COMBINED MATRICES AND TISSUE-ENGINEERED CONSTRUCTS MADE OF BIOPOLYMERS IN RECONSTRUCTIVE SURGERY OF ENT ORGANS

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Microtia is a combined congenital malformation with the prevalence of one case per 10,000–15,000 newborns, which accounts for 50% of all congenital malformations. Treatment of microtia is a challenging task. Numerous solutions have been proposed, however, none of these options guarantee good functional and aesthetic outcome. High hopes for solving the problem are placed on advances in reconstructive surgery. The study was aimed to determine the possibility of using advanced biocompatible endoprostheses manufactured using the tissue engineering technologies. Two closely related male 2-year-old minipigs of the Sus salvanius breed underwent implantation of bioengineered implants manufactured by combined 3D bioprinting with application of the collagen solution containing autologous cartilage tissue cells under the temporal fascia. The samples were collected 3 months later. Histological examination and immunohistochemistry showed that the implanted endoprosthesis initiated the development of regenerated connective tissue and its own vasculature in 100% of cases, thereby ensuring cell viability and integrity of biological structures; furthermore, no facts of the endoprosthesis rejection or resorption were reported. We have concluded that the developed implant manufacturing method is promising and can provide the basis for creation of domestic porous ear implants based on biocompatible polymeric materials, hydrogels, and autologous cellular material. It is necessary to further test the auricular implant using biological models.

Keywords: outer ear reconstruction, cell engineering, cartilage tissue, minipigs

Author contributions: Daikhes NA — concept, planning the experiment, management, manuscript editing; Diab KhM — manuscript writing, data provosion; Nazaryan DN — surgical stage of the experiment, manuscript editing; Vinogradov VV, Reshulsky SS — manuscript writing, data acquisition; Machalov AS — planning the experiment, data acquisition, manuscript editing; Karshieva SSh — creating the endoprosthesis, cell culture maintenance; Zhirnov SV — creating the endoprosthesis, printing the substrate; Osidak EO — creating the endoprosthesis, developing the hydrogel; Kovalev AV — histological assessment; Hesuani YuD — creating the resulting tissue-engineered construct.

Compliance with ethical standards: animal handling was compliant with the common ethical standards of Basel Declaration.

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Received: 20.12.2023 **Accepted:** 14.02.2024 **Published online:** 25.03.2024

DOI: 10.47183/mes.2024.007

КОМБИНИРОВАННЫЕ МАТРИЦЫ И ТКАНЕВЫЕ ИНЖЕНЕРНЫЕ КОНСТРУКЦИИ ИЗ БИОПОЛИМЕРОВ В РЕКОНСТРУКТИВНО-ВОССТАНОВИТЕЛЬНОЙ ХИРУРГИИ ЛОР-ОРГАНОВ

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Микротия — это врожденный комбинированный порок, встречается с частотой один случай на 10 000–15 000 новорожденных и составляет 50% всех врожденных пороков. Лечение микротии — сложная задача, предложено множество вариантов ее решения, однако ни один из них не гарантирует высоких функциональных и эстетических результатов. Большие надежды в решении проблемы возлагаются на достижения регенеративной медицины. Целью исследования было определить возможность применения современных биосовместимых эндопротезов, изготовленных с помощью технологий тканевой инженерии. Двум близкородственным самцам-минипигам двухлетнего возраста породы *Sus salvanius* под височную фасцию были имплантированы биоинженерные импланты, изготовленные методом комбинированной трехмерной биопечати с нанесением на них раствора коллагена с аутогенными клетками хрящевой ткани. Через 3 месяца образцы были изъяты. По результатам их гистологического и иммуногистохимического исследования, в 100% случаев имплантированный эндопротез инициировал развитие соединительнотканных регенератов и формирование собственной сосудистой сети, тем самым обеспечивая жизнеспособность клеток и сохранность биологических структур, при этом отторжения и явлений резорбции эндопротеза не выявлено. Сделан вывод, что разработанный метод изготовления имплантатов перспективен и может послужить основой для создания отечественных пористых ушных имплантатов на основе биосовместимых полимерных материалов, гидрогелей и аутологичного клеточного материала. Необходима дальнейшая апробация импланта ушной раковины на биологических моделях.

