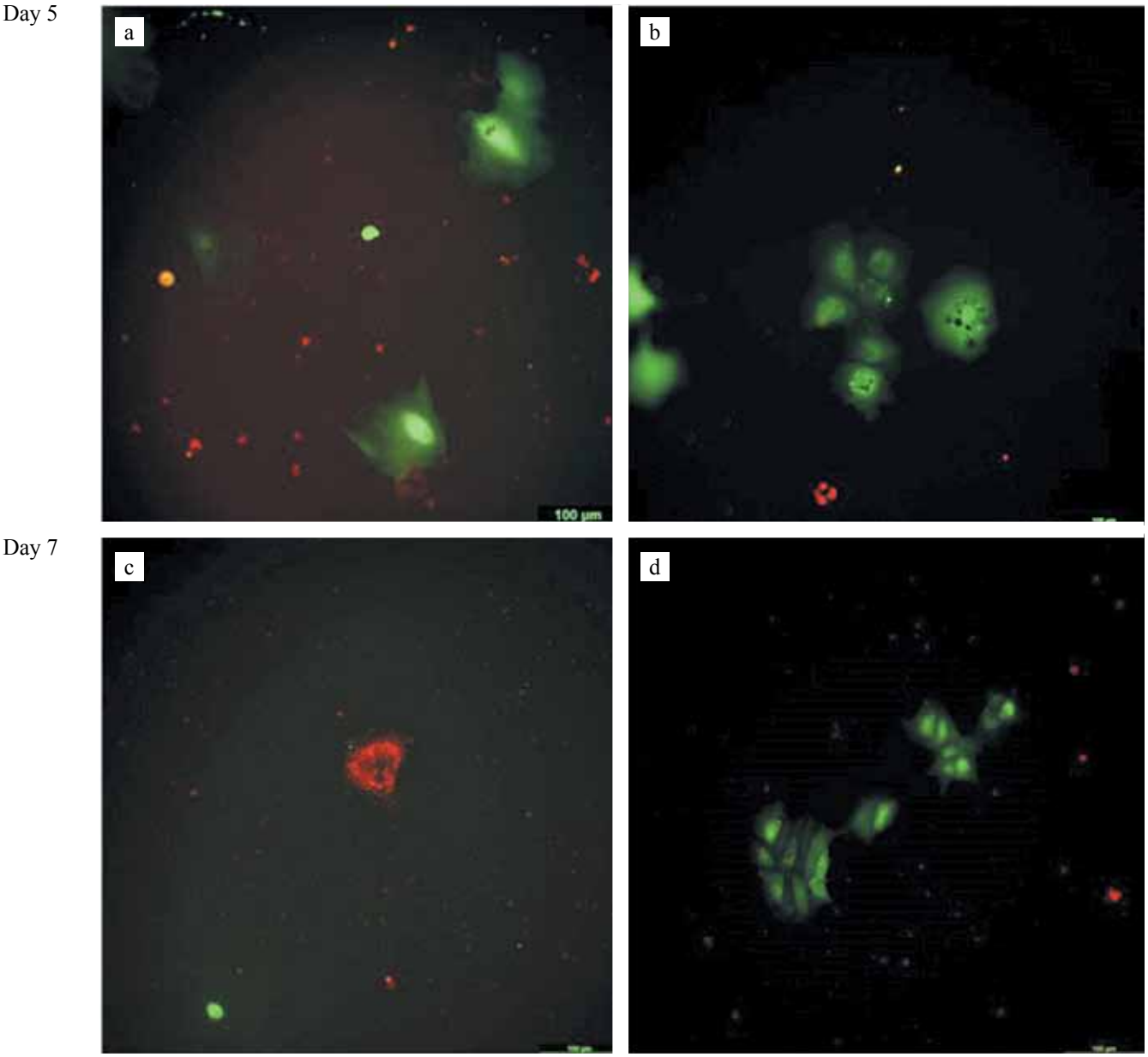


Fig. 3. Fluorescence microscopy of liver cells at day 5 (A and B) and day 7 (C and D) after isolation. Live/Dead™ staining. a, c – control cells (without addition of small-molecule cocktail), b, d – experimental cells (with addition of the small-molecule cocktail); b – group of newly formed cells; d – formation of cell clusters. Green color – live cells, red color – cells in a state of cell death. 200× magnification

expressed on various cell types, including mesenchymal and epithelial cells, as well as stem/progenitor cells of different origin [16]. So, CD29, along with CD44 and CD90, is often considered a marker of mesenchymal stem/stromal cells (MSCs) isolated from different tissue sources, both in humans [17] and in rodents [18]. In the adult liver, integrin β1 is expressed by mature hepatocytes [19], as well as by hepatic stem cells and hepatoblasts [20].

Using flow cytofluorimetry, we analyzed the expression of Integrin beta 1 (CD29) on the cell surface (Fig. 9, a).

As seen in Fig. 9, a, CD29 expression was observed on more than 60% of the cells. Morphologically, hepatocyte-like cells predominated in this culture (Fig. 9, b).

Deletion of integrin beta 1 in the embryonic liver leads to disruption of the normal development of hepatocyte polarity, lack of specification of intercellular contacts, and failure to form tubules. Directed deletion of integrin beta 1 in adult hepatocytes prevents restoration of normal hepatocyte architecture after liver injury followed by development of fibrosis [21]. So, CD29 expression on cells derived from rat hepatocytes after culturing them in the presence of small molecules may also ensure maintenance of the characteristic architecture of the forming clusters and promote proliferation.

The expression of intracellular markers in cells was analyzed using two methods. Using ultrahigh resolution fluorescence microscopy, the expression of such liver-associated proteins as cytokeratin 18, alpha-fetoprotein, HNF4α, and the proliferation cell marker Ki-67 was assessed.

Proliferation marker Ki-67 is expressed in the nucleus during the G1, S and G2-M phases of cell cycle in proliferating cells [22]. We showed that almost 100% of the cells express this protein (Fig. 10, a).

Cytokeratin 18 is a cytoskeleton protein and a major intermediate filament expressed in the liver [23]. Cytokeratin 18, along with cytokeratin 19, has been shown to be expressed in human hepatic stem cells similar to rodent oval cells [24]. We have shown that cytokeratin 18 expression is characteristic of all cells in culture that have hepatocyte-like morphology (Fig. 10, b).

Hepatocyte nuclear factor 4α (HNF4α) is an orphan nuclear receptor that is known to be a master regulator of hepatic differentiation processes because it regulates a large number of hepatocyte-specific genes [25]. HNF4α regulates important liver functions such as glycolysis, gluconeogenesis, fatty acid metabolism, bile acid synthesis, drug metabolism, apolipoprotein synthesis, ureogenesis and blood coagulation by regulating the transcription of multiple genes involved in each of these functions [26–28]. In addition to mature hepatocytes, HNF4α is expressed by human and rodent bipotent hepatoblasts during embryogenesis [29], as well as by hepatic stem cells in the adult organ [30].

We also detected HNF4α expression predominantly in cells forming characteristic clusters (Fig. 11, a).

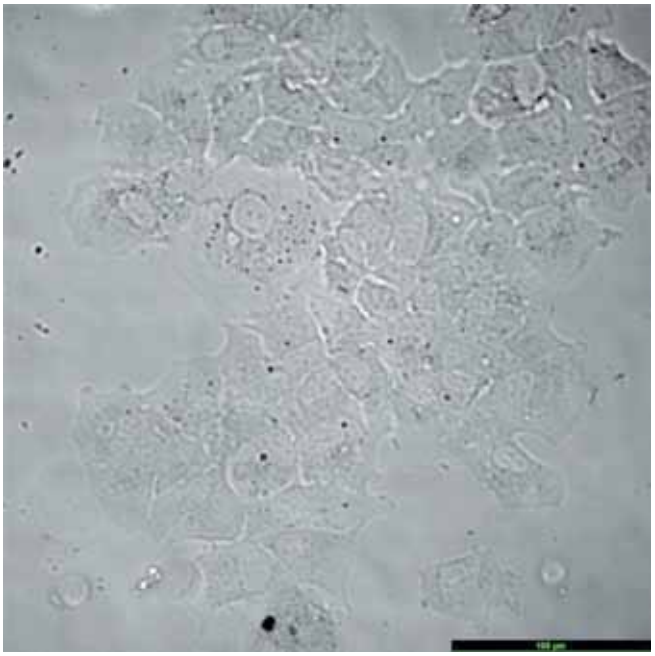

Fig. 4. Phase-contrast microscopy of a dense hepatocyte-like cell cluster at day 10 in the experimental culture. 200× magnification

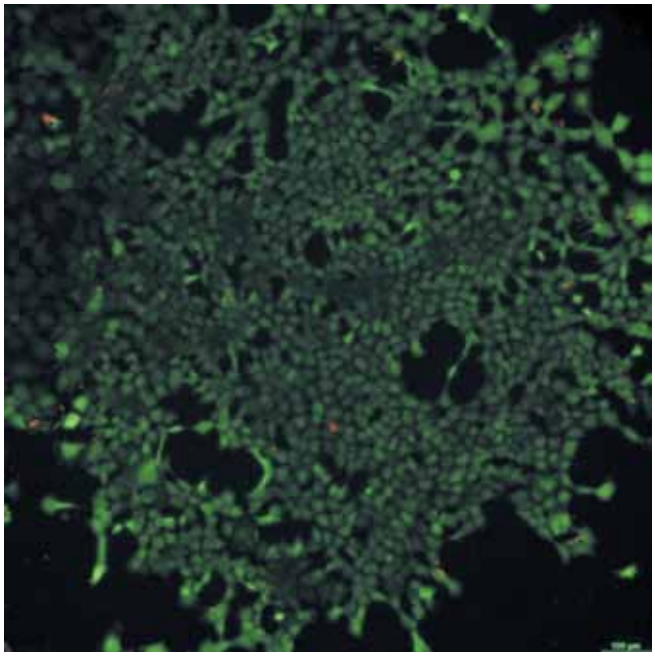

Fig. 5. Fluorescence microscopy of cells on day 15 after isolation. Live/Dead™ staining. Expansion of cells in clusters leading to formation of large cell clusters. Green color – live cells, red color – cells in a state of cell death. 100× magnification

The obtained images clearly show the nuclear localization of HNF4α, which is characteristic of normal hepatic cells. HNF4α expression in the cells cultured in the presence of the small-molecule cocktail indicates their hepatocytic origin and progenitor potential.

Alpha-fetoprotein (AFP) is one of the most studied markers of cell differentiation and tumor development. This protein is expressed by fetal and malignant hepatocytes and is not expressed by normal mature hepatocytes [31]. AFP is a major marker of oval cells [32] and immature hepatocytes [33]. Due to the fact that AFP expression strongly correlates with expression of fetal genes during ontogenesis, this protein serves as an ideal candidate

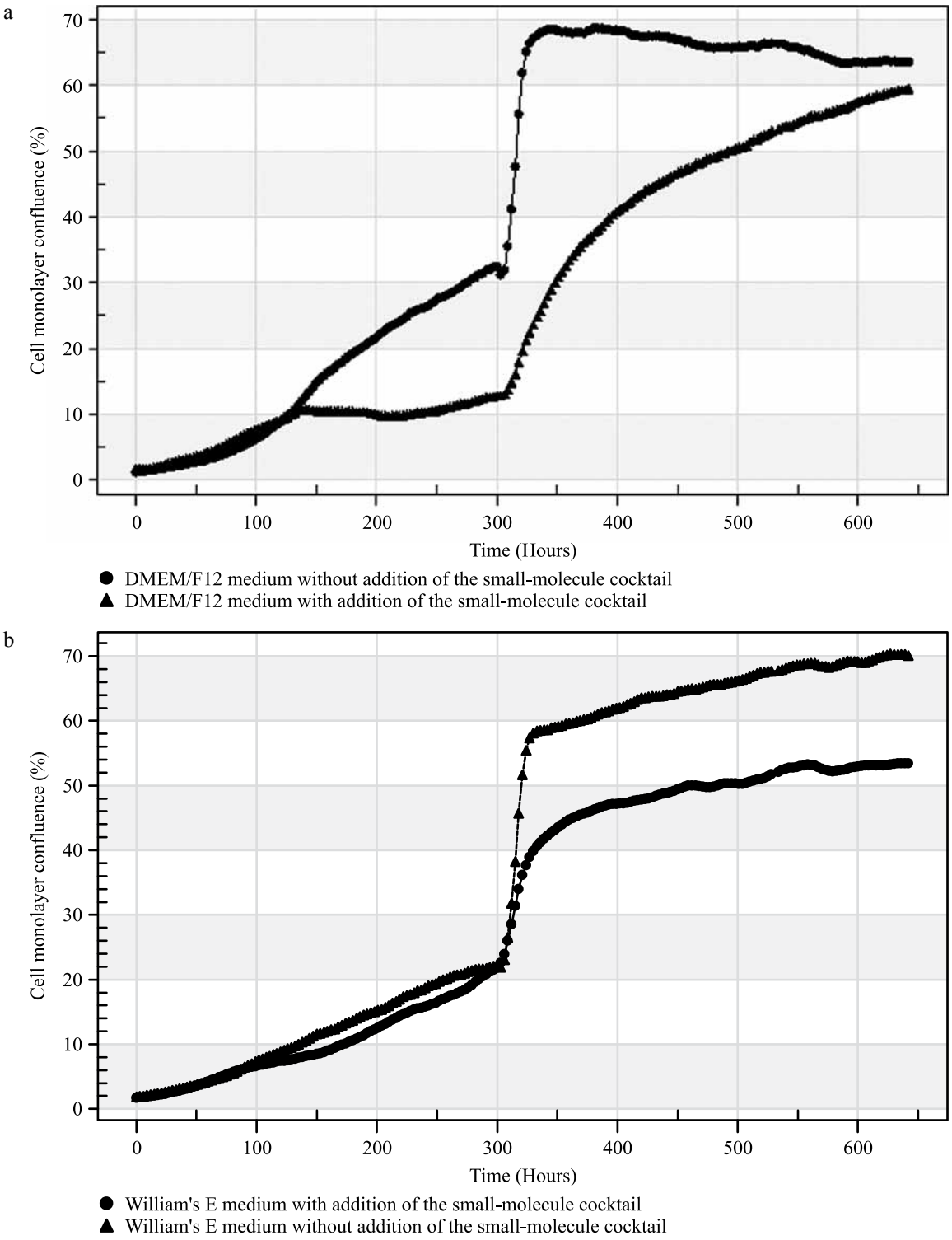


Fig. 6. Growth curves of cells at passage 1 cultured on different media for 26 days. The growth curves were constructed based on estimation percentage of the cell monolayer confluence due to photofixation of visual fields every 4 hours. a – cells were cultured on a DMEM/F12 medium; b – cells were cultured on William's E medium.

