DOI: 10.15825/1995-1191-2021-4-119-131

BIOACTIVE COATING FOR TISSUE-ENGINEERED SMALL-DIAMETER VASCULAR GRAFTS

V.A. Surguchenko[1], E.A. Nemets[1], V.Yu. Belov[1, 2], V.I. Sevastianov[1]

[1] Shumakov National Medical Research Center of Transplantology and Artificial Organs, Moscow, Russian Federation

[2] Institute of Biomedical Research and Technology, Moscow, Russian Federation

Objective: to develop a method for modifying composite small-diameter porous tubular biopolymer scaffolds based on bacterial copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and gelatin modified with a double-layered bioactive coating based on heparin (Hp) and platelet lysate (PL) that promote adhesion and proliferation of cell cultures. **Materials and methods.** Composite porous tubular biopolymer scaffolds with 4 mm internal diameter were made by electrospinning from a 1 : 2 (by volume) mixture of a 10% solution of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer, commonly known as PHBV, and a 10% solution of gelatin, respectively, in hexafluoro-2-propanol. The structure of the scaffolds was stabilized with glutaraldehyde vapor. The scaffolds were modified with a bioactive Hp + PL-based coating. The surface morphology of the samples was analyzed using scanning electron microscopy. Biological safety of the modified scaffolds *in vitro* (hemolysis, cytotoxicity) was evaluated based on the GOST ISO 10993 standard. Interaction with cultures of human endothelial cell line (EA.hy926) and human adipose-derived mesenchymal stem cells (hADMSCs) was studied using vital dyes. **Results.** We developed a method for modifying small-diameter composite porous tubular biopolymer scaffolds obtained by electrospinning from a mixture of PHBV and gelatin modified with double-layered bioactive coating based on covalently immobilized Hp and human PL. The modified scaffold was shown to have no cytotoxicity and hemolytic activity *in vitro*. It was also demonstrated that the developed coating promotes hADMSC adhesion and proliferation on the external surface and EA.hy926 on the internal surface of the composite porous tubular biopolymer scaffolds *in vitro*. **Conclusion.** The developed coating can be used for the formation of *in vivo* tissue-engineered small-diameter vascular grafts.

Keywords: heparin, platelet lysate, biopolymer matrix, gelatin, poly(3-hydroxybutyrate-co-3-hydroxyvalerate), electrospinning, small-diameter vascular grafts, biological safety.

INTRODUCTION

Minimization of thrombosis and intimal hyperplasia processes is one of the key tasks for successful development and application of small-diameter vascular grafts (sdVGs) [1–3]. Endothelialization is considered the most preferable option for optimization of the internal surface of vascular grafts [4–6]. The monolayer of functionally active endothelium, similar to that present in native blood vessels, increases thromboresistance and long-term patency of the prosthesis due to ability of endothelial cells to synthesize a wide range of bioactive factors preventing platelet aggregation, regulating excessive proliferation, migration and contractile activity of smooth muscle cells, homeostasis, and inflammation [7–9].

In order to increase the specificity of interaction with endothelial cells (ECs), endothelial progenitor cells (EPCs) and other cell types responsible for blood vessel regeneration, as well as to achieve rapid endothelization, various approaches for modifying sdVGs surface are used [10–15]. A widely used modification method is the use of coatings based on extracellular matrix proteins, such as collagen, its partially hydrolyzed form gelatin, elastin, fibronectin [12, 14, 16] or immobilization on the surface of peptides simulating adhesion sites. The best known of the family of such synthesized compounds is RGD peptide (Arg-Gly-Asp), which repeats the adhesive fragment of the fibronectin molecule [8, 17, 18]. Incorporation of bioactive compounds, such as antibodies or growth factors, into the structure of sdVGs can help to attract EPCs and mature ECs in situ. CD34 and vascular endothelial growth factor receptor 2 (VEGFR-2, CD309) are present on the surface of circulating EPCs. CD31 and VEGFR-2 antibodies are used to bind ECs [11, 18]. Factors such as stromal cell-derived factor-1 (SDF-1) and granulocyte colony-stimulating factor (G-CSF) have been found to enhance EPC mobilization from bone marrow [11, 18–21]. Basic fibroblast growth factor (bFGF)

Corresponding author: Valentina Surguchenko. Address: 1, Shchukinskaya str., Moscow, 123182, Russian Federation. Phone: (499) 196-26-61. E-mail: valent.egorova@gmail.com

can stimulate resting ECs, causing their proliferation and organization into tubular structures [11, 22].

The most frequently used vascular endothelial growth factor (VEGF) is a cytokine that is highly specific to ECs and EPCs, activating and supporting their migration and proliferation [22–24]. One of the significant disadvantages of using growth factors, particularly VEGF, is the rapid loss of biological activity and high cost [22]. Immobilization of growth factors such as bFGF, transforming growth factor beta 2 (TGF-β2) or VEGF through the heparin-binding domain has been shown to increase resistance to denaturation and enzymatic cleavage under physiological conditions, prolonging their bioactivity [18, 25].