Ключевые слова: реконструкция наружного уха, клеточная инженерия, хрящевая ткань, минипиги

Вклад авторов: Н. А. Дайхес — идея, планирование эксперимента, руководство, редактирование рукописи; Х. М. Диаб — подготовка рукописи, предоставление данных; Д. Н. Назарян — хирургический этап эксперимента, редактирование рукописи; В. В. Виноградов, С. С. Решульский — подготовка рукописи, сбор данных; А. С. Мачалов — планирование эксперимента, сбор данных, редактирование рукописи; С. Ш. Каршиева — создание эндопротеза, ведение клеточных культур; С. В. Жирнов — создание эндопротеза, печать подложки; Е. О. Осидак — создание эндопротеза, разработка гидрогеля; А. В. Ковалев — проведение гистологических исследований; Ю. Д. Хесуани — создание итоговой тканеинженерной конструкции.

Соблюдение этических стандартов: все манипуляции с животными были проведены в соответствии с едиными этическими нормами Базельской декларации.

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Статья получена: 20.12.2023 **Статья принята к печати:** 14.02.2024 **Опубликована онлайн:** 25.03.2024

DOI: 10.47183/mes.2024.007

The combined malformation referred to as microtia or aural deformity with the external auditory canal atresia, which includes malformations of the outer and middle ear, has the prevalence of one case per 10,000–15,000 newborns. The patients with this abnormality suffer not only from severe conductive hearing loss, but also from the gross cosmetic defect [1–5].

Treatment of microtia is a challenging task. Numerous solutions have been proposed, from the use of ear prosthesis to the reconstructive surgical procedure involving the use of various surgical techniques and reconstructive materials. Reconstructive head and neck surgery has to address a complex set of challenges including not only restoration of the lost organs, their parts or tissues, and restoration of function, but also aesthetic rehabilitation [6–8].

Three main methods to eliminate microtia are used in surgical practice: the use of autologous rib cartilage, porous polyethylene implant and intraosseous implants for removable prosthetics. The treatment method is selected based on the microtia severity, functional goals of surgical correction, patient's age, wishes of the patient or his/her representatives. Auricular reconstruction involving the use of autologous rib cartilage graft is traditionally carried out on a step-by-step basis, as reported by Tanzer, Brent, Nagata, and Firmin. The alloplastic implants have gained greater acceptance as one more option for ear reconstruction, since alloplastic reconstruction can be performed in younger individuals without any damage to the donor site. Ear reconstruction using porous polyethylene (Su-Por, Omnipore, Medpor, Porex Surgical) is currently considered to be athe standard method for microtia correction in children under the age of 3 years [3, 4, 9].

The National Medical Research Center for Otorhinolaryngology of FMBA of Russia has accumulated rich experience in elimination of microtia since 2014: more than 516 cases involving the use of various methods and materials. The best outcome was obtained when using the auricle made of porous polyethylene heteromaterial as an endoprosthesis. We managed to achieve satisfactory surgical outcome in 80.48% of cases; complications developed in 19.52% of cases, among which in 12.19% of cases partial extrusion of the endoprosthesis helix was reported, and in 7.31% partial resection of the endoprosthesis was required to close the defect due to migration of the defect and its eruption through soft integumentary tissues.

We assessed aesthetic outcomes of auricular reconstruction using the porous polyethylene heteromaterial. In 24.39% of cases, no prominent postauricular fold or sufficiently protruding ear was obtained in the late postoperative period due to scarring of skin grafts and, consequently, pressing the shaped auricle to the skull, which was considered as unsatisfactory outcome. In all other 75.61% of cases, the auricle shaped had clear contours of the helix and antihelix, correctly positioned earlobe, and was arranged symmetrically with respect to the contralateral ear. Thus, we can say that surgical complications occur in 19.52% of cases when using porous polyethylene heteromaterial as the auricular endoprosthesis, while satisfactory aesthetic outcome can be achieved in 75.61% of cases only. The findings suggest the need to search for new methods and materials for elimination of microtia [10, 11].

Reconstructive surgery has been developing rapidly over the years and recently. The area is interdisciplinary, especially in the field of regenerative medicine. Tissue engineering that involves developing the constructs made of specific materials (matrices, scaffolds) and culturing stem cells or tissue-specific cells on these constructs is an important instrument of regenerative medicine [12–19].

The advances of modern regenerative medicine have found use in cardiovascular surgery, traumatology, orthopedics, trachea surgery, abdominal surgery, urology, plastic and aesthetic surgery, otolaryngology, and maxillofacial surgery [7, 18, 20–22].