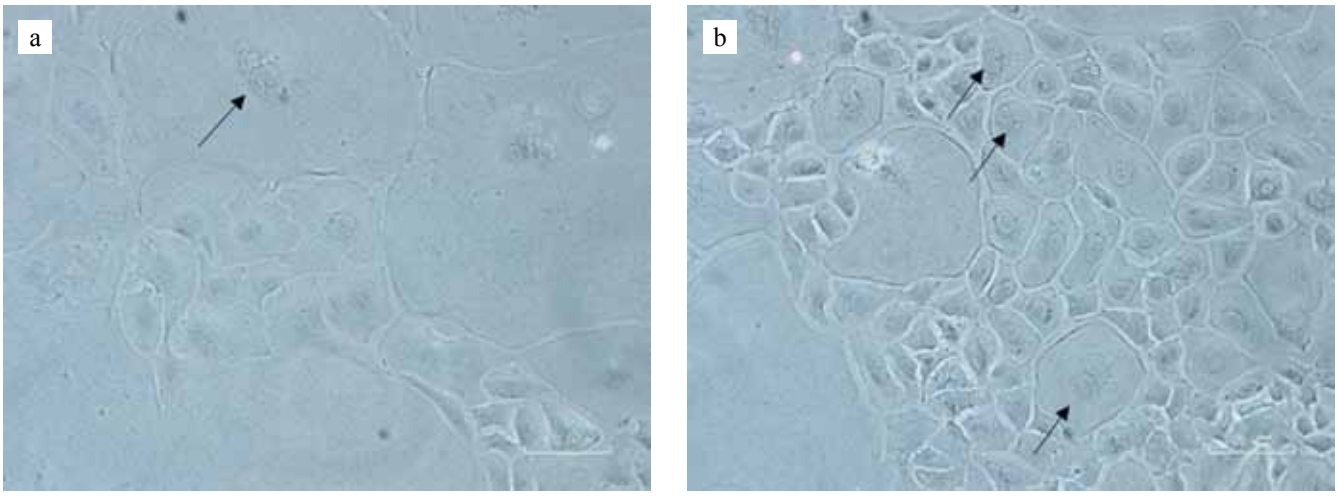


Fig. 7. Phase-contrast microscopy of hepatocyte-like cell clusters forming in the presence of a small-molecule cocktail. a – central binucleated cell, presumably giving rise to cluster growth. b – cell proliferation in a cluster; arrows indicate binucleated cells. 200× magnification

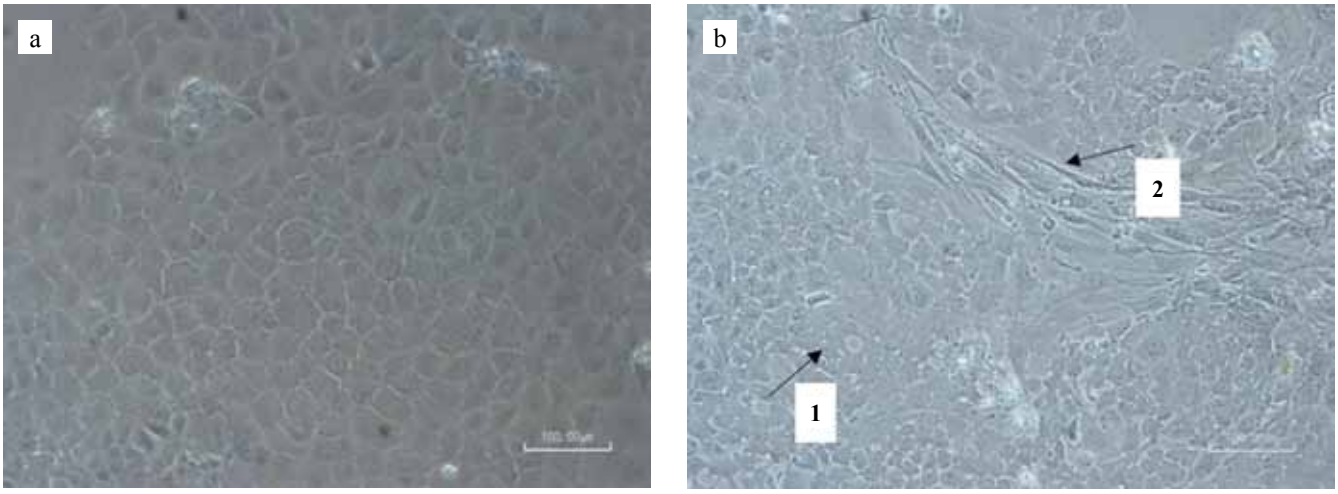


Fig. 8. Phase-contrast microscopy of the cell layer at late stages of culturing (47 days) in the presence of a small-molecule cocktail. a – dense hepatocyte-like cell cluster; 200× magnification. b – contact zone of different cell types within a single cell layer; 1 – hepatocyte-like cells, 2 – fibroblast-like cells. 100× magnification

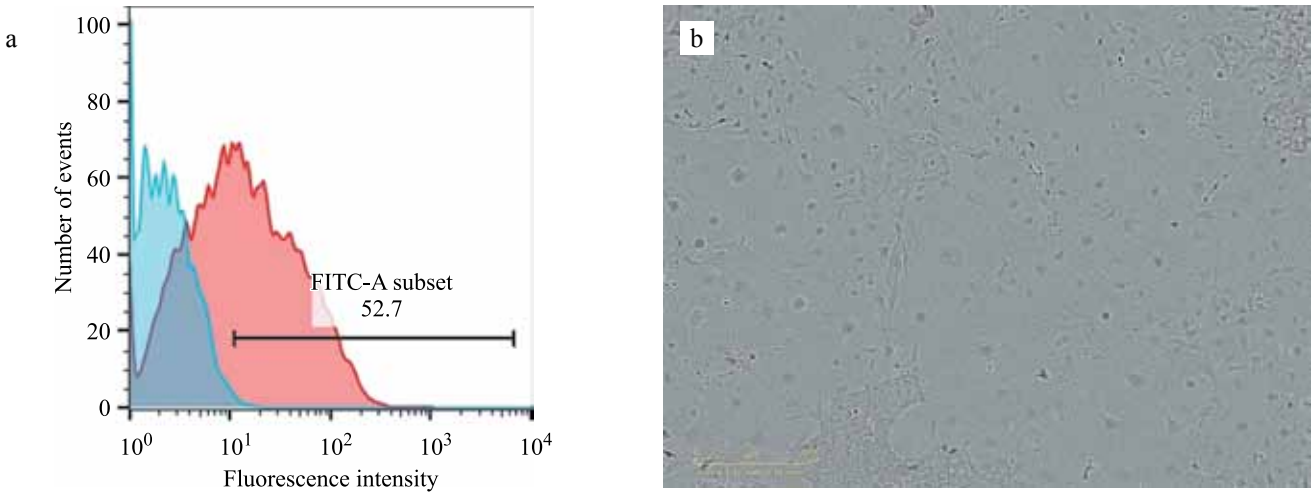


Fig. 9. a – cytofluorimetric analysis of CD29 expression. Blue peak – autofluorescence of control cells not stained with antibodies. Red peak – fluorescence of anti-CD29-FITC bound to the cells. b – phase-contrast microscopy of the culture. 100× magnification

marker for tracking and studying stem cell development, differentiation and redifferentiation pathways.

We analyzed α-fetoprotein expression using two methods, fluorescence microscopy and flow cytometry. For this purpose, we performed intracellular staining of cells with antibodies specific to AFP. The staining results are shown in Figs. 11, b and 12. When performing fluorescence microscopy, we observed a not very distinct pattern of AFP expression in cells, namely, very few cells were stained with antibodies to this protein, and its localization in most cells was predominantly perinuclear, and only in some cells AFP was distributed in the cytoplasm, which is typical for this protein (Fig. 11, b).

To confirm the presence of AFP expression, cytofluorimetric analysis of intracellular staining of cells with appropriate antibodies was performed (Fig. 12).

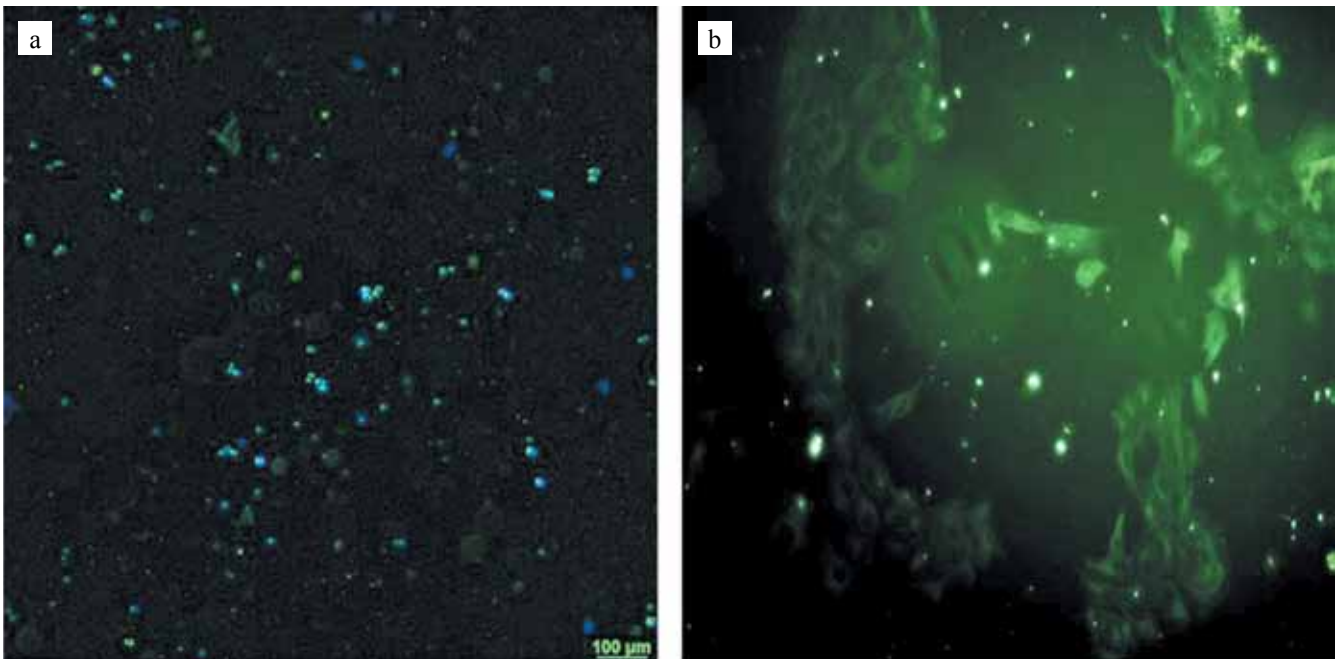


Fig. 10. Fluorescence microscopy of cells after culturing in the presence of small molecules. a – cells were stained with Ki-67 antibodies. Nuclei are stained with DAPI. 100× magnification. b – cells were stained with cytokeratin 18 antibodies. 200× magnification

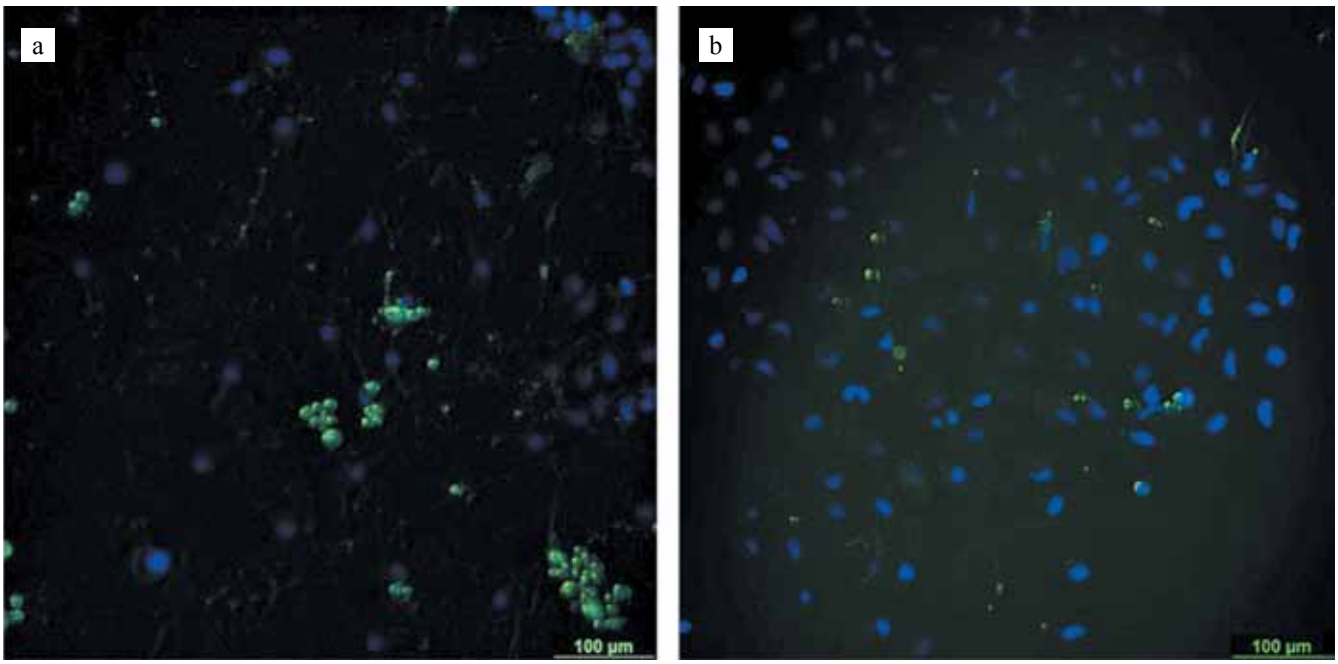


Fig. 11. Fluorescence microscopy of cells after culturing in the presence of small molecules. a – cells stained with anti-HNF4α. b – cells stained with AFP antibodies. Cell nuclei were stained with DAPI. 200× magnification

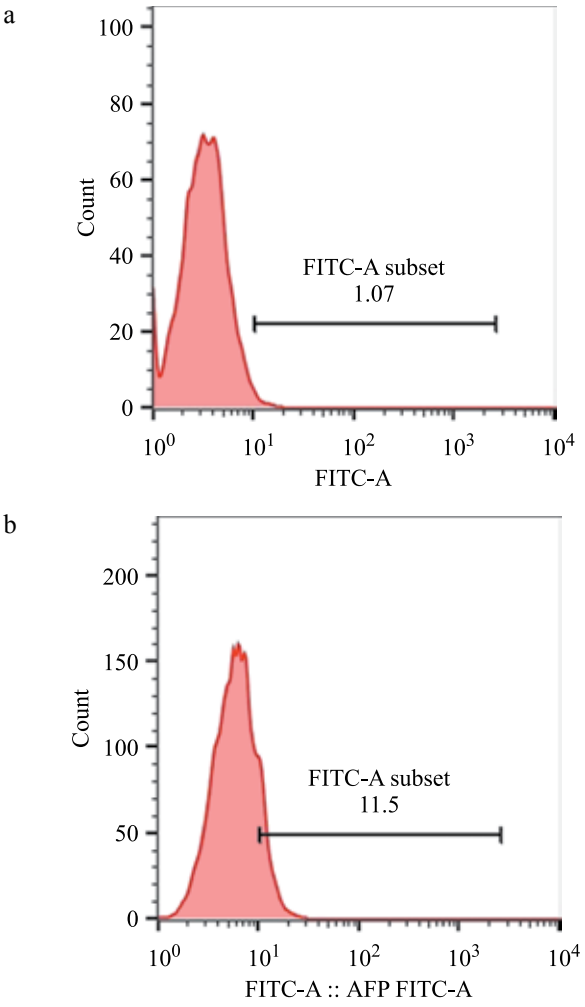


Fig. 12. Cytofluorimetric analysis of AFP expression in cells. a – control cells stained with anti-species secondary antibodies. b – cells stained with AFP antibodies

As follows from Fig. 12, about 10% of cells express α-fetoprotein, which confirms the result obtained by fluorescence microscopy. Thus, the presence of AFP expression in cells derived from mature rat hepatocytes after culturing in the presence of the small-molecule cocktail indicates their dedifferentiation/reprogramming into an immature progenitor state.

## CONCLUSION

Induction of mature hepatocytes by a small-molecule cocktail may serve as one approach to reprogramming terminally differentiated cells into a progenitor state. It has been demonstrated that the presence of three small molecules Y-27632, A-83-01 and CHIR99021, in the medium allows to culture rat hepatocytes for a long time (over 2 months). At the same time, significant changes in their morphology occur and liver progenitor cell markers, such as AFP and HNF4α, start to be expressed. This result opens up new opportunities for studying the mechanism of dedifferentiation/reprogramming of mature hepatocytes. In our opinion, it is a starting point for further studies aimed at studying the properties of human hepatic progenitor cells, developing their production methods with subsequent application in the treatment of end-stage liver diseases, such as cirrhosis of various etiology and malignant tumors.

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*The authors declare no conflict of interest.*

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# TOLERANCE AND MINIMIZATION OF IMMUNOSUPPRESSIVE THERAPY AFTER LIVER TRANSPLANTATION

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In this review of current publications, we look at the molecular mechanisms of tolerance of the liver and its allografts in terms of minimization and possibilities of withdrawing immunosuppressive therapy, mainly in the long-term period after liver transplantation. Information about clinical trials with regulatory T cells (Tregs) for the purpose of tolerance induction is presented. Data from a new consensus study on individualization of immunosuppressive therapy regimens are presented. Options for possible withdrawal of immunosuppression both in the early and in the long term after liver transplantation (LT) are considered. We suggest a way to study the lymphoproliferative potential of a liver transplant recipient to be investigated, since not only rejection determines life expectancy, but also the degree of immunosuppression effect on bone marrow depending on patient age.