It is clear that these technologies can be used for treatment of microtia. This motivated us to carry out an experimental study aimed to search for and create new ear endoprostheses using the today's capabilities of domestic regenerative medicine and tissue engineering. The study was aimed to assess the possibility of using the combined matrices and tissue-engineered constructs made of biopolymers for auricular reconstruction.

METHODS

Two closely related minipigs of the *Sus salvanius* breed were used as biological models in the *in vivo* experiment. The average body weight of minipigs was 37.5 kg. Minipigs were kept at the Research Center of Biomedical Technologies of the Federal Medical Biological Agency under stress-free conditions with *ad libitum* access to food and water. The experiment included several phases (Fig. 1).

A modified Cartesian FDM printer was used to manufacture the implant. The technological process of creating the implant consisted of five major steps: 1) creating a 3D model of the auricle; 2) transformation and adjustment of ear topology; 3) preparation of the cellular component; 4) preparation of the collagen hydrogel; 5) manufacturing the implant.

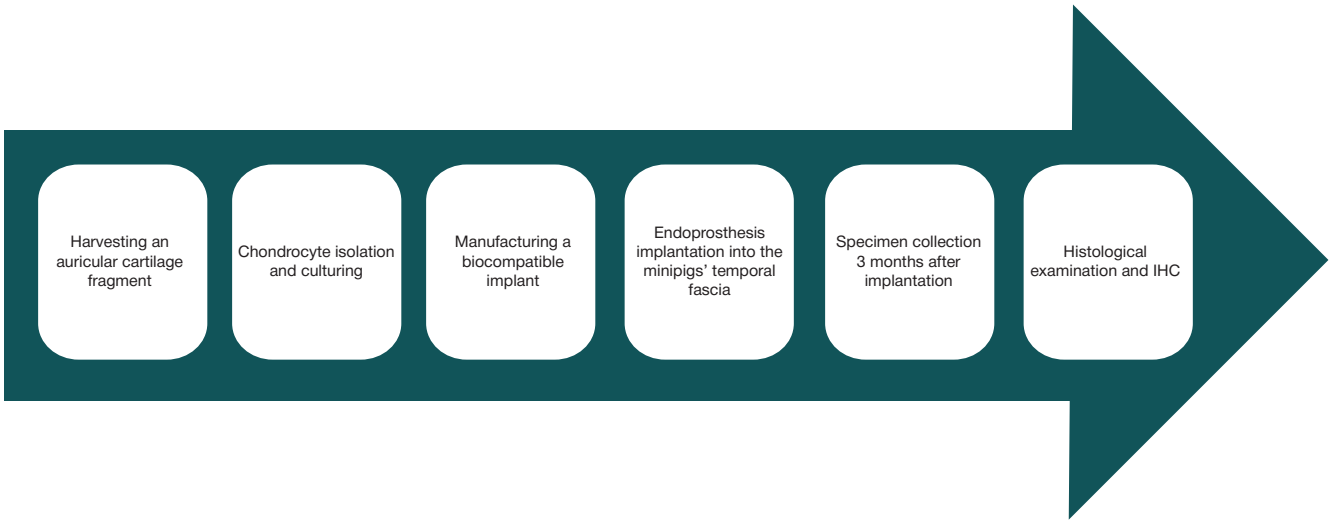

Fig. 1. Flowchart showing phases of the experiment

To obtain autologous chondrocytes from the elastic cartilage, two surgical teams harvested auricular cartilage from the left ears of two animals under total intravenous anesthesia. The cartilage piece size was 1.5 × 1.5 cm. The cartilage pieces were placed in different test tubes with transport medium (buffer). Then chondrocyte isolation from the auricular cartilage was performed followed by cell culturing and cell population expansion; this process took a month. The cells were cultured in the DMEM medium (Gibco; USA) containing 2 mL of L-glutamine (Paneco; Russia) and the single-dose antibiotic antimycotic solution (Gibco; USA) supplemented with 10% (v/v) fetal bovine serum (Gibco; USA) at 37 °C and 5% CO_2. The Versene solution (Paneco; Russia) and the 0.25% trypsin–EDTA solution Gibco; USA) were used to take the cells off the substrate.