*Keywords: apoptosis, immune tolerance, immunosuppression, mesenchymal stem cells, regulatory T cells, liver transplant, NK cells.*

The effects of immunosuppressive therapy in the long-term period after liver transplantation (LTx) are associated with a set of adverse effects limiting recipient survival. And if the survival rate in the first year after LTx seems satisfactory, the life expectancy of patients in the late period remains significantly lower than in the general population (Fig. 1). Some of the leading causes of negative outcomes are malignancy, infections, cardiovascular and nephrological problems [1]. Therefore, the search for rational ways to overcome undesirable effects remains relevant. Based on analysis of modern literature, the main approaches to modifying immunosuppression after LTx, include intraoperative and delayed induction of tolerance, individualization and rationalization of regimens to reduce frequency of side effects of drugs, and, finally, minimization of immunosuppression up to complete withdrawal.

Most researchers consider the liver to be an immune privileged organ that can be tolerant to various influences. Immune tolerance in the liver is mediated by specialized antigen-presenting cells (APCs), including dendritic cells (DCs), Kupffer cells, sinusoidal endothelial cells and hepatic stellate cells. By providing autoantigens to their own T cells, these cells promote their apoptosis, anergy or differentiation of T lymphocytes into regulatory T cells. The tolerogenic role of the liver immune system was first demonstrated back in 1969 by Calne R.Y. et al. [2], who found that porcine liver allografts that had mismatches in the major histocompatibility complex (MHC) survived without immunosuppression.

Explanation for the relative tolerogenicity of the liver is multifactorial: the large size of the organ results in a much larger endothelial surface area on which antibodies are distributed, thus their effects are weakened; the liver has a natural regenerative capacity, so liver tissue damage is potentially reversible in case of rejection. Expression of MHC class II antigens on liver cells is more variable than that observed in the kidneys and heart. Tolerance in the liver has an evolutionary basis because 75% of blood flow in the liver comes from the portal vein, which collects blood from the gastrointestinal tract enriched with microbial antigens. The immune system of the liver has evolved to tightly regulate immune responses to harmless intestinal microorganisms and to avoid unwanted inflammatory-type reactions [3].

Achieving immune tolerance after LTx would potentially eliminate the need for long-term immunosuppression. Currently, the mechanisms of tolerance of transplanted liver are still being actively studied and clarified. Mesenchymal stem cells (MSCs), regulatory T cells (Tregs), and donor NK cells are mainly involved in the formation of tolerance (Fig. 2). Tregs are recognized as central regulators of immune response, they express FOXP3, a transcription factor regulating the transcription of genes responsible for T cell differentiation and the expression of cytokines and other factors involved in suppression of immune response. An important marker of regulatory T cells is the expression of CD25, receptor of the IL-2 cytokine, on their surface.

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Although presentation of donor DC antigens is the main rejection factor, tolerogenic phenotypes of DCs have also been found in the liver [6, 7]. DCs are also capable of inhibiting T cell proliferation by providing a small number of costimulatory molecules, which along with high expression of PDL1 (a PD1 ligand, a membrane protein preventing T cell activation) cause anergy or deletion of the alloreactive T cell clone. DCs secrete IL-10 and TGF-β, which induce Treg differentiation. CTLA4 receptors on the Treg surface bind to the B7 protein on DCs with higher affinity than CD28, disrupting interactions between DCs and T cells. Tregs also promote a tolerogenic microenvironment by secreting TGF-β, IL-10, and IL-35, binding IL-2 to CD25 with higher affinity than effector T cells, and through direct cytotoxicity mediated by two known pathways via granzyme, perforin, and Fas-FasL interactions. Unlike recipient NK cells, which can mediate rejection, donor-derived NK cells transplanted as passenger cells can directly lyse alloreactive recipient immune cells through NKG2D-MIC-A and TRAIL-TRAILR interactions. There is evidence that recipient NK cells may also have tolerogenic potential. MSCs inhibit T cell proliferation and differentiation by secreting indoleamine-2,3-dioxygenase (IDO), an enzyme capable of metabolizing the amino acid tryptophan to kynurenine. T cells require this amino acid for activation, and tryptophan deficiency induces apoptosis or inhibits proliferation and differentiation during PD-L1-mediated cell-to-cell contact [4, 5]. Kupffer cells can be polarized by the M2 phenotype, producing IL-10 and TGF-β and thus also promoting tolerance. They can also release nitric oxide (NO) with IFN-γ to inhibit T cell proliferation. Liver sinusoidal endothelial cells (LSECs) act as 'amateur' APCs with typically low levels of MHC class II antigen expression. LSECs, together with liver stellate cells, induce T cell apoptosis through PDL1-PD1 interactions [3–8].

The liver retains a tolerogenic potential even when alloreactive T cells have gained access to the parenchyma and tissue damage has begun. According to some data, alloreactive T cells cause hepatic cell death either through apoptosis or lysosomal degradation of hepatocytes. This process also partly depends on Treg, which inhibits rejection reaction [9, 10].

The study of these mechanisms seems to be important for justification of the possibilities of tolerance induction after LTx.

Treg-based cell therapy is a promising alternative approach to promote allograft engraftment, potentially minimizing the use of traditional immunosuppression [11, 12]. In 2020, the Lancet journal published data from the ONE study demonstrating the safety of Treg in kidney donor recipients [13]. Other centers are nearing completion of clinical trials, which are in different phases, focusing on the study of alloantigen-specific Tregs, as they have a better suppressive function against alloreactive effector T cells than polyclonal Tregs [14]. Treg expansion in vivo represents another interesting therapeutic strategy. Tregs exhibit a higher affinity for IL-2, so the use of low concentrations of this molecule

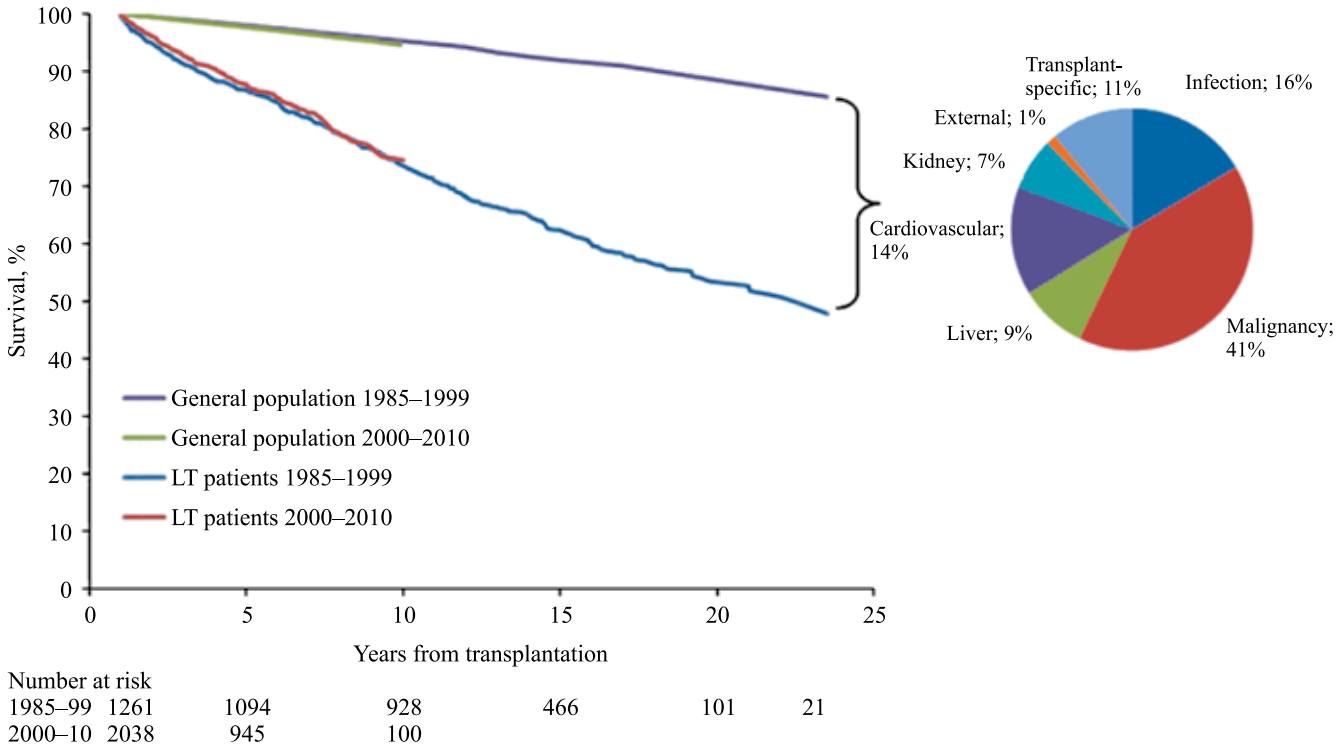



Fig. 1. Differences in long-term survival among liver transplant recipients and the general population [1]

can increase the Treg pool in vivo several times without significantly increasing the count of effector T cells [15].

Several clinical trials using a number of Treg drugs in LTx are currently underway worldwide (Table). Polyclonal regulatory T cell therapy with ex vivo cell augmentation is used in the ThRIL study at King's College Hospital (UK), the DeLTA and ARTEMIS studies at the University of California (San Francisco, USA) using donor alloantigen, reactive regulatory T cells (darTregs), and at Nanjing Medical University (China) using donor antigen-specific Tregs in patients in the early and late periods after liver transplantation.

In the case of related LTx, the cell product can be produced before the transplant surgery and infusion can be performed in the early post-transplant period. However, LTx from a deceased donor is prevalent worldwide, making it difficult to access and obtain donor tissue. In addition, production of Tregs is very resource-intensive. There are very few licensed laboratories capable of producing Tregs under GMP conditions [16]. There are still many technical problems in terms of production, scaling, and storage of the cell product. However, this direction seems to be the most inspiring for tolerance induction both in the early and late periods after LTx.

Since the donor liver is a foreign organ continually providing APCs, while tolerance induction has not become widely available, immunosuppressive therapy remains necessary for prevention and treatment of graft rejection.

In real clinical practice, the range of drugs for immunosuppression is not so large. Tacrolimus remains the mainstay. Regimens consisting of a combination of mycophenolates and calcineurin inhibitors (CNIs), or, more recently, everolimus, are commonly used as a maintenance immunosuppression regimen after LTx.

The mechanisms of suppression of T cell activation by these drugs are well studied. Nevertheless, the effects of these immunosuppressants on hepatocyte apoptosis have not been investigated. Hepatocyte apoptosis stimulates fibrogenesis in various liver injuries [17]. From this point of view, a study by Lim E.J. et al. [18] is of interest. The authors evaluated hepatocyte death based on apoptosis markers in biopsy by immunohistochemical method against immunosuppression and was compared with the state of liver cells in people without liver disease, operated on for other reasons. The level of hepatocyte apoptosis markers was significantly higher in liver transplant recipients compared to the control. Apoptotic hepatocytes are engulfed and utilized by both

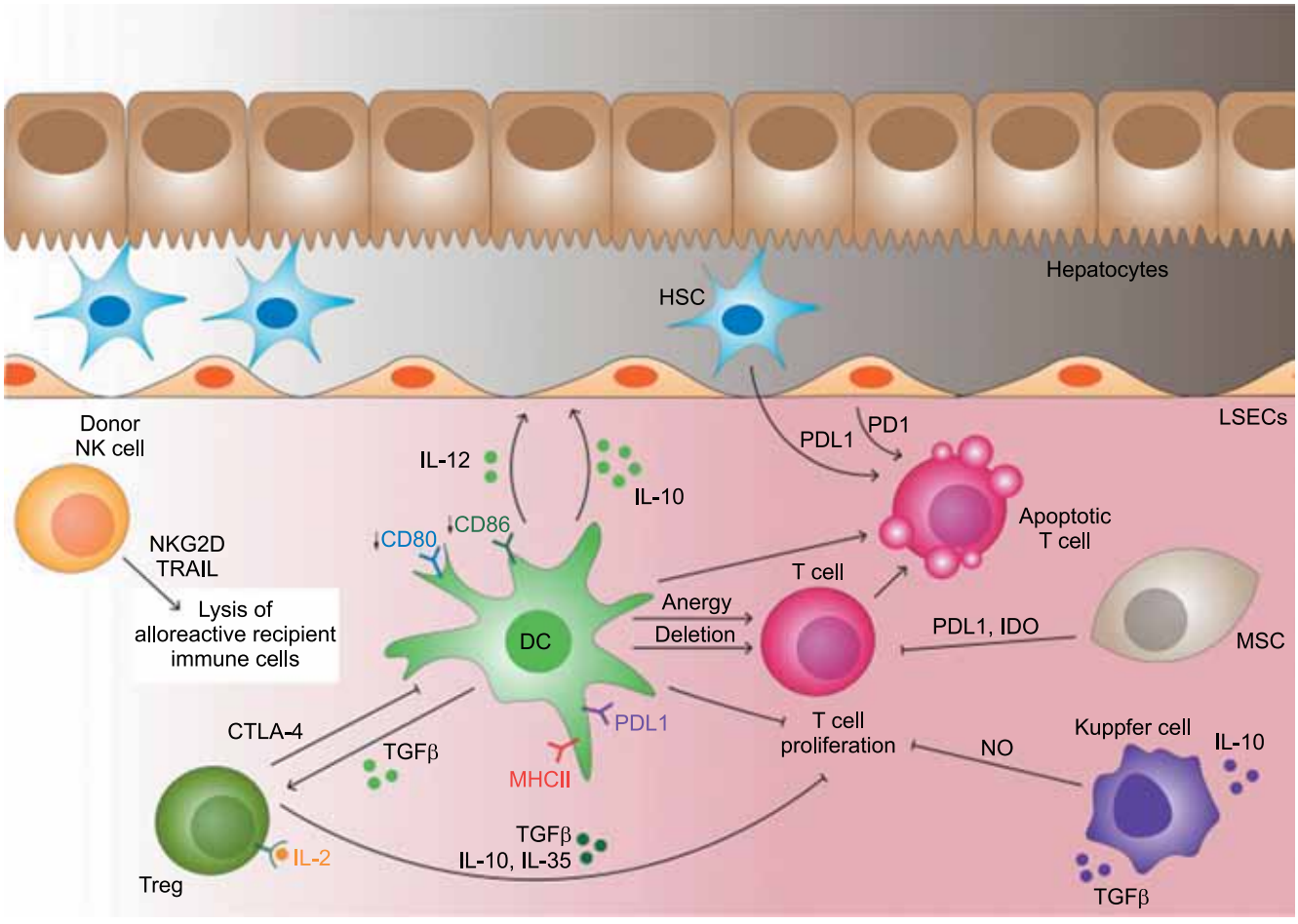



Fig. 2. Cellular mechanisms of liver allograft tolerance (description in text) [3]

Kupffer cells and stellate liver cells. Kupffer cells that have engulfed apoptotic hepatocytes secrete TGF-β, a major fibrogenesis factor [19]. On murine hepatocyte culture, it was shown that combinations of cyclosporine (CyA) and mycophenolate (MMF), tacrolimus (Tac) and MMF reduced cell viability and increased hepatocyte apoptosis, while isolated Tac and CyA did not cause such effects. Sirolimus as a monocomponent had no effect on hepatocyte viability and apoptosis, and in combination with MMF, these effects were expressed minimally [20]. Thus, it is possible to choose a rational immunosuppression regimen taking into account the effect of drugs on hepatocyte apoptosis, and, consequently, on fibrogenesis. The CNI/mTOR and mTOR/MMF regimens in the long-term period after LTx appear to be reasonable [18].

Although much experience has been accumulated in the use of immunosuppressive agents after LTx, the regimens are nevertheless heterogeneous and not ideal. Long-term recipient survival is associated not only and not so much with rejection reactions, but also with accumulation of adverse events. For their correction, two approaches can be implemented: preliminary individual selection before LTx and standard regimens, which are modified as complications develop [21]. In 2020, the Italian consensus on the use of everolimus in clinical practice after LTx was published, where algorithms for modification of immunosuppressive therapy were proposed. The choice of immunosuppressive therapy regimen should take into account a set of clinical variables, including the primary disease, patient transplant status, type of surgery, characteristics of the early postoperative period, events and expected complications associated with the long-term use of CNIs, and the risk of de novo malignancy. Strategies to prevent or limit CNI-related adverse events are worth using as early as possible after LTx. Currently, the most effective nephroprotective strategies are recognized as reducing CNIs exposure, which is possible with early administration of everolimus or mycophenolate if everolimus is not indicated. The presented algorithms allow minimizing the risks of adverse events and toxicity of immunosuppressants (Fig. 3, 4) [22]. These recommendations are also consistent with the above experimental data on rational immunosuppression regimens based on the effects of known drugs on liver fibrogenesis.

Unfortunately, results from experimental studies on tolerance mechanisms are not conclusive enough for us to establish certain factors for effective minimization and withdrawal of maintenance immunosuppressive therapy. In the absence of reliable tolerance biomarkers [23], with the complexity and inconsistency of molecular mechanisms, the only reliable way to confirm tolerance is if there is no graft rejection after deliberate cessation of immunosuppression.

From a clinical point of view, tolerance is defined as stable graft function in an immunosuppressed recipient with no clinically significant immune response.

In 2015, Chi-Xian Zhang et al. presented a review of data on minimization and possible withdrawal of immunosuppressive therapy after LTx [24], based on evidence of congenital liver tolerogenicity and careful evaluation of clinical parameters. Successful weaning from immunosuppression was possible in nearly 20% of selected liver transplant recipients.

In a later study [25], 95 recipients 1–2 years after LTx were randomly selected for withdrawal (n = 77) and continuation (n = 18) of maintenance therapy. Inclusion criteria were: monocomponent immunosuppression; ade-

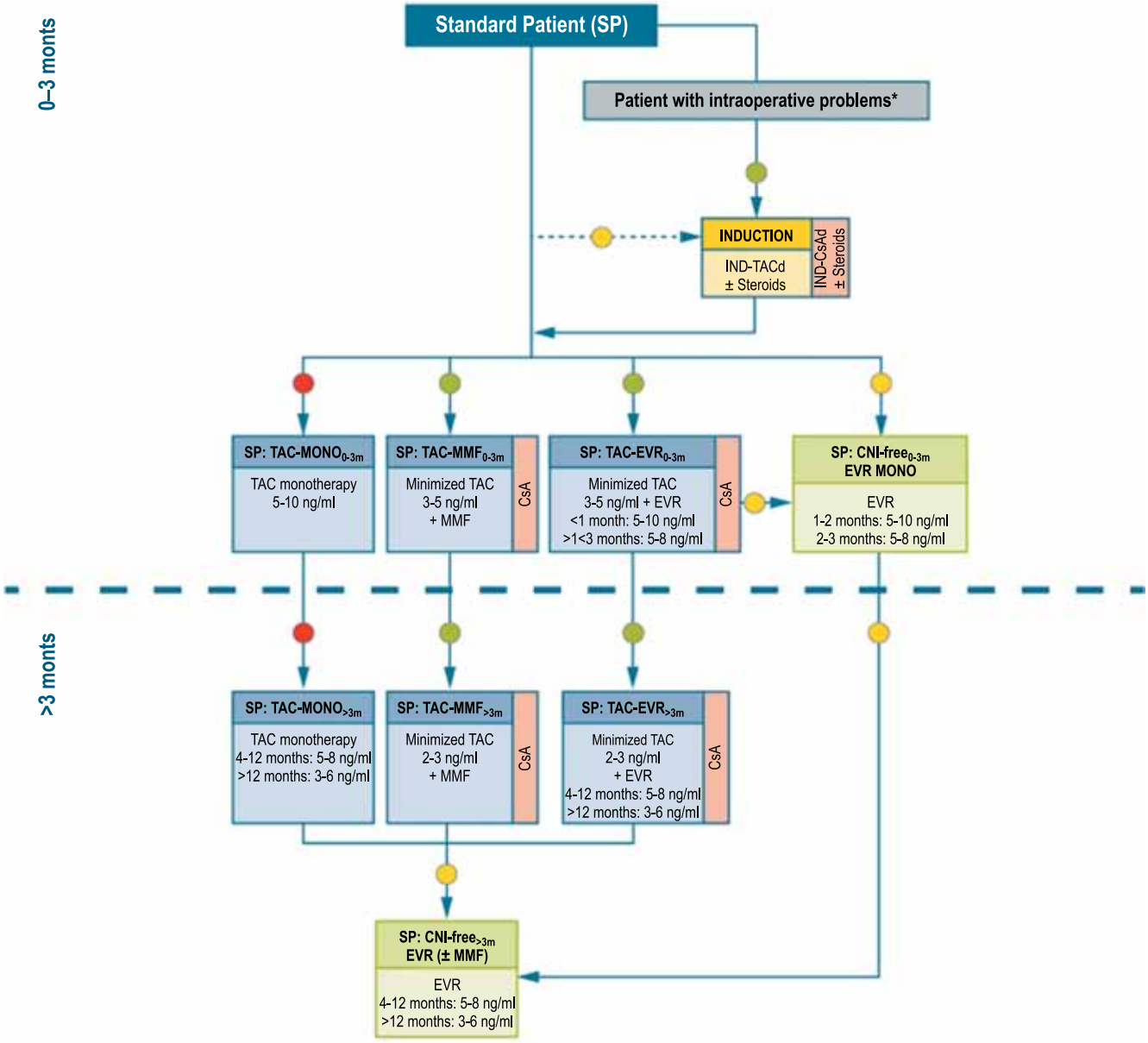
Table

**Clinical trials using Treg *ex vivo* in liver transplantation [16]**

| Test | Liver transplant setting | Country | Study design | Age (years) | Treg dose | Repeated administration | Immunosuppression withdrawal |
|---|---|---|---|---|---|---|---|
| NCT 01624077 | Chronic rejection | China | Open-labeled single group assignment | 10–60 | $1 \times 10^6$ cells/kg donor | Yes | Yes |
| ARTEMIS (NCT 02474199) | Induction of tolerance in living-related LT | USA | Open-labeled single group assignment | 18–70 | $300–500 \times 10^6$ cells donor | No | Yes |
| ThRIL (NCT 02166177) | Induction of tolerance in living-related LT | UK | Open-labeled single group assignment | 18–70 | Autologic polyclonal<br>Cohort 1: $0.5–1.0 \times 1 \times 10^6$ cells/kg<br>Cohort 2: $3.0–4.5 \times 1 \times 10^6$ cells/kg<br>Cohort 3: $5.0–6.5 \times 1 \times 10^6$ cells/kg | No | No |
| dELTA (NCT 02188719) | Induction of tolerance in deceased-donor LT | USA | Nonrandomized open-labeled parallel assignment | 21–70 | Donor alloreactive<br>Cohort 1: 0<br>Cohort 2: $25–60 \times 10^6$ cells<br>Cohort 3: $100–240 \times 10^6$ cells<br>Cohort 4: $400–960 \times 10^6$ cells | No | Yes |

quate liver and kidney function; ≤2 Ishak fibrosis stage; no rejection on biopsy. Immunosuppression was reversed according to an 8-step reduction algorithm at 8-week intervals. Fifty-two of 77 recipients (67.5%) reduced the dose to ≤50% of baseline, and 10 of 77 (13.0%) completely discontinued immunosuppression for ≥1 year. Immunosuppression was intensified in cases of rejection or veering of laboratory tests. Bolus injection of methylprednisolone was required in 5 of 32 cases of rejection. A composite end point (death or graft loss; grade 4 secondary malignancy or opportunistic infection; Ishak stage ≥3; or >25% decrease in glomerular filtration rate within 24 months of randomization) was achieved in 12 of 66 (18%) and 4 of 13 (31%) in the withdrawal and minimization groups, respectively. Thus, early minimization of immunosuppression was feasible in selected liver recipients, while complete withdrawal was successful in only a very small proportion.

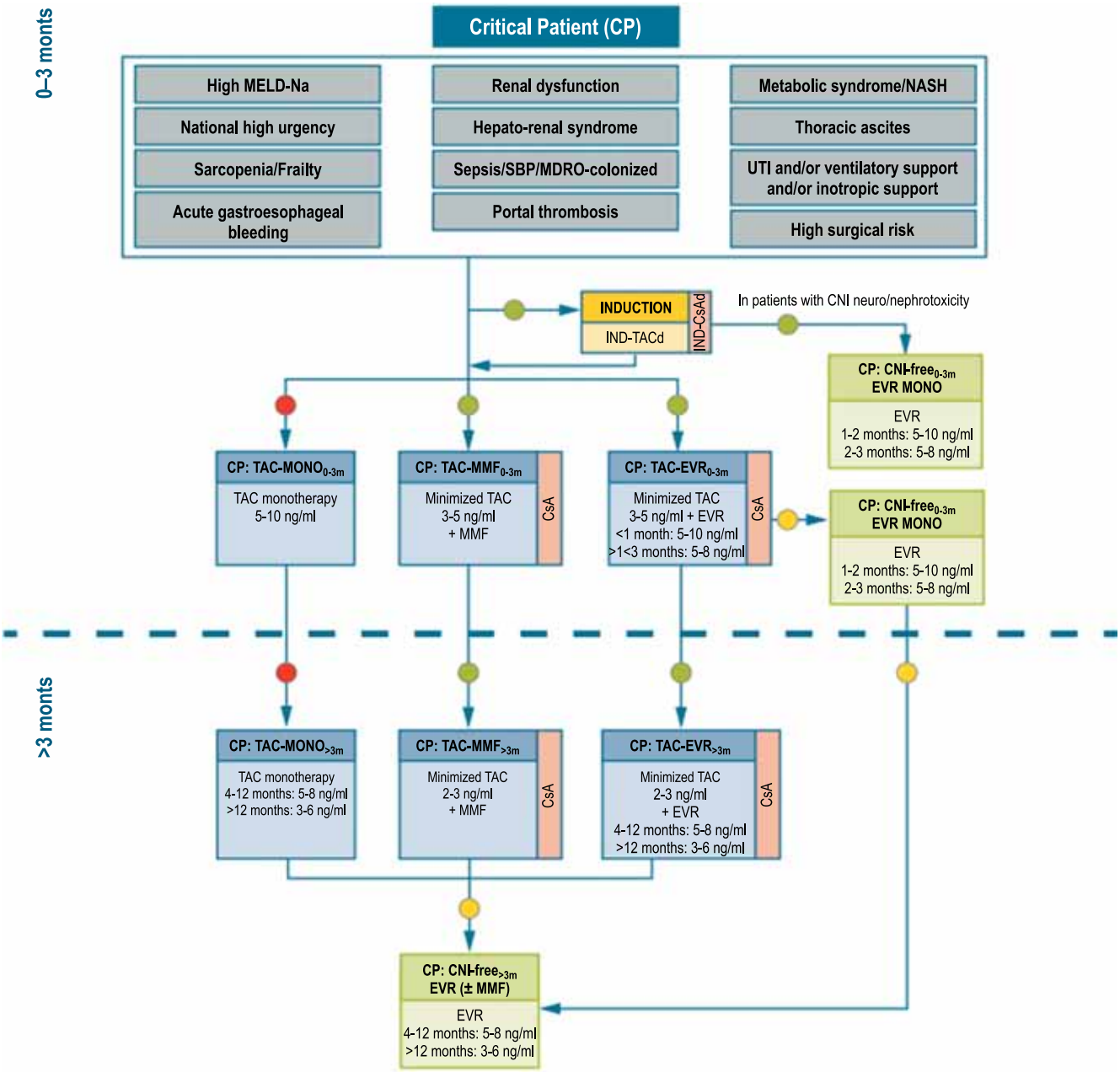
Data from another prospective randomized trial [26], suggest that over 40% of carefully selected liver recipients can have their immunosuppression reversed several years after LTx. 102 stable liver recipients 3 years or more after LTx were selected for withdrawal. Medications were gradually discontinued over several months. The primary endpoint was development of tolerance, defined as successful discontinuation of immunosup-




*Se grave evento intraoperatorio considerare switch a "Paziente critico – Alto rischio chirurgico".
CNI = Calcineurin inhibitors; CsA = Cyclosporin A; d = delayed; EVR = Everolimus; IND = Induction; MMF = Mycophenolate mofetil; TAC = Tacrolimus;

Fig. 3. Immunosuppression algorithm for standard patients. Key: green circle = recommended; yellow circle = caution advised; red circle = not recommended [22]

pressive drugs when graft function remained stable for at least 12 months and there were no histological signs of rejection. Of the 98 recipients, 57 retained and 41 successfully discontinued all immunosuppressive drugs. In tolerant patients, medications were discontinued for an average of 8.0 ± 4.6 months. Twenty-three tolerant patients had one episode of temporary allograft dysfunction unrelated to rejection. All episodes resolved spontaneously without increasing doses of immunosuppressants. Two tolerant patients died with normal graft function 16 and 26 months after complete cessation of medication because of postoperative complications of colorectal cancer resection and metastatic ovarian cancer, respectively. None of the tolerant recipients developed rejection during follow-up. In 57 intolerant patients, acute rejection was detected at 6.4 ± 4.4 months after the start of minimization therapy. All intolerant patients received immunosuppressants at the time of rejection. Liver biopsy was performed in 89% of cases and rejection was classified in most cases as borderline or mild according to Banff criteria. No cases of chronic rejection were observed. In 21 patients, rejection was cured




CNI = Calcineurin inhibitors; CsA = Cyclosporin A; d = delayed; EVR = Everolimus; IND = Induction; MDRO = Multidrug-resistant organism; MMF = mycophenolate motetil; MELD-Na = Model for End-stage Liver Disease – Sodium; NASH = Non alcoholic steatohepatitis; SBP = Spontaneous bacterial peritonitis; TAC = Tacrolimus; UTI = Urinary tract infection;

Fig. 4. Immunosuppression algorithm for critically ill patients. Key: green circle = recommended; yellow circle = caution advised; red circle = not recommended [22]

by returning to the baseline immunosuppression. In the remaining patients, restoration of baseline immunosuppression was combined with low or moderate doses of oral steroids, and only once was their bolus injection required. In 55 recipients (96.5%), all parameters were completely normalized 5.6 ± 5 months after rejection. There were no graft losses.

At the end of the study, successful reversal of immunosuppression was significantly more likely to occur in men ($p = 0.009$), in those older at the time of LTx ($p = 0.05$), and in those who lived longer after LTx ($p < 0.0001$). Time after LTx was identified as the strongest predictor of clinical tolerance. Among patients living over 10 years after LTx, 79% were successful in withdrawing immunosuppression, compared to patients living for more than 5.7 years, where 38% were successful in withdrawing all medications. It was significantly easier to wean off immunosuppression for those who lacked CNIs in the study enrollment regimen ($p = 0.005$).

Nevertheless, there are very few randomized trials of stepwise minimization and complete withdrawal of immunosuppressive therapy in the long-term period, and withdrawal still seems inappropriate at 1–2 years after LTx.

There is another not quite traditional view on the problem of long-term survival or maintenance of the functional state of a liver transplant. In some recipients, loss of a liver transplant may be due to not graft rejection but depletion of the proliferative potential of the lymphocytic growth of the bone marrow (the product of the number of stem/progenitor blood lymphocytes and mitotic activity), whose value limits the life span during natural aging [27]. Individual monitoring of lymphoproliferative potential could contribute to timely dose adjustment and modification of immunosuppressive therapy regimens. The study of the least studied functional relationship of own regulatory T lymphocytes with hematopoietic stem cell pool, seems relevant to objectivize the individual monitoring of immunosuppression in the remote period after LTx. This aspect can be considered as one of the approaches to minimize, and possibly cancel immunosuppression.

In conclusion, based on further studies on molecular mechanisms of tolerance, ways of inducing it in clinical practice may be determined, and then immunosuppressive therapy may lose its relevance. Prospective and promising is the introduction of allogeneic Tregs in order to induce immune tolerance. In the long-term post-LTx period, preservation of immunosuppression regimens, taking into account the recipient's individual profile, remains relevant. Dose minimization and immunosuppression cancellation in carefully selected recipients with stable graft function seem possible but require careful monitoring of liver graft status. Additional prospective studies are now needed to confirm the safety and efficacy of complete withdrawal of immunosuppression compared with maintenance therapy, as well as to verify tolerance biomarkers.

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*The authors declare no conflict of interest.*

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# IMMUNOSENESCENCE AS A REASON FOR INDIVIDUALIZED IMMUNOSUPPRESSIVE THERAPY IN KIDNEY TRANSPLANTATION

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Transplantation in elderly patients is obviously more challenging due to existing underlying diseases, changes in pharmacokinetics of immunosuppressive drugs, polypragmasy, and transformation of immunoreactivity (immunosenescence). Our review presents data on modification of adaptive and innate immunity during aging. It also considers the possibility of both reduced and adapted immunosuppressive therapy in elderly renal transplant recipients in achieving an optimal balance between efficacy and complications.