After the chondrocyte cultivation, a bioengineered implant printed using two polymers and collagen was manufactured. CT/MRI scans of a healthy patient and the ImageJ (USA) and 3D Slicer (USA) software tools were used to create a 3D model of the auricle. The ear topology transformation and adjustment needed to create a model of the implant were performed using the Autodesk Fusion 360 (USA) and/or KOMPAS-3D (Russia) software tools. The Prusa Slicer and/or Super Slicer (USA) software was used to prepare the model for printing.

The implant was manufactured by combined 3D printing, for which printer was installed in sterile environment. The printer extruders were previously heated to 200 °C, and the stage was heated to 50 °C. Polylactide was used for stiffeners (Ingeo 4032D, China; Natureworks LLC, USA), and the Elastollan 1170 A 10FC000 thermoplastic polyurethane was used for porous scaffold. The polymeric scaffold gyroid pores were filled with the Viscoll collagen gel (Imtek; Russia). We printed layer-by-layer, first with polylactide, then with polyurethane, ensuring that the two polymers were imprinted into each other. After creating the scaffold, a distinct nozzle was used to imprint the hydrogel composition into it. To prepare the collagen hydrogel, dilution and neutralization were performed at a temperature of +4 °C: first, the syringe containing the culture medium (DMEM, 10% fetal bovine serum, 100 mM Tris-HCl) was hermetically connected to the syringe containing the 4% type 1 collagen solution (Viscoll; Russia), in a ratio of 1 : 4. Then the collagen solution was mixed thoroughly with the culture medium to obtain the neutralized homogenous collagen solution.

Furthermore, two pairs of cylinders were prepared (diameter 10.5 mm, length 18 mm). “Cylinder 1” (collagen + autologous chondrocytes) was filled with the specially prepared collagen solution with the swine autologous cartilage tissue cells (chondrocytes, concentration of cells about 30 million per milliliter). “Cylinder 2” (collagen) was not filled; it represented a collagen backbone.

After manufacturing the bioengineered auricles and cylinders 1 and 2 these were implanted under the temporal fascia. Two surgical teams operated both animals simultaneously. The right temporal fascia was detached under total intravenous anesthesia; the auricular implant was installed in the muscle bed and wrapped in the temporal fascia. The left temporal fascia, under which “Cylinder 1” (collagen + autologous chondrocytes) was implanted, was detached in both animals in the same way; “Cylinder 2” (collagen) was implanted under the mobilized frontalis muscle. Thus, three distinct myofascial compartments with implanted bioengineered materials not connected to each other were formed in two animals (Fig. 2).

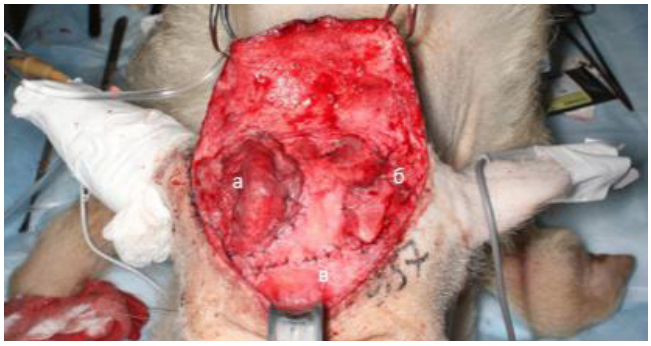

Fig. 2. Surgical wound after implantation of bioengineered ear, “Cylinder 1” and “Cylinder 2”. a — site of the bioengineered ear implantation in the region of the right temporal fascia; b — site of the “Cylinder 1” (collagen + autologous chondrocytes) implantation in the region of the left temporal fascia; c — site of the “Cylinder 12” (collagen) implantation in the region of frontalis muscle

The follow-up period was 3 months. Then both animals were humanely withdrawn from the experiment through intravenous euthanasia. The fragments of implants and disks were collected and fixed in the 10% neutral formalin solution in phosphate buffer. All the implanted materials were sent for histological examination.

RESULTS

After harvesting the implants, initial macroscopic assessment of their condition was performed. Both animals showed complete integration of grafts with their myofascial sheaths, along with no signs of tissue infection or necrosis (Fig. 3).