*Keywords: kidney transplantation, aging, immunosenescence, immunosuppression therapy.*

## INTRODUCTION

Kidney transplantation (KTx) remains the optimal treatment method for renal replacement therapy (RRT), providing the best patient survival [1].

Immediate and long-term transplant outcomes depend on several factors related to age, underlying disease, duration of dialysis, infections, duration of graft function and the cause of loss of first graft (for repeat transplantation), presence of preexisting antibodies, type and quality of donor organ, and co-existing diseases [2–5]. Some of these factors have a direct impact on the recipient's immune system prior to transplantation.

For example, it is known that young people under the age of 30 have a more reactive immunity; then, the T cell-mediated immunity suffers most of all with age. This is primarily associated with age-related thymic involution, starting from 15–20 years, and accompanied by a decrease in its mass, weakening of its function and synthesis of regulatory factors. This leads to a natural progressive suppression of this thymus-dependent immunity. The ratio of regulatory lymphocyte subpopulations changes against the background of general lymphopenia. Humoral immunity also undergoes negative changes: in the elderly there is a drop in normal antibodies, including isohemagglutinins, which should be taken into account when determining the blood group and organ transplantation in the elderly [6, 7].

Over the past decades, the number of elderly people has increased significantly and is expected to grow even more from 8% of the total world population in 2015 to 16% in 2050 [8]. Moreover, the fastest growing age group of recipients is patients over 65 years of age [9]. For example, in the United States, the number of operations on patients over 65 years of age increased from 17% to 33.3% of the total number of kidney transplants between 2012 and 2018 [10]. In Australia, 14% of kidney transplants performed in 2015 were in patients aged 65 years or older [11]. The 2012 European Kidney Transplant Registry report indicated that the prevalence of transplants was 22% in the age group over 65 years and 20% in the age group over 75 [12].

There is no age distribution of recipients in the Registry of the Russian Transplant Society; only data on kidney transplantation in minors are reported separately [13]. According to data from Sklifosovsky Research Institute of Emergency Care, the proportion of patients over 60 years of age on the kidney transplant waitlist is 9–13% of the total number of potential recipients, which is somewhat lower than in Europe and the United States [14].

This increase in the frequency of kidney transplantation in the elderly can be explained by the aging population, improved transplant outcomes, and introduction of expanded kidney donor criteria [15, 16]. However, transplantation in elderly recipients is obviously more challenging due to existing comorbidities, changes in the pharmacokinetics of immunosuppressive drugs, polypragmasy and transformation of immunoreactivity (immunosenescence).

Despite this, studies show that kidney transplantation in elderly patients is associated with reduced mortality compared to dialysis [9, 17]. Elderly recipients have a lower risk of acute rejection due to decreased immune reactivity, but they are the most likely age group to die with a functioning graft [18]. While the short-term outcomes in elderly transplant recipients are similar to those in younger recipients, the long-term graft survival and survival of elderly recipients is inferior to that of younger recipients. The most common causes of death in elderly

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recipients are infection, malignancy, and cardiovascular disease, each of which can be partially attributed to immunosuppressive agents [19–21].

Clearly, multifaceted modifications of adaptive and innate immunity with aging can justify both reduced and adapted immunosuppressive therapy in elderly kidney transplant recipients to achieve an optimal balance between efficacy and toxicity. By minimizing side effects, an individualized strategy can provide the optimal level of immunosuppression for elderly transplant recipients to minimize or prevent infections, malignancies and chronic kidney disease, as well as cardiovascular complications related to diabetes, hypertension and hyperlipidemia [22, 23].

The study of indicators such as cytokine production, lymphocyte proliferation or activation antigen expression on T cells as biomarkers can be used to monitor and evaluate immune system activity, since for some of them there is a statistically significant association with the frequency of acute rejection or immunosuppressant toxicity, and this is an obvious unmet clinical need [24].

## IMMUNOSENESCENCE AND ORGAN TRANSPLANTATION

Immunosenescence (immune aging or deterioration of the immune system, derived from the Latin term senescere, meaning "to grow old") is a gradual suppression of immune reactivity in the elderly due to a decrease in the number of naive T lymphocytes, accumulation of memory T cells and changes in B cells, causing a reduced antibody response [18]. Immune aging is characterized by impaired function of both adaptive and innate immunity and can affect all immunological components and cause a shift in both regulation and function of the entire immune system [25, 26]. Obviously, the mechanisms underlying transplant rejection differ in young and elderly transplant recipients, and clinical outcomes in elderly recipients should be accompanied by individualization of immunosuppression [27].

Immunosenescence is a complex and continuous remodeling of certain cell subpopulations, rather than uniform changes [28]. The effect of aging on T cell-mediated immunity is most prominent, while the changes in B cells are considered by scientists to be less pronounced [29].

T cells play a key role in both the development of tolerance and transplant rejection. In an aging population, the dynamics of T cell-mediated immunity includes internal and systemic changes in T cells with a change in the ratio of naive T cells and memory T cells and thymic involution, which certainly entails clinical significance [30].

Thymic involution begins as early as 1 year of age, with the naive T cell count decreasing by 50% over any 15 years of life and leading to significant decrease in the production of naive T lymphocytes in the thymus in the population over 60 years of age [31]. Remarkably, memory T cells are long-lived, and memory T lymphocyte responses show a half-life of 8–15 years. The lifespan of memory T cell subsets is mainly due to self-renewal rather than the lifespan of individual T cells [32]. Naive T cells can divide and generate daughter T cells with a naive phenotype. Such increased homeostatic proliferation can compensate, at least partially, for the decreased thymic activity with aging. However, this new T cell population, consisting mainly of memory T cells, has a reduced ability to recognize and eliminate new pathogens [27]. Thus, the response of elderly T lymphocytes is mainly based on less effective memory T cell responses, which lack the ability of young T cells to migrate and naïve de novo production [28].

A major review by Russian authors substantiates the need to introduce into clinical practice a comprehensive monitoring of immune blood cells and cytokines in patients with transplanted organs in order to be able to select individual immunosuppressive therapy tactics, assess its effectiveness and predict the outcomes. It is emphasized that special attention should be paid to the characteristics of CD4+ T lymphocytes and determination of the ratio of their individual populations in the peripheral blood, since they are the main players in the immune system response to the graft [33].

The ratio of CD4+ (T-helpers) to CD8+ (T-suppressors) cells in peripheral blood is called the immunoregulatory index and in most elderly people undergoes an inversion along with increased activated T cells and T lymphocytes expressing NK-cell markers [34]. Schaenman J.M. et al. showed that older kidney transplant recipients demonstrated decreased frequency of naïve CD4+ and CD8+ T cells, and increased frequency of terminally differentiated, immune senescent, and NK T cells. The authors also observed a trend towards increased frequency of T cell immune senescence in patients experiencing infection in the first year after transplantation, which reached statistical significance. They noted the potential for risk stratification and customization of immune suppression to prevent infection and rejection after transplantation [35].

Aging of T cells is also accompanied by loss of costimulatory molecule CD28 on CD4+ and CD8+ T cells [36]. CD28 is a key costimulatory surface receptor that plays a crucial role in antigen-dependent activation, proliferation and survival of T cells and prolongs graft survival. Virtually all human T cells express CD28 during birth. In contrast, by age 80, 10–15% of CD4+ T cells in peripheral blood and 50–60% of CD8+ T cells lack CD28 expression. As an alternative and compensatory pathway for classical T cell receptor/CD28 activation, aging T cells increase de novo expression of cytotoxic NK cell receptors [37]. These changes indicate that in-

creased NK-cell receptor expression will influence alloimmune responses in the elderly, potentially reflecting the relevance of an enhanced innate immune response. Although the overall significance of NK cell receptors in kidney transplantation remains poorly understood, recent work has demonstrated phenotypic changes in the NK cell repertoire induced by immunosuppressive treatment [38]. Such age-related changes in T cells provide grounds for exploring the potential of new immunosuppressive approaches [28].

In our 2020 study, interesting age-associated cellular immunity features were also noted – in healthy volunteers, the functional activity of peripheral blood mononuclear cells was statistically insignificant but gradually decreased with increasing age. Meanwhile, in patients with chronic kidney disease (CKD) undergoing dialysis, this dependence was of the same direction, but expressed significantly stronger and was statistically significant. It can be concluded that older CKD patients after kidney transplantation are subject to significant changes in the morphofunctional state of peripheral blood mononuclear cells and subpopulation composition of lymphocytes, while the severity of changes in the functional state of these cells is strongly related to age, which is not observed in healthy volunteers. This should be considered when choosing immunosuppressive therapy in older kidney transplant recipients [39].

Additional in vitro studies have shown that loss of CD28 is accompanied by increased expression of its antagonist gene, the CTLA-4 receptor, which potentially enhances the already inhibitory effect [40].

Some authors found a very significant correlation between age and the percentage of CTLA-4+ in CD4+ cells, as well as between age and mean CTLA-4 fluorescence intensity. CTLA-4 levels also correlated with immune system activation as determined by CD3+ HLA-DR+ cell levels. Consequently, age-related immune system aging is in part caused by chronic immune system activation with a corresponding decrease in CD28 costimulatory molecules and increase in CTLA-4 inhibitory molecules [41].

It is important to note that immune aging should be viewed as a multitude of complex modifications of immunological functions and regulations with broad implications for alloimmune responses. There is little evidence for the effect of age on B cell function. Reports demonstrate that older age affects humoral immune response through decreased naïve B cell count combined with decreased T cell count, which are integral to B cell activation [42]. In contrast to T cells, B cell homeostasis is maintained in the periphery by decreased turnover of mature B lymphocytes. Despite this, a decrease in B cell count causes a change in antibody specificity and a decrease in plasma cells in the bone marrow [26]. However, a number of authors, on the contrary, believe that aging apparently leads to decreased diversity of naive B cells rather than to significant changes in peripheral B cell count, which suggests that the effect of aging on the B cell component may be primarily qualitative [43].

The narrowing of the B cell repertoire with age correlates with susceptibility to infection. A correlation has been reported between Epstein–Barr virus seropositivity and B cell clonal expansion in the very elderly (80 years and older) without association with persistent cytomegalovirus (CMV) infection [44]. It remains clinically unclear whether humoral response is age-dependent and whether humoral rejection requires a different therapeutic approach in the elderly. Most humoral immune responses require the assistance of related T lymphocytes, and, as noted above, immunosenescence is associated with changes in the CD4+ compartment. However, how immune age-associated changes in CD4+ cells initiate changes in B cells in older adults has not been directly investigated.

Several reports also associate aging with a decrease in the Th1/Th2 cytokine ratio, whereas the total number of type 1 and 2 cytokine-producing T cells appears to increase with age [45]. Impaired IL-2 production in older T cells may also be associated with age-related loss of CD-28, since costimulatory signaling is critical for T cell activation and their subsequent IL-2 production [46]. One study showed that both IL-2 cytokine capacity and CD4+ T cell sensitivity decline with age, at least in mouse models [45]. Maintenance immunosuppression relies heavily on calcineurin inhibitors specifically targeting IL-2 production in T cells. Taken together, loss of CD28 and decreased IL-2 production may represent critical factors in impaired alloimmune response in the elderly, affecting immunosuppression and tolerance protocols.

Regulatory T cells (Tregs) play a crucial role in maintaining immune tolerance and preventing exacerbation of immune responses to foreign antigens. Numerous studies have proved that $FoxP3^+$ Tregs accumulate with age [47, 48]. As a result, immune response activity or formation of immunological tolerance depends on the balance of T-helpers (Th) initiating active immune response and triggering rejection, and on Tregs having the opposite effect.

## EFFECT OF IMMUNE AGING ON KIDNEY TRANSPLANT OUTCOMES

Elderly renal transplant recipients have a higher overall mortality rate, and nearly 50% of graft losses are associated with death in a working graft, compared with 15% in younger recipients. Immunosuppression complications such as cardiovascular disease, infection, or malignancy, especially among older patients, represent important components of morbidity and mortality [20]. Nevertheless, predicted life expectancy has increased to

10 years in kidney recipients older than 65 years compared to the control group of corresponding age remaining on dialysis [49].

Weaver-Pinzon O. et al. conducted a retrospective study of 52,995 adult kidney transplant recipients and came to logical conclusions: mortality among recipients aged over 60 years was significantly higher, mortality among younger recipients was due to acute rejection, coronary vasculopathy and graft failure, while mortality among older recipients was due to infection, malignant tumors and kidney failure [50]. Another large study also confirmed a decrease in both innate and adaptive immune reactivity with age, which contributes to a lower incidence of acute rejection and increased infectious mortality in older recipients [51].

Jackson-Spence F. et al. conducted a retrospective single-centre analysis of 1140 consecutive patients receiving kidney-alone allografts in different age groups. They noted that elderly kidney transplant recipients had increased risk of complications associated with immunosuppression, but rejection rates and death-censored graft losses were similar. Therefore, the authors consider clinical trials of age-adapted immunosuppression to be necessary [52].

In a study by Tullius S.G. et al., elderly recipients (>50 years) had a lower incidence of acute rejection compared to younger recipients, despite the more obvious immunogenicity of elderly donor kidneys [53].

Other authors also note that in kidney transplants, less than 25% of failures in older recipients are due to rejection, compared to 50% in recipients younger than 45 years of age. However, acute rejection in the elderly has a more pronounced deleterious effect on patient and graft survival. Age-related internal organ changes and immunogenicity aspects may be relevant in this context, since older recipients are more likely to receive organs from older donors [49].

Thus, in transplant recipients, aging of the immune system probably reduces the risk of acute rejection but increases the risk of side effects associated with immunosuppression, especially infections and malignancies [54].

The relevance of metabolic disorders also increases with aging. Indeed, recurrent diabetes mellitus is more common in the elderly and is associated with the immunosuppression used. For example, the risk of its occurrence after kidney transplantation increases 1.5-fold during each decade of life. Incidence of pre-transplant diabetes mellitus also increases from 7% to 31% with age, as shown in a study of over 12,000 liver transplant recipients [55]. In turn, the presence of diabetes mellitus is associated with increased incidence of acute rejection, infections, late cardiovascular complications, and poor outcomes.

The risk of death from infection increases exponentially with age among renal transplant recipients, while among kidney and lung recipients older than 60 years, infection is the leading cause of increased mortality seen in the first postoperative year [54]. Cytomegalovirus infection is considered an environmental contribution to immunosenescence, as the CMV-specific CD8 T cell count is highest in the elderly. It has been suggested that filling the "immunological space" with CMV-specific T cells may narrow the T cell repertoire and strongly influence the memory component. The peripheral naïve T-cell population in people not infected with CMV showed a higher naïve T cell count and a lower immunoregulatory index [56].

Cancer incidence is known to increase steadily with age, reaching its highest level in transplant recipients over the age of 50. Skin cancers and lymphoproliferative diseases are the most common malignancies among transplant recipients. In addition, de novo malignancies are a major cause of death, accounting for one-third of deaths among liver transplant recipients, unrelated to liver disease [57].

## DISCUSSION

Aging induces a series of modifications in T cell-mediated immunity. In general, T cell compartments undergo a shift toward a less effective response throughout life. In organ transplantation, impaired T cell-mediated immunity with aging is associated with less acute rejection and improved graft survival. At the same time, older transplant recipients are more likely to experience side effects of immunosuppression with higher rates of infections and malignancies.

The complex balance between under- and over-immunosuppression becomes even more vulnerable in older recipients due to changes in pharmacokinetics and pharmacodynamics of drugs [58]. Also, existing rejection treatments can have detrimental effects in the elderly and often lead to over-immunosuppression. It is becoming apparent that immunosuppression protocols for elderly transplant recipients must balance the risk of acute rejection with the risk of adverse cardiovascular, infectious and other complications.

Decisions to adjust immunosuppressant doses are based on clinical experience, and patients often deviate from target concentrations, being exposed to alternative risks of toxicity and graft rejection. A five-year survival rate of 78% has been achieved for recipients using this strategy, but these success rates only indicate that there is still room for improvement [59]. Current data on recommended standard immunosuppressive therapy are mostly derived from studies in which elderly patients were excluded or were a minority. To date, there have been only a few well-designed prospective studies in the elderly that demonstrate the need to correct immunosuppression in the first months after transplantation [49].

Many authors emphasize the need to reduce maintenance doses of immunosuppressive drugs in elderly recipients to possibly minimize side effects and consider further research in this direction necessary. For example, it is suggested that a reduced dose of thymoglobulin or IL-2R antibody is preferable as an induction treatment for this group of recipients, a reduced dose of tacrolimus or immunosuppression without a calcineurin inhibitor (CNI) can be considered as maintenance therapy. Based on experimental data, mTORi (in particular, Betalasept) appears to be a promising candidate to replace CNIs in elderly patients [28].