Almost the same pattern was revealed by further histological examination of bioengineered ears of both animals using the hematoxylin and eosin stain. A moderately dense, thickened area of muscle tissue with abundant fibrovascular connective tissue and blood vessels of varying diameter filled with blood is visible in both specimens. No inflammatory infiltrate has been revealed. The dense, acellular eosinophilic tissue (presumably collagen) adheres closely to the muscle tissue in a multifocal manner. The central part represents a labyrinthine structure of intertwined muscle fibers, among which large fields of eosinophilic fibrous tissue are visible. It should be noted that there is prominent vascularization increasing along the periphery of the central part in both specimens, which is indirect evidence of the graft integration with their myofascial sheaths. Multiple dense lymphoplasmacytic infiltrates showing predominance of plasmacytes are seen in the central part. No neutrophilic inflammation, including the rejection reaction, has been revealed.

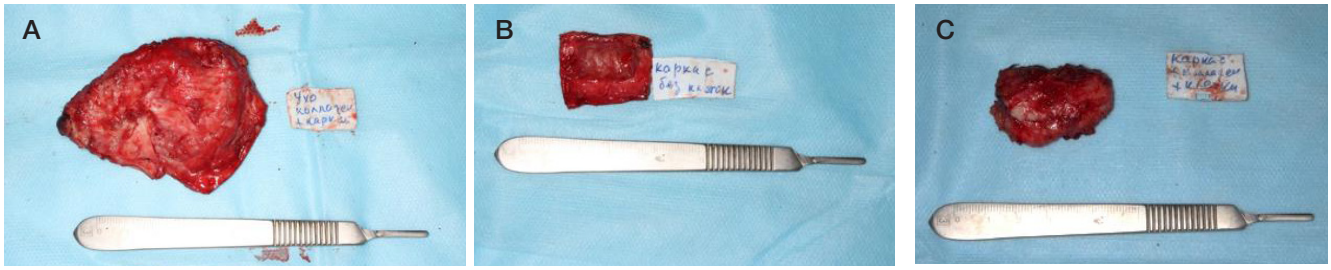

Fig. 3. Bioengineered ear **(A)**, “Cylinder 1” (collagen + autologous chondrocytes) **(B)** and “Cylinder 2” (collagen) **(C)** after harvesting, 3 months after implantation

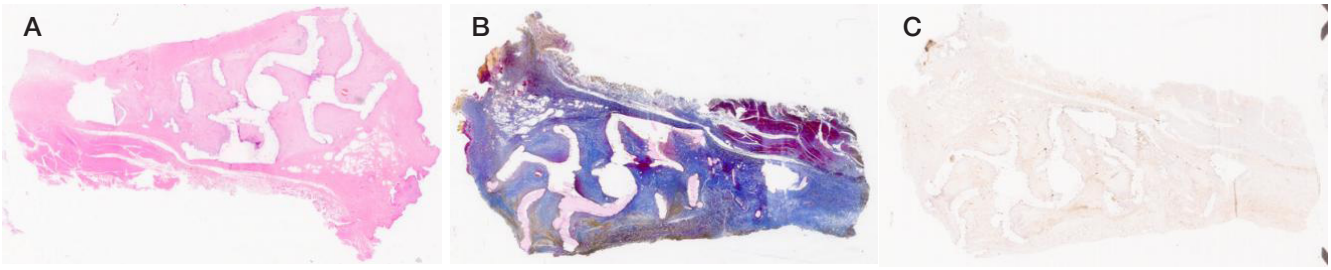

Fig. 4. Histological specimen of bioengineered ear, hematoxylin and eosin stain **(A)**; Mallory stain **(B)**; immunohistochemistry specimen **(C)**

The similar histological pattern is observed when applying Mallory stain, when large parts of specimens become deep blue, which, according to the staining method, corresponds to collagen fibers. In certain areas, collagen adheres closely to the muscle fibers (brown stain). Immunohistochemistry testing for markers of neoangiogenesis (VEGF and CD31) has revealed weak expression in both specimens (Fig. 4).

Histological examination of the "Cylinder 1" specimen supplemented with type 1 collagen-based hydrogel containing autologous chondrocytes has shown that the specimens consist primarily of loose fibrous tissue with the maze-like voids. In some places there are narrow fields of fibrosis and small roundish fields of acellular eosinophilic tissue. The fibrous tissue is moderately vascularized, with numerous large, slit-like blood vessels filled with blood. Multifocal inflammatory lymphoplasmacytic infiltrates have been revealed, along with the development of lymphoid follicles consisting mainly of small-sized lymphocytes. It is noteworthy that a narrow layer of mature cartilage tissue can be seen between the muscles. When applying Mallory stain, no more than 75% of both specimens become deep blue. The color intensity suggests that the collagen content is higher on the periphery. In the center light blue, red, and blue colors can be seen, which suggests the presence of collagen fibers between the muscle bundles, on the periphery of specimen, outside the implant area. Immunohistochemistry testing for expression of VEGF and CD31 markers has revealed strong expression in the vascular endothelium (Fig. 5).

Histological examination of the "Cylinder 1" stained with hematoxylin and eosin has revealed predominance of the tissue fragments consisting entirely of the moderately dense, fibrous tissue and containing the maze-like voids. The fibrous tissue and scanty vascularization are visible in the middle part of these tissues, along the periphery. Dense lymphoplasmacytic sheaths can be seen around the blood vessels. In the central part, there are multiple dense mixed-cell infiltrates showing predominance of plasmacytes and the presence of sporadic lymphocytes, even smaller neutrophil counts and varying siderophage counts. When applying Mallory stain, large parts of both specimens acquire blue color of varying intensity, depending on the field of view, which suggests high predominance of collagen fibers. Immunohistochemistry has revealed weak expression of the neoangiogenesis markers (VEGF and CD31) (Fig. 6).

Comparative characteristics of the histological specimens of bioengineered ear, "Cylinder 1" and "Cylinder 2" obtained from both animals are provided in Table 1.

The analysis of data provided in Table 1 suggests that the construct manufactured contributed to the development of the regenerated connective tissue having its own vasculature, while the structure of the regenerated tissue fiber backbone was adjusted to the construct shape, which ensured the joint response to the exposure to external mechanical forces. There were no signs of scaffold rejection. Filling the scaffold with cells and collagen affected the regenerated tissue structure; the regenerated tissue filled the entire implant in all cases.

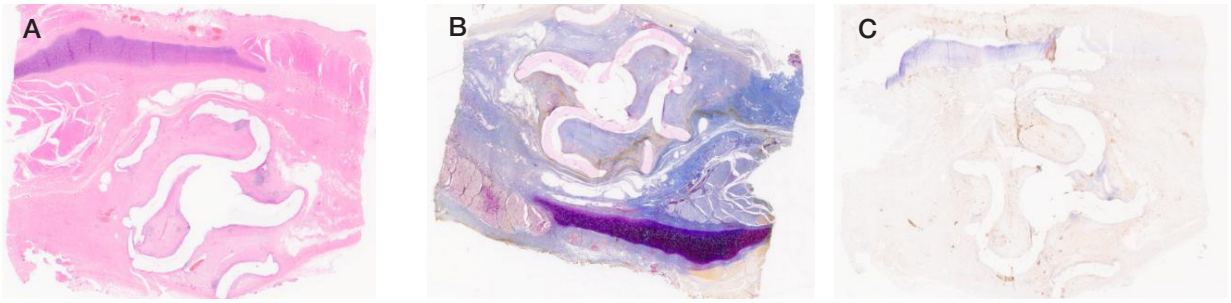

Fig. 5. Histological specimen of "Cylinder 1" based on type 1 collagen with autologous chondrocytes: hematoxylin and eosin stain **(A)**; Mallory stain **(B)**; immunohistochemistry specimen **(C)**

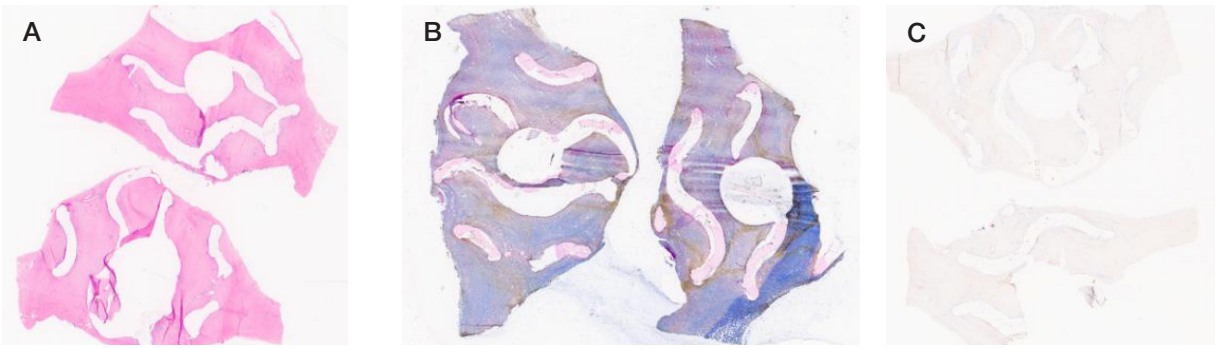

Fig. 6. Histological specimen of "Cylinder 2": hematoxylin and eosin stain **(A)**; Mallory stain **(B)**; immunohistochemistry specimen **(C)**