Because of age-related changes in T cell differentiation, the pharmacodynamics of immunosuppressants also change, and this is one reason why the dose of these drugs may be reduced. In addition, simultaneous administration of several drugs can lead to the side effects of pharmacodynamic interactions. It is well known that CNIs can cause acute and chronic nephrotoxicity. A recent study by Khan S. et al. focused on acute kidney injury in elderly patients and the cumulative or synergistic nephrotoxicity of CNIs with nonsteroidal anti-inflammatory drugs, aminoglycosides, angiotensin-converting enzyme inhibitors and antimicrobials. The authors concluded that the use of nephrotoxic drugs should be minimized, since elderly patients are more prone to acute kidney injury after transplantation [60].

Amelia R. Cossart et al. in their 2019 review also examined currently known evidence on the pharmacokinetics and pharmacodynamics of commonly prescribed immunosuppressants (tacrolimus, cyclosporine, mycophenolate, and prednisolone) in older kidney transplant recipients and noted that older recipients may have higher dose exposure or lower clearance of calcineurin inhibitors. There have also been reports of a 50% reduction in the efficacy of tacrolimus in the elderly, a lack of increased mycophenolic acid dosing on the graft of elderly recipients, and unclear effects of aging on the pharmacokinetics of prednisolone [19].

Meier M. et al. believe that individualized immunosuppression strategies, such as calcineurin inhibitor withdrawal and mycophenolic acid withdrawal, can improve patient and graft survival in the case of an aged recipient. The authors consider the benefits of steroid withdrawal less obvious, but perhaps more important in the elderly, in whom age-associated bone mass loss, glucose intolerance, and other metabolic changes complicate steroid therapy [61].

It is becoming apparent that the aging immune system may not only require reduction, but also individualization of immunosuppression. Thus, clinical trials evaluating graft and recipient survival are urgently needed to implement age-adapted immunosuppressive protocols to meet the needs of this vulnerable group of kidney transplant recipients [62].

The use of minimum drug concentrations as the current "gold standard" for monitoring immunosuppressive therapy levels is also a disadvantage of modern immunosuppressive drug therapy for the elderly. However, monitoring blood concentrations may not adequately reflect the effects of aging immune system or age-associated organ dysfunction. Consequently, diagnostic use of biomarkers is necessary to adjust drug therapy for age-related changes. Scientists are beginning to think about finding pharmacodynamic, pharmacogenetic or immunological markers of individualized immunosuppression. Instead of dosing immunosuppressive drugs based on pharmacokinetic measurements, an immunological biomarker would better reflect the activity of the drug (or combination of drugs) rather than just its concentration [54]. Indeed, monitoring of peripheral blood cells and cytokines in the pre- and post-transplant period reveals changes in the processes of developing organ rejection or engraftment, which may provide grounds for individualization of immunosuppressive therapy [63].

Coming up with and implementing a comprehensive individualized immunotherapeutic strategy in kidney transplantation will allow to minimize the complications of immunosuppressive drugs used, their nephrotoxicity, various infectious and cancer diseases, and post-transplant diabetes, thereby improving kidney transplant outcomes and reducing the transplant waitlist with simultaneous rational use of expensive immunosuppression.

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# THE ROLE OF TGF-β1 GENE POLYMORPHISMS IN THE DEVELOPMENT OF POST-TRANSPLANT COMPLICATIONS

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Transforming growth factor beta 1 (TGF-β1) is an immunosuppressive and profibrogenic cytokine capable of influencing the development of graft rejection and graft fibrosis in solid organ recipients. The TGF-β gene has a significant polymorphism that may cause individual protein expression levels and be associated with post-organ transplant complications. It is believed that three TGFB1 polymorphic variants (rs1800469, rs1800470 and rs1800471) may be associated with the development of graft rejection, graft fibrosis and chronic dysfunction of a heart, liver or kidney transplant. A review of current literature presents the results of studies on the relationship between TGF-β1 gene polymorphisms and post-transplant complications in solid organ recipients. The findings of various studies of TGF-β1 gene polymorphism in solid organ recipients are not always unambiguous, and their results are often difficult to generalize even with the help of meta-analysis. Samples included in studies vary in terms of ethnicity, gender, age, and underlying medical conditions, while results are highly dependent on sample structure or latent relatedness. Currently available data suggest that TGFB1 polymorphism may determine a predisposition to the development of graft rejection, graft fibrosis and graft dysfunction in solid organ recipients, but this is not conclusive and requires further, larger studies.

*Keywords: single-nucleotide polymorphism, graft rejection, graft fibrosis, graft dysfunction.*

Transplantation of vital organs is the only effective method of treatment for patients with end-stage chronic diseases leading to organ failure/irreversible loss of organ functions. Transplantation allows achieving long-term survival and rehabilitation. The post-transplant period may be accompanied by such complications as graft rejection, fibrosis and graft dysfunction. Prevention of these is an urgent task in transplantology [1].

The role of the major histocompatibility complex (MHC) in the occurrence of acute and chronic graft rejection is well studied. However, apart from MHC, other factors may influence the development of post-transplant complications. Today, there are a wide range of cytokines, including interleukins, interferons, various growth factors, and their receptors known to be important regulators of the immune response after organ transplantation [2].

Organ recipients can develop long-term post-transplant complications, including graft fibrosis, which can lead to structural and functional remodeling of the organ and subsequent dysfunction. Acute and chronic graft rejection, arterial hypertension, metabolic syndrome, diabetes mellitus, renal dysfunction, etc. contribute to fibrotic changes in the transplanted organ [3, 4].

Biopsy is mainly used to verify the pathology of transplanted organs. It is associated with all the risks of invasive interventions. The search for new molecular genetic markers and the development of minimally invasive techniques for detecting post-transplant complications based on these markers is a priority task that occupies an essential place in the concept of personalized medicine.

Transforming growth factor beta 1 (TGF-β1), a cytokine, an important component of the immune system that has immunosuppressive and profibrogenic effects, can have a significant impact on development of post-transplant graft rejection, graft fibrosis and infectious processes [5–8].

As studies by various authors, including the work by our laboratory have shown, cytokine levels correlate with liver transplant function and may have prognostic and diagnostic significance [9–12]. TGF-β1 content in the recipient's blood and tissues may depend on various factors, such as clinical, pharmacological, including genetic.

It is now known that the TGF-β1 gene has a significant genetic polymorphism that may cause individual levels of protein expression and be associated with various diseases [13–17] and post-transplant complications after organ transplantation [16, 18–22].

Cytokine TGFB1 is encoded by the TGFB1 gene, has sequence number 190180 in the Online Mendelian Inheritance in Man (OMIM) genetic database. The gene is located on the chromosome 19 long arm at locus 19q13.2, consists of 7 exons and 6 introns with a total length of about 23,000 base pairs. The size of the regula-

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tory part of the gene is about 3 base pairs and is located at positions –2665 to +423 in exon 1 (+1 is the translation start site) [23]. Currently, eight potentially significant single nucleotide polymorphisms (SNPs) and one deletion/insertion polymorphism (registered in gene databases as rs2317130, rs11466313, rs1800468, rs1800469, rs11466314, rs1800470, rs1800471 and rs11466316) that affect the TGF-β1 expression and regulate its transcription have been identified in the TGFB1 gene [24]. All of the single nucleotide substitutions studied are associated with characterization of human immune status and are on the list of those recommended by the 15th International Histocompatibility and Imunogenetics Workshop held in Brazil in 2008 for clinical diagnostic purposes.

Studies of the genetic polymorphism of the TGFB1 gene in solid organ transplantation have been carried out mainly in kidney and liver recipients [25–28], and considerably less in heart recipients, due to the number of heart transplants performed [29–31]. As a rule, the outcomes studied included acute and chronic rejection, mid-term graft function, tacrolimus levels, new-onset diabetes after transplantation, development of graft fibrosis, and incidence of infectious diseases.

The greatest interest to researchers in the field of solid organ transplantation comes from 3 polymorphic variants in the TGFB1 gene: rs1800469, which is a cytosine to thymine substitution C(–509)T in the promoter region, localized in exon 1 in codon 10 (T+869C), rs1800470, leading to leucine to proline substitution, and rs1800471 in codon 25 (C+915G), leading to arginine to proline substitution in the protein product. Presumably, the above SNPs can lead to different levels of TGF-β1 production and may be associated with graft rejection, fibrosis, and chronic graft dysfunction [20, 27, 30].

In heart recipients, two TGFB1 gene polymorphisms have been mainly investigated: rs1800470 and rs1800471. A number of studies have found an association between rs1800471 and acute cellular rejection and/or development of coronary heart disease after heart transplantation [32–34]. However, other studies have failed to find a reliable association between acute cellular rejection and rs1800471 [35, 36], and this polymorphism has not been found to be associated with chronic rejection either [37]. Data on association of rs1800471 with kidney function after transplantation are also inconsistent [38, 39]. For the rs1800470 polymorphism, it has been shown that it may be associated with accelerated development of CHD [37] and impaired renal function after heart transplantation [40].

Linkage analysis of TGF-β1 gene polymorphism in liver recipients showed predisposition of a certain genotype to liver fibrosis and kidney failure after transplantation [41, 42]. D. Eurich et al [42] investigated two types of polymorphism and their relationship with the development of liver fibrosis in 192 liver recipients. It was shown that the C allele substitution at codon 25 was associated with liver fibrosis. On the other hand, a study by H. Xie et al. [26] could not find an association between TGF-β1 polymorphism and acute rejection or recurrent hepatitis B virus infection in liver recipients.

Results and conclusions from various studies of TGFB1 genetic polymorphism in solid organ recipients are not always unambiguous, which may be due to the insufficient number of cases studied, ethnic heterogeneity of the sample, as well as differences in definition of the studied phenotypes and application of different analysis techniques. Meta-analyses and systematic reviews are often used to summarize heterogeneous studies, which are conducted according to certain standards and are thought to bring disparate data to a common denominator [18, 28, 43].

For example, a meta-analysis of 18 studies looking for an association between the TGF-β1 + 869 T/C and TGF-β1 + 915 G/C gene polymorphisms are not associated with acute rejection susceptibility in kidney recipients [28].

A meta-analysis of 23 case-control studies with 795 acute kidney rejection cases and 1,562 non-rejection controls also sound no significant association between the TGF-β1 codon 10 polymorphism (rs1982073) and an increased risk of acute kidney rejection in the general population [44]. Moreover, stratified analysis revealed no significant association between TGF-β1 polymorphism and susceptibility to acute rejection depending on the ethnicity of the recipient and donor. The researchers concluded that TGF-β1 polymorphism rs1982073 was not significantly associated with increased susceptibility to rejection. However, the authors conclude that studies with a large number of subjects from different ethnic groups are required to further validate the results.

The above examples show that it is difficult to generalize the results of heterogeneous studies even with meta-analysis. The samples included in the studies vary in terms of ethnicity, gender, age, and underlying diseases, while the resulting evidence may be highly dependent on sample structure or latent relatedness. It is possible that larger studies involving genome-wide genetic variation could uncover new loci and confirm known genetic variations associated with organ transplant outcomes.

Independence from the physiological state, immutability and one-time test represent some of the important advantages of gene diagnostics over other laboratory methods of analysis. The results of such a study provide information about a patient's weaknesses and allow targeted prevention of diseases by selecting medications according to the individual characteristics of the patient's body. Meanwhile, complex conditions such as acute and chronic graft rejection or dysfunction can be influenced by multiple genetic polymorphisms, which individually contribute only a small proportion to the overall risk and whose significance is difficult to assess when analyzing small groups. Various pathogenetic pathways are

involved in the development of these conditions, and in many cases, it is unclear which processes are involved or how important a particular pathway is. Therefore, studies on candidate genes based on prior knowledge of gene function do not always lead to identification of genetic variants associated with clinical outcomes of organ transplantation.

Analysis of stable combinations of several polymorphisms composing a haplotype (a cluster of alleles inherited together) may be more informative than single polymorphisms and help identify the genetic basis of susceptibility to gene-associated diseases [24]. For the TGFB1 gene, co-carriage of several polymorphisms is assumed, which can lead to cumulative association that is determined by unidirectional changes in protein levels [45]. However, the study of more than one polymorphism requires multivariate analysis of a large sample, otherwise the study may lead to false-positive results.

TGF-β1 levels may be determined not only by the polymorphism of a single gene, but also by genetic variants of other factors included in the cellular pathways of the cytokine, such as binding proteins and its receptors. For example, it has been shown that in liver recipients, the risk of developing hepatitis C is associated with the frequency of rs868 single-nucleotide polymorphism in the TGFBR1 gene of TGF-β cytokine receptor gene, which is located in the untranslated region of the gene [46].

Interactions between different genes may also be of clinical importance. For example, in patients with type 1 diabetes, which is a multifactorial autoimmune disease where the interaction and polymorphism of the HLA and insulin genes are important, an interaction between the HLA genes and various cytokines, particularly TGF-β1, has also been found [47].

In addition, predisposition to various polygenic diseases may depend on the ethnic origin of the individual, which necessitates studies in genetically homogeneous groups. It should be noted that, at present, there are virtually no studies on the role of gene polymorphism outside the MHC system in the development of post-transplant complications in solid organ recipients in the Russian population.

Genome-wide association studies (GWAS), which include millions of genetic variants, are considered the most appropriate research design for polygenic diseases. Importantly, such studies use an agnostic approach, meaning that there is no bias, unlike candidate gene studies based on prior knowledge of gene function. Whole-genome studies require large sample sizes, which are necessary to ensure statistical power and significance of the result, but are limited by the number of transplants performed [29]. To solve the problem, an international network of genetic and translational research in transplantation (www.igenetrain.org) was established, which currently conducts about 30 genomics studies. Whole-genome studies in transplantology are aimed at improving transplant outcomes through more accurate dosing and individual selection of immunosuppressive drugs and/or possible stratification of the risk of adverse organ transplant outcomes. It has been shown that the use of genetic typing data of recipient and donor polymorphisms in prognostic models can improve the accuracy of calculating the risk of graft fibrosis following liver transplantation [48].

## CONCLUSION

Presented evidence suggests that in organ transplantation, TGF-β1 levels in blood may be genetically determined and may determine the predisposition to fibrosis, graft dysfunction and infectious diseases in solid organ recipients. However, currently available evidence on the role of TGFB1 polymorphism in post-transplant complications is not enough to draw unequivocal conclusions; further, more extensive research is required [18, 42].

Finding out the role of polymorphism of genes encoding the activity of pro- and anti-inflammatory cytokines, including TGF-β, in the pathogenesis of post-transplant complications is an important task that, on one hand, will allow to predict the risk of developing a pathology or its severity and, on the other hand, will allow to individually select a specific therapy for a particular patient.

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# LYMPHATIC CIRCULATION AND HEART FAILURE

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**Objective:** to summarize current knowledge about the interactions between the lymphatic/cardiovascular systems and interstitial tissue, which are associated with heart failure (HF). The authors attempt to answer the fundamental question of whether lymphatic insufficiency is a cause or consequence of HF. Understanding lymph formation processes in HF will allow finding new ways of treating HF.

*Keywords: lymphatic system, heart failure, Starling equation, interstitial fluid, lymph, lymphatic drainage.*

## INTRODUCTION

One of the main physiological functions of the body is water metabolism to maintain fluid homeostasis. Three types of fluids are involved in this process: blood (plasma), interstitial fluid and lymph. The complex mechanisms of movement of water molecules between these compartments have the ultimate goals of delivering nutrients and oxygen to all cells of the body and expelling toxic products of metabolism back through the kidneys, lungs and liver.

The cardiovascular system supplies blood to all organs and tissues, where, by convection, ultrafiltration or diffusion, water, nutrients and oxygen reach the cells through the endothelial capillary wall into the interstitial space (IS). From the IS, the fluid returns back through the lymphatic system (LS) to the circulatory system. These systems, which regulate water exchange between blood, interstitium and lymph, work together to maintain the body's homeostasis. Moreover, each system has a certain buffer capacity, and the failure of one of them affects the relative fluid balance between the sectors.

Of the three fluid types, most is known about blood and the circulatory system, as the heart and large vessels are relatively easy to visualize, and have been studied in detail. In contrast, LS and formation processes of interstitial fluid (IF) have been much less studied and so is their role in development of signs, symptoms and organ dysfunction in heart failure (HF), which can be seen as the inability of LS to adequately compensate for increased fluid flow from the circulatory system to IS. This paper presents current evidence on lymphatic formation and the mechanisms by which lymph returns to the circulatory system, and considers the consequences of inadequate lymphatic flow (LF). Lymph drainage techniques will be explored as an effective therapeutic measure in refractory HF to reduce edema.

The development of new LS imaging technology would expand our understanding of the interaction between the circulatory system and LS in HF and begin to develop new methods for normalizing LF into the venous system.

## INTERSTITIAL FLUID FORMATION

In 1894–96, Starling [1, 2] found, based on his experiments on an isolated canine hind limb, that saline solution introduced into the interstitial tissue is absorbed into the bloodstream. He thus concluded that hydrostatic pressure is responsible for fluid ***filtration,*** oncotic pressure is responsible for fluid ***reabsorption***, and that there is a balance between hydrostatic and osmotic pressures in the capillaries and the surrounding tissue, which determine the filtration and reabsorption processes between the capillaries and the tissue.

On this basis, he proposed a classical model that describes the process of fluid filtration and reabsorption in the capillaries as a process of interaction between hydrostatic and osmotic pressure on both sides of the capillaries, which he expressed through the equation, which later became known as Starling's equation (1).

$$Jv = LpS([Pc - Pi] - \sigma[\pi p - \pi i]), \quad (1)$$

where Jv is transcapillary fluid transport; Lp is hydraulic conductivity of the capillary wall; S is surface area of the capillary where fluids are exchanged; Pc is hydrostatic pressure of blood in the capillary; Pi is hydrostatic pressure in the interstitium; σ is reflection coefficient, which reflects protein permeability in the capillaries; πp is capillary colloid osmotic pressure; πi is interstitial colloid osmotic pressure.

When Jv is positive (i.e., when the difference in hydrostatic pressure exceeds the difference in osmotic pressure), the process of fluid ***filtration*** from capillaries into the IS occurs. When Jv is negative, the liquid passes

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from the IS back to the capillaries When Jv is positive (i.e., when the difference in hydrostatic pressure exceeds the difference in osmotic pressure), the process of liquid filtration from capillaries into the IS occurs. When Jv is negative, the liquid passes from the IS back to the capillaries – a ***reabsorption*** process.

In accordance with this, E. Starling argued that the difference between hydraulic pressure in the arterial side of the capillaries and osmotic pressure in the tissues determines fluid filtration from the capillaries in the IS, and that reverse reabsorption of fluid from the IS occurs in the venous side of the capillaries.

A diagram illustrating Starling's hypothesis is shown in Fig. 1.

At the arterial end of the capillary, hydrostatic pressure in the capillary exceeds oncotic pressure in the plasma, resulting in fluid filtration from the vessel into the tissue.

At the venous end of the capillary, hydrostatic pressure drops, oncotic pressure in the plasma turns out to be higher than hydrostatic pressure, and as a result there is a backflow of fluid (*reabsorption*).

Elsewhere, Starling postulated that increased venous pressure (VP) in HF patients is the result of increased blood volume, most likely due to renal fluid retention, and not just due to a mechanical increase in VP with poor cardiac function [3]. In the same work, he proposed the following mechanism of edema in patients with HF: "It seems likely that obstruction of lymph flow from the thoracic duct (TD) into the blood, as well as stretching of the TD, due to a significant increase in lymph formation in the liver, may contribute to the occurrence of edema in the rest of the body".

Starling's work subsequently triggered so many preclinical studies aimed at understanding the mechanisms of lymph formation and interaction between blood, interstitium and lymph.

About 60 years later, A. Guyton, measured the internal hydrostatic pressure, in a perforated capsule implanted into the tissue, corresponding to the pressure in the IS close to the atmospheric pressure [4]. This was later confirmed by other researchers and naturally is equal to 2 mmHg [5, 6].

Interstitial oncotic pressure was also measured in the fluid accumulating in the capsule and was found to be 9–15 mmHg, much higher than previously thought [7].

Since in normal conditions, hydrostatic pressure on the arterial side of the capillaries is ~35 mmHg, whereas on the venous side of the capillary, it is ~15 mmHg, which indicates a positive gradient of filtration from capillary to IS along the entire length of the capillary. Therefore, an important conclusion was made that in norm there is no venous reabsorption along the entire capillary length, although in certain types of pathology, associated, for example, with a drop in blood pressure (BP) in hypovolumic shock, fluid reabsorption from the venous side of the capillary is possible [8–10].

Thus, Starling's concept, which had existed for more than 100 years, was found to be erroneous. Recent discoveries have completely changed the idea of the mechanism of interstitial capillary fluid evacuation. This is based on the fact that in normal conditions, even in venous stasis, all interstitial ultrafiltrate is completely removed through capillaries in the IS and further removed exclusively by the LS, i.e. there is no venous reabsorption (Fig. 2).

It is important to understand that Starling forces leading to IF formation differ in different parts of the body due to differences in hydrostatic pressures at capillary level. For example, sinusoidal pressure in the liver is ~5 mmHg and capillary pressure in soft tissues is ~35 mmHg. The only variable in Starling's equation for all organs and tissues is the oncotic pressure of plasma in the capillary (πp), which is ~24 mmHg.

Therefore, for a positive balance of filtration from the capillaries into the IS, the oncotic pressure in the interstitium (πi) must also be different for different organs. In

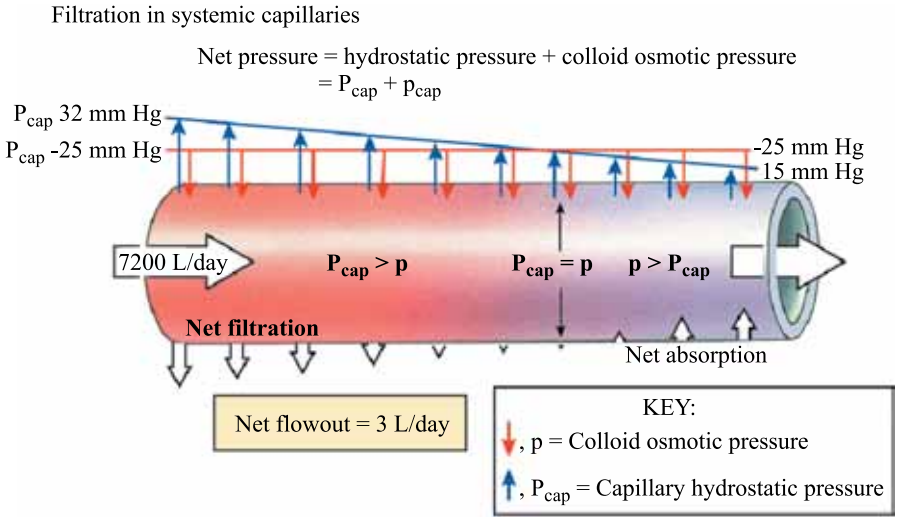


Fig. 1. Fluid movement according to Starling's law

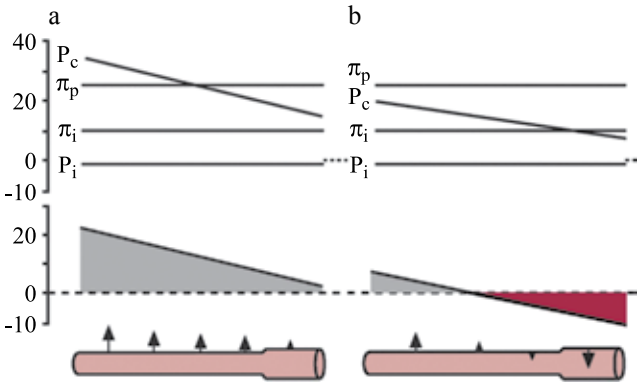


Fig. 2. a) New concept of liquid filtration in the capillary; b) Filtration/adsorption concentration of fluid in the capillary according to Starling's law

addition, the permeability of capillary endothelium for proteins (σ) must differ to allow regulation of flow of proteins from the capillaries into the IS. Consequently, lymph composition and flow, which reflect the composition of the interstitial ultrafiltrate, may differ significantly in different organs.

## THE PROCESS OF FLUID REMOVAL FROM THE INTERSTITIAL SPACE TO THE LYMPHATIC SYSTEM

Tissue edema, one of the main clinical manifestations of HF, involves accumulation of fluid in interstitial tissues. The volume of this fluid depends on the balance between capillary filtration and lymph outflow into the venous system (Fig. 3).

In this case, LS is practically the only mechanism for IF removal and transport into systemic bloodstream. As stated above, capillary filtration is determined by the balance of hydrostatic and oncotic pressure (modified Starling equation) and capillary permeability.

Hydrostatic interstitial pressure in most tissues is lower than atmospheric pressure [11], while central venous pressure (CVP) is approximately 5 mmHg and energetically active mechanisms are required for lymphatic transport.

It has been established that lymph movement in vessels is provided both by external factors (compression from surrounding tissues, intestinal peristalsis, external respiratory pressure fluctuations, massage, pulsation of blood vessels, etc.) and internal factors (contractions of lymphatic vessels, the walls of most of which contain a muscular layer that has general biochemical and functional characteristics comparable to those of vascular and cardiac muscles). A segment of the lymph vessels are between the two valves, called the lymphangiome (Fig. 4), which contracts similarly to cardiac muscle [12].

Besides, lymphatic valves are an important mechanism for ensuring unidirectional lymph flow. The pressure differential required to close the lymphatic valve depends largely on the vessel diameter: less than 1 cm $H_2O$ at a small diameter to several cm$H_2O$ when the vessel diameter is close to maximum [13]. Thus, the valves become less effective in dilated vessels, potentially contributing to reverse lymphatic flow when the vessels are chronically dilated, such as in HF [14, 15].

It has been shown that at rest, $^1/_3$ of lymph flow (LF) in human lower extremities depends on compression of skeletal muscle contractions (external pump) and $^2/_3$ of active pumping of collecting vessel network (internal pump), and that active contraction of lymphatic vessels can create from 20 to 120 mmHg pressure [16, 17].

At the same time, the main pathway of LF is the thoracic duct (TD) through which $^3/_4$ of all lymph (from the lower extremities, pelvic walls and organs, abdominal cavity, left half of the chest cavity, left upper extremity, left half of the head and neck) moves to the venous system (Fig. 5). $^1/_4$ of LF is formed by the fusion of the right bronchomediastinal, subclavian and jugular trunks and flows into the right venous system [18].

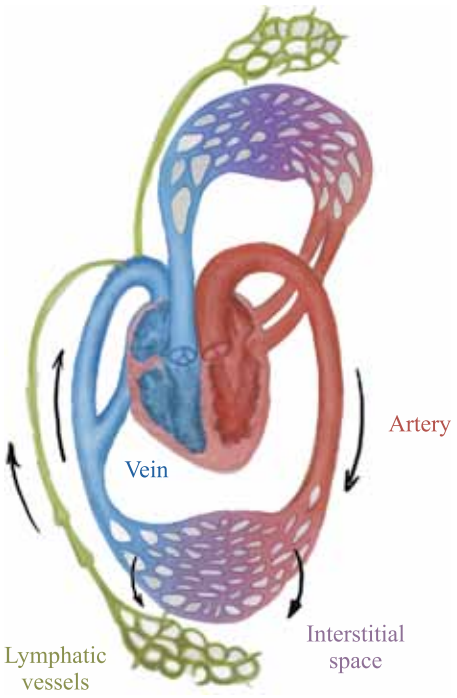


Fig. 3. Interaction between the circulatory system and the lymphatic system

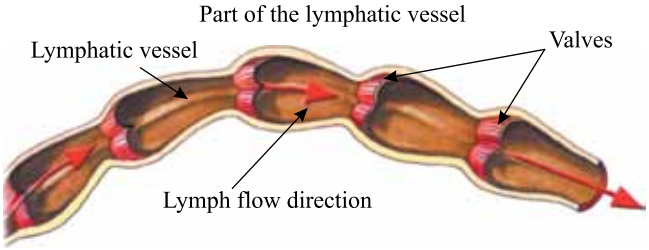


Fig. 4. Structure of the lymphatic vessel

## RELATIONSHIP BETWEEN HF AND LF

Loop diuretics are the primary antiedema therapy in patients with HF to maintain euvolemic status. This is despite the lack of high-quality data demonstrating clinical benefit with respect to cardiovascular outcomes. However, in acute HF, patients may exhibit decreased sensitivity to diuretics, which is often referred to as diuretic resistance and is associated with a higher risk of rehospitalization and death [19, 20]. Features of LF in HF, which have linked the pathophysiology of pulmonary edema, have been the subject of many studies, who have shown significant increases in LF and TD pressure despite increased CVP in the lymph outlet area [21]. These studies have prompted several researchers to hypothesize that TD decompression may be one of the treatments for HF. Allen et. al [22] showed that external TD drainage in sheep with increased left atrial pressure reduces pulmonary edema and pleural effusion. Dumont et al. drained TD in 5 patients with HF [23].

Initial lymph drainage from TD ranged from 4–17 liters per day. There was immediate significant improvement in HF symptoms in all patients, a significant decrease in CVP and weight loss.

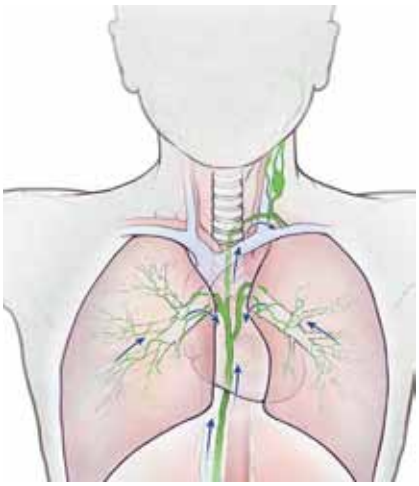

Fig. 5. Lymph flow from the thoracic duct to the vein

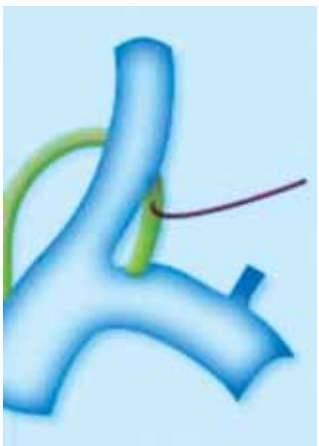

Fig. 6. Lymph drainage from the thoracic duct

In a subsequent study, Witte et al. [24] reported external TD drainage in 12 patients with CHF (four patients with compensated HF and 42 healthy patients; diuretics were discontinued during the whole lymphatic drainage period.

In doing so, they showed that LF from TD was significantly increased from ~1 mL/min to ~8 mL/min. TD pressures were elevated and correlated with elevated CVP. They also noted that HF symptoms improved within hours of initiating TD drainage. In contrast to diuretic treatment, which often leads to a worsening of renal function, they demonstrated a significant improvement in renal function in four oliguric patients. This effect could potentially be explained by decompression of the renal LS, resulting in decreased renal insufficiency and improved diuresis.

After these observations, Clauss and Breed [25] concluded that thoracic duct drainage can be used as therapy in CHF and the significant therapeutic effects of external drainage observed in these works can be explained simply by fluid removal, similar to the effects of diuresis.

Based on previous studies, Hraška V. [26] surgically anastomosed TD to the left atrium, the lowest pressure point in children with Fontan physiology, who had protein loss enteropathy and plastic bronchitis and which is known to result from lymphoedema. He reported significant improvement in symptoms in most of these patients, thus proving that such "internal decompression" of an overloaded LS can potentially eliminate the symptoms of LS edema.

## CONCLUSION

There has been tremendous progress in the study of HF mechanisms over the past few decades. However, there are still many questions in understanding the pathophysiological mechanisms of patients' symptoms, which cannot be explained by cardiac muscle dysfunction and vascular system response alone. For many years and up to the present time, even with new basic scientific knowledge about ultrafiltrate formation and the important contribution of LS to fluid removal from tissue, this area remains relatively understudied in modern clinical medicine. The role of LS in chronic HF is still poorly defined.

The general consensus is that edema in heart failure should be seen as the inability of the lymphatic system to remove excess amounts of interstitial fluid. The process of significant increase of lymphatic flow in heart failure remains poorly investigated. Assuming that the only mechanism of fluid movement from the circulatory system to the interstitial space is filtration in the capillaries, the question arises about a significant increase in lymph flow in heart failure, which, in our view, may be associated with increased number of functioning capillaries. We put forward the idea that heart failure leads to decreased

capillary blood flow, which results in oxygen deficiency in the distal ends of capillaries and therefore in the tissue surrounding these ends of capillaries. The natural reaction to this is the opening of the sphincters in this area and the inclusion of additional functioning capillaries into the blood flow and, accordingly, this should lead to increased filtration flow in the interstitial space.