Table. Comparative characteristics of histological specimens obtained from both animals

	Animal 1			Animal 2		
	Auricle	Cylinder 1 (collagen + chondrocytes)	Cylinder 2 (polylactide)	Auricle	Cylinder 1 (collagen + chondrocytes)	Cylinder 2 (polylactide)
Vascularization	++–	++–	+– –	++–	++–	+– –
Integration and adhesion	+++	+– –	+– –	+++	+– –	+– –
Angiogenesis markers VEGF and CD31	+– –	+++	+– –	+– –	+++	+– –
Signs of inflammation and rejection	– – –	+– –	+– –	---	+– – –	+– –

Note: +++– — parameter is strongly expressed; ++– – — parameter is moderately expressed; +– – — parameter is weakly expressed; – – – — parameter is not expressed.

DISCUSSION

The volumetric product representing a combined tissue-engineered scaffold in the shape of the auricle was designed based on the digital 3D model of human auricle. The scaffold was effectively reproduced by polymer 3D printing and used in the experiment involving minipigs. The product unique nature and dimensionality result from the features of the arrangement of polylactide stiffeners following the contours of the auricular tragus, antitragus and helix, as well as from the shape and arrangement of polyurethane gyroid meshes. Imprinting of mesh threads into polylactide has made it possible to avoid sagging of the meshes and obtain the mechanically strong polymeric scaffold suitable for implantation into a living organism. The interconnected open spaces are located between the threads of gyroid meshes. These open spaces can be artificially filled with the collagen-based hydrogel, hydrogel with living cells; the open spaces turned out to be accessible for natural spontaneous ingrowth of regenerated surrounding tissues after the scaffold implantation under the temporal fascia of the living body.

The use of such technologies has made it possible to solve a series of technical problems reported in many foreign studies, which are related to the contour path control, mechanical strength, and stability of the bioengineered endoprosthesis shape [21].

Scaffold implantation under the temporal fascia of the model animal initiated the development of the regenerated connective tissue growing from the connective tissue anatomical structures (primarily temporal fascia) surrounding the implant. The regenerated tissue inside the implant had its own vasculature ensuring viability of cells and preservation of biological structures throughout the entire volume of the auricular scaffold.

The scaffold structure determined the possibility of growth and the features of the regenerative and adaptive remodeling of live tissues and blood vessels filling the scaffold. Successful integration of the non-absorbable scaffold obtained into the living organism results largely from mechanical characteristics of the implant (the scaffold functions as the inner “skeleton” of the ear) matching the characteristics of the live tissues it is attached to. The findings suggest the effectiveness of combining stiffeners with gyroid meshes. It has been found that the structure of the regenerated tissue fibrous backbone adapts to the construct shape, thereby ensuring the joint response of the tissue-engineered construct that makes it possible to preserve integrity of the live tissues and scaffold when exposed to external mechanical forces.

It has been found that filling the scaffold with cells and collagen affects the regenerated tissue structure. This can be taken into account when further improving the tissue-engineered construct of the auricle and developing new methods for implantation of the construct and auricular reconstruction. The regenerated tissue filled the entire interpolymeric volume of the scaffold in all cases, and ensuring filling the construct with the elastic cartilage may constitute the goal of further research.

CONCLUSIONS

The *in vivo* studies involving biological models have shown that the scaffold shaped like a full-size auricle is superior to the control samples due to ensuring better integration and interplay with live tissues, no inflammation, and vascularization that is sufficient for survival of tissues located within the tissue-engineered construct. It is necessary to further test the auricular implant using biological models. The implant manufacturing method developed is promising and can form the basis for developing domestic porous ear implants based on biocompatible polymeric materials, hydrogels, and autologous cellular material.

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