As many studies have shown, in heart failure conditions, increase in lymph production is not compensated by lymph outflow into the venous system, which leads to edema in the organs and tissues. Therefore, one of the promising methods of mechanical support for lymph outflow into the venous system in heart failure involves a local reduction of elevated venous pressure in the area of lymph outlet into the venous system.

The relatively recent clinical introduction of lymphatic system imaging technique based on magnetic resonance lymphangiography provides the basis for a more in-depth analysis of interaction between the lymphatic system and the system in heart failure [27–29].

*The authors declare no conflict of interest.*

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# POST-TRANSPLANT MALIGNANCIES IN SOLID ORGAN RECIPIENTS: DEVELOPMENT MECHANISMS AND RISK FACTORS

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According to the International Agency for Research on Cancer, there were an estimated 19,292,789 new cancer cases in various localizations and 9,958,133 cancer deaths worldwide in 2020. These frightening figures clearly show that malignancies among the population is a pressing matter. The risk of post-transplant malignancy in solid organ recipients is 2–6-times higher than in the general population. Given the steadily increasing number of solid organ transplants worldwide and the gradual increase in life expectancy among organ recipients, studying the issues concerning risk factors and development mechanisms becomes a crucial task.

*Keywords: malignant tumor, transplantation, solid organs.*

## INTRODUCTION

According to the International Agency for Research on Cancer, the year 2020 had about 19,292,789 new cancer cases and 9,958,133 cancer deaths worldwide. Breast cancer is the most commonly diagnosed cancer in the world, accounting for 2,261,419 cases (11.7%), followed by lung cancer 2,206,771 cases (11.4%), colorectal cancer 1,931,590 (10%), prostate cancer 1,414,259 (7.3%) and stomach cancer 1,089,103 (5.6%). Lung cancer tops the mortality chart with 1,796,144 (18%), colorectal cancer 935,173 (9.4%), liver cancer 830,180 (8.3%), and stomach cancer 768,793 (7.7%). There are some gender differences in cancer incidence in men and women. Among men, lung cancer is the most common (14.7%), followed by prostate cancer (14.1%) and colorectal cancer (10.6 per cent). In women, breast cancer (24.5%), followed by colorectal cancer (9.4%) and lung cancer (8.4%). These frightening figures clearly show the urgency of the problem of malignant tumors among the population [31].

According to reports, the risk of malignant tumors in solid-organ transplant recipients is 2–6 times higher than in the population. Given the steadily increasing number of solid organ transplants worldwide (113,363 solid organ transplants were performed in the world in 2020 alone) and the gradual increase in life expectancy of donor organ recipients, the relevance of studying malignancies would only increase. Issues of cancer development in this category are covered in sufficient detail in foreign literature, but we did not find any domestic sources [1–13, 30].

## EPIDEMIOLOGY

Since the first donor organ transplants, there has been increased risk of cancer cases among solid-organ transplant recipients [1, 2, 6–10]. Most authors conclude that within the next decade, malignant tumors will become the leading cause of death in this patient category [3, 4, 11–13]. As life expectancy increases, so does the importance of the problem of cancer [2, 14].

The literature contains numerous data on the occurrence of malignant tumors in solid-organ transplant recipients. The authors, analyzing the groups of recipients diagnosed with cancer, differing by age, sex, transplanted organ, country of residence, etc., confirm the relevance of this problem. For example, Wareham N.E. et al., who studied the data of 212 solid-organ transplant recipients with malignant tumors found that cancers were the second among liver recipients, third among kidney recipients, and fourth most common cause of death among lung recipients in 5 years after transplantation. The authors concluded that the causes of cancer are primarily related to immunosuppressive therapy, as well as the presence of precancerous diseases [6, 7].

Based on analysis of 8,520 cancer cases among 164,156 solid-organ transplant recipients, Hall E.C. et al. found that with the exception of nonmelanoma skin cancer, non-Hodgkin's lymphoma (NHL) (one of the adverse outcomes of post-transplant lymphoproliferative disorder (PTLD)) is the most common malignant tumors in solid-organ transplant recipients [1, 11, 12].

According to E. Yanik et al., among 187,384 solid-organ transplant recipients, including kidney (58%), liver (22%), heart (10%) and lung (4%), 9,323 cancers (4.97%) were identified. The most common malignant tumors were lung cancer (N = 1993), NHL (N = 1846) and prostate cancer (N = 1544) [15].

The risk of cancer in solid-organ transplant recipients, according to Guillemin A. et al., is 2.6 times higher than

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in the general population and is the third most common cause of death among this category of patients [5].

Vajdic C. et al. reports that solid organ recipients have a 3-fold higher risk of developing cancers compared to the general population [16].

In a study by S. Acuna et al. who evaluated 11,061 solid-organ transplant recipients, malignant tumors were the second cause of death after cardiovascular diseases. Of the recorded deaths in this study cohort, 20% were attributed to the development of cancer, the majority (68%) of which occurred in de novo cases, 11% (1,267 patients) developed a de novo malignancy within 5 years of transplantation. Mortality from nonmelanoma skin cancer (squamous cell and basal cell cancer) in solid-organ transplant recipients is 30-fold higher than in the general population, and the mortality rate from NHL is 10 times higher than in the population [17].

According to reports from E. Neval et al., among the 1656 solid-organ transplant recipients studied, the most frequent type of cancer was skin cancer (121 patients), then lymphoma (non-Hodgkin and Hodgkin), 37 patients) and lung cancer (19 patients) [2, 16].

Z. Huo et al. found that the risk of lung, liver, and kidney cancers was mainly increased in heart and/or lung recipients, liver recipients, and kidney recipients, respectively [18].

In a cohort study that analyzed the frequency of malignancy in 175,000 solid organ transplant recipients, Brennan D.C. et al. noted a more than 5-fold increase in incidence of Kaposi sarcoma, nonmelanoma skin cancer, non-Hodgkin lymphoma, liver cancer and anogenital cancer. And there was also a significant increase in other cancers: lung, kidney (in lung and kidney recipients, respectively), colorectal, pancreatic, Hodgkin's lymphoma, and melanoma. In contrast, the incidence of breast cancer and prostate cancer was lower [19].

According to A.-M. Noone et al., among the 221,962 solid organ recipients studied, 15,012 developed cancer (6.76%). Lung cancer was the largest contributor to mortality (3.1%), followed by non-Hodgkin lymphoma (1.9%), colorectal cancer (0.7%), and kidney cancer (0.5%). NHL was the largest contributor among children (4.1%), and lung cancer among 50+ year-olds (3.7–4.3%). Mortality attributable to cancer increased steadily with longer time since transplant, reaching 15.7% of deaths 10+ years post-transplant [20].

Thus, despite some inconsistency in the data obtained by different authors, the risk of malignant tumors such as lymphomas (Hodgkin and non-Hodgkin lymphomas), lung cancer, skin cancer and colorectal cancer is higher in solid organ transplant recipients than in the population.

## PATHOGENESIS

There are three main mechanisms of cancer development in solid organ transplant recipients: development of de novo malignancy; transplantation of an organ containing malignant tumor (primary or metastasis); progression of a neoplastic process existing in the recipient before transplantation [8, 18]. According to many authors, there are a number of factors associated with development of malignant tumors in solid-organ transplant recipients, including: the nature and duration of immunosuppressive therapy, concomitant viral infection, sun exposure (for skin cancer), a history of pre-transplant dialysis in kidney recipients (the risk of renal cell carcinoma is associated with formation of acquired polycystic kidney disease, occurring in patients on hemodialysis for a long time), and episodes of acute rejection [1, 7, 11, 16–22]. Additional risk factors common to the general population also include smoking, alcohol abuse, and age of the recipient [23]. It is important to note that we have an incomplete understanding of how immunosuppressive drugs actually contribute to the development of malignancies. The high risk of PTLD associated with the use of anti-T-cell antibody induction is almost certainly associated with intense immunosuppression and a consequent decline in immunosupervisory functions [24, 25]. Immunosuppressants lead to immune suppression in recipients, thereby reducing antitumor and antiviral defense, and they can also have a direct damaging effect on the recipient's DNA (e.g. azathioprine increases the risk of malignant tumors and especially squamous cell skin cancer due to its mutagenic effect and potentiation of the adverse effect of ultraviolet light) [7, 17, 26]. Immunosuppressive therapy directed against T-lymphocytes (antithymocyte globulin) predisposes to the development of Epstein–Barr virus-induced PTLD [7, 15]. On the contrary, therapy with antibodies targeting B-lymphocytes (rituximab) does not increase the incidence of malignancies [8].

There is a correlation between the likelihood of developing malignant tumors and increased levels of immunosuppression. For example, heart transplants often maintain high levels of immunosuppression, unlike kidney transplants, because of the risk of death associated with organ rejection. In a study of more than 50,000 kidney and heart transplants, the incidence of PTLD was highest in the first year when immunosuppression was the highest, it declined by 80% thereafter. Incidence of malignant neoplasms was significantly higher in heart recipients, which was consistent with a greater degree of immunosuppression in these patients [18]. Episodes of transplant rejection in the first year after transplantation increase the likelihood of malignancy, apparently due to high doses of immunosuppressive drugs used in the event of a rejection crisis [16, 17, 22]. The different incidence of cancers in different solid organ recipients may be related to the direct effect of immunosuppressive drugs [1, 8, 12, 16, 18, 19]. There is a dose-dependent association between calcineurin inhibitors and malignant tumors, such as lymphoproliferative disorders or skin cancer. In contrast, sirolimus and other M-TOR inhibitors have a direct antitumor effect [19]. Some authors have reported

reduced risk of malignancies against the background of taking sirolimus (in comparison with controls, sirolimus use reduced the risk of malignant neoplasms by 40% and reduced the risk of nonmelanoma skin cancer by 56%) [7, 11, 14, 18, 19], others note no such effect [19]. In vivo experimental studies have shown that azathioprine increases the risk of malignant neoplasms and, especially, squamous cell skin cancer due to its mutagenic effect and potentiation of the adverse effect of ultraviolet light [3, 5, 7, 8]. Cyclosporine increases the risk of Kaposi's sarcoma and metastatic lung injury in patients with glandular cancer [3, 5, 7, 8]. Brennan D.C. et al. cites data from a study of 231 renal allograft recipients. Patients receiving low-dose cyclosporine (75 to 125 ng/mL) for 12 months had a lower incidence of all malignancies (23 versus 37 diseases), especially skin cancer (17 versus 26 diseases) compared to the group of patients receiving conventional doses of cyclosporine (150 to 250 ng/mL). The median follow-up was 66 months [19]. According to Brennan D.C. et al., the use of tacrolimus increases the risk of malignant tumors after kidney transplantation. The carcinogenic effect of calcineurin inhibitors is achieved mainly due to stimulation of transforming growth factor beta (TGF-β) secretion by cells and increased expression of vascular endothelial growth factor [8]. Solid organ recipients receiving mycophenolate mofetil have a lower risk of cancer, which may be associated with reduced incidence of acute rejection, which in turn leads to lower need for increased doses of immunosuppressive drugs [5, 7]. The role of corticosteroids in development of malignancies is difficult to assess because they are almost always prescribed simultaneously with other immunosuppressants [5].

According to the majority of authors, the presence of oncogenic viruses, or their transmission with the donor organ is a significant risk factor for cancer development against the background of prolonged immunosuppression. For example, Epstein–Barr virus is the main provoking factor of non-Hodgkin's lymphoma and Hodgkin's lymphoma, which are malignant forms of PTLD. Anogenital cancer is associated with human papillomavirus and Kaposi's sarcoma with herpesvirus type 8. In these situations, immunosuppression reduces immune control of these viral infections [1, 7, 16, 18, 19, 21, 22]. According to Z. Huo et al., the presence of hepatitis C or B virus is considered a risk factor for liver cancer, and the presence of human papilloma virus can provoke cervical, vulvar, vaginal, penile, anus and oropharyngeal squamous cell cancer. Among solid organ recipients, 90% of patients with squamous cell cancer had human papillomavirus, whereas in the population, the prevalence of human papillomavirus among patients with squamous cell skin cancer ranges from 11% to 32% [18]. According to J. Liao et al., the development of cervical, vulvar and vaginal cancers in solid-organ transplant recipients is also associated with persistent human papillomavirus [28].

If a liver transplant has been performed for primary sclerosing cholangitis or a lung transplant for cystic fibrosis, recipients have an increased risk of colorectal cancer, as up to 80% of recipients with primary sclerosing cholangitis have inflammatory bowel disease (most often non-specific ulcerative colitis, which is a risk factor for colorectal cancer), and in cystic fibrosis there are changes in the gastrointestinal epithelium due to mucus secretion disorders [24]. With unilateral lung transplantation or kidney transplantation, there is increased risk of malignant tumors in the preserved native organ according to the nosological form of the original disease that led to transplantation [24]. According to Wareham N.E. et al., elderly age and chronic inflammation were the risk factors for cancer, especially lung cancer [12, 21]. Besides, bad habits leading to end-stage disease in solid-organ transplant recipients (for example, smoking in lung recipients and heart-lung complex) also increases cancer incidence [3, 8].

Several authors believe that immunosuppressive therapy may contribute to cancer development from undetected neoplasms prior to transplantation [1, 3, 5, 7, 9]. For example, increased risk of melanoma in solid-organ transplant recipients within 5 years after transplantation may be associated with the presence of a neoplastic process in the donor, undetected before transplantation. In turn, immunosuppression leads to rapid growth and progression of the tumor [24]. Malignant tumors in donor organs (liver, lungs and kidneys), if these organs were obtained from donors with a history of cancer, are diagnosed after transplantation in almost half of cases [1, 5, 9]. Acuna S.A. et al. report that among 22 of those that received heart and/or lung from such donors, malignant tumors developed in 45% of the recipients [19].

Acuna S.A. et al. found that a significant number of patients in need of solid organ transplantation had a history of cancer, representing a group at increased risk of developing a malignant tumor of new localization, as well as pre-existing malignant tumors [29]. According to the literature review and meta-analysis by S. Acuna et al., the overall rate of cancer recurrence after solid organ transplantation in patients with a history of cancer prior to transplantation is 6%. The lowest risk of cancer recurrence was in liver transplant recipients, and the highest was in kidney transplant recipients [29].

Despite the fact that report from the IPITTR (Israel Penn International Transplant Tumor Registry) study showed that the rate of cancer recurrence after kidney transplantation in patients with a history of cancer before transplantation was 21% (the majority (53%) had surgery in the first 2 years after diagnosis or treatment of cancer). S. Acuna et al. argues that we should be skeptical about these data. As in IPITTR, patients are registered voluntarily, without the obligation of further long-term monitoring [29]. Current English-language guidelines for selection of kidney transplant candidates recommend

different waiting times before transplantation for patients with a history of cancer before transplantation. The waiting period can range from none at all for some malignancies in situ, to more than 5 years for melanoma, bladder cancer, colorectal cancer, and breast cancer. For recipients of other solid organs, transplantation is recommended after complete cure of the tumor, provided that the expected cancer survival exceeds the expected survival after transplantation [29].

## CONCLUSION

Malignancies are one of the leading causes of death worldwide, regardless of gender. Despite often contradictory data from different authors, most agree that there is an increased risk of certain types of malignancies in solid organ transplant recipients who routinely take immunosuppressive medications (e.g., skin cancer, colorectal cancer, lung cancer, lymphoproliferative diseases). Besides the well-known risk factors typical for the general population (smoking, viral infections, alcohol abuse, age, gender, etc.), there are specific factors in solid organ transplant recipients, such as the effect of immunosuppressive therapy, not only immune suppression but also the direct effect of immunosuppressive drugs. It is also necessary to mention specific mechanisms of development of malignant tumors in solid organ transplant recipients, such as donor-transmitted tumors.

Analysis of reports shows that as the life expectancy of solid-organ transplant recipients increases, and given the constantly growing number of solid organ transplants, the relevance of early diagnosis of malignant tumors will only increase. Without good knowledge of risk factors and mechanisms of tumor progression in solid-organ transplant recipients, it would be impossible to timely diagnose and prevent cancer in this patient category.

*The authors declare no conflict of interest.*

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