Heparin (HP), a glycosaminoglycan with well-studied and characterized anticoagulant properties, is also often used to functionalize biomaterials and matrices intended for contact with blood in order to increase their thromboresistance [26]. HP also plays an important role in endothelial cell adhesion and homeostasis, it improves attachment to the matrix, providing trophic and differentiation cell signaling, while inhibiting the proliferative activity of smooth muscle cells [1]. Joint immobilization of heparin and angiogenic growth factors can simultaneously suppress thrombosis and stimulate endothelization [2] At the same time, platelet lysate (PL) obtained from platelet-rich plasma (PRP) can serve as a promising, accessible, and inexpensive source of growth factors (bFGF, TGF-β2, VEGF) [27–30]. PL can become an alternative to the widely used recombinant growth factors, which lead to a significant increase in the cost of the resulting products and can also provoke immunogenic reactions [29].

We have previously developed composite small-diameter porous tubular biopolymer scaffolds based on bacterial copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) and gelatin with adjustable resorption rate, possessing the necessary complex of physical and mechanical properties characteristic of native small-diameter blood vessels [31]. However, stabilization of the scaffold structure using glutaraldehyde vapor resulted in increased cytotoxicity. Taking into account both the properties of HP and the rich set of growth factors, including VEGF and bFGF contained in PL, we made an assumption that biofunctionalization of the surface of the tubular scaffolds we developed earlier by immobilizing HP in combination with a PL-based bioactive coating may help to eliminate cytotoxicity and impart adhesion to the scaffold both high hemocompatibility, including thromboresistance, and specific affinity for endothelial cell.

The aim of this work was to develop a method for modifying PHBV- and gelatin-based small-diameter composite porous tubular biopolymer scaffolds with a two-layer bioactive heparin and platelet lysate coating that promotes surface endothelization.

MATERIALS AND METHODS

Fabrication of composite porous tubular biopolymer scaffolds

Composite porous tubular biopolymer scaffolds (CPTB scaffolds) with 4 mm internal diameter were made by electrospinning from a 1:2 (by volume) mixture of 10% PHBV copolymer solution (PHBV, Sigma-Aldrich, USA) and a 10% acidic gelatin solution (Gelatin from porcine skin Type A, Sigma-Aldrich, USA) respectively in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, P&M-Invest, Russia) on electrospinning machine NANON-01A (MECC CO, Japan) at 25 kV voltage between electrodes, 2 mL/h solution feed rate, 100 mm distance to the collector, 1000 rpm rotation speed of the substrate rod, using a 27G needle. After the end of the solution application process, the sample scaffolds were dried in the thermostat at 37 °C for 2 hours followed by vacuuming at 10–20 mmHg residual pressure and 37 °C temperature for 24 hours.

Stabilization of CPTB scaffold structure

The structure of CPTB scaffolds was stabilized with glutaraldehyde (GA) vapor in a closed container without direct contact of the samples with 25% GA solution at room temperature for 48 hours. After the fixation process, the samples were washed thoroughly in three changes of distilled water and dried for 24 hours at room temperature.

Application of the bioactive coating

Immobilization of heparin (HP) (Sigma-Aldrich, USA) was performed by incubating the CPTB scaffold samples in an aqueous anticoagulant solution with 1 mg/ml (150–200 units/ml) concentration for 2 hours at room temperature, resulting in covalent binding of HP amino groups to unreacted GA aldehyde groups. The unbound HP was removed by washing three times in distilled water. The resulting heparinized scaffolds were sterilized by gamma-irradiation at 1.5 MiRad dose.

The required volume of human platelet lysate solution (hPL, Renam, Russia) was obtained by diluting dry (lyophilized) hPL in a 1:9 ratio with Hanks' solution containing no Ca^{2+} and Mg^{2+} ions (HBSS, Gibco® by Life Technologies™, UK). The lysate solution was sterilized by filtration through a 0.22 μm membrane filter, 0.22 μm

pore size. Sterile heparinized samples were treated with hPL solution under aseptic conditions for 1 hour at 37 °C immediately before the experiment. Binding of positively charged growth factors contained in hPL to negatively charged HP was polyionic.

Surface morphology of CPTB scaffolds

The surface structure of the modified CPTB scaffold samples was analyzed using a JSM-6360LA scanning electron microscope (SEM) (JEOL, Japan) at 5 kV accelerating voltage and 100×–1000× magnification. Gold was sputtered to create a conductive coating.

Cell cultures

Cultures of mouse embryonic fibroblast cell line NIH/3T3 cells (ATCC®CRL-1658™) and human umbilical vein endothelial cell line EA.hy926 ATCC®CRL-2922™) from the ATCC (American Type Culture Collection) were stored in liquid nitrogen at –196 °C before use. After thawing, NIH/3T3 fibroblasts and EA.hy926 ECs were seeded into 25 cm^2 or 75 cm^2 standard culture vials (CELLSTAR® Greiner Bio-One, Germany) and cultured in appropriate complete growth medium DMEM, high glucose (4.5 g/L, DMEM high glucose with HEPES, PanEco, Russia) supplemented with 10% calf serum (CS, Biosera, Germany) or fetal calf serum (FCS, HyClone, USA), respectively, antibiotic and antimycotic Anti-Anti (Gibco® by Life Technologies™, UK) and 2 mM alanyl-glutamine (PanEco, Russia) in a CO_2 incubator under standard conditions: 37 °C, in a humid atmosphere containing (5 ± 1) % CO_2. Before the experiment, cells were removed from the surface of the culture plate using TrypLE™ Express Enzyme dissociation reagent (Gibco® by Life Technologies™, UK) and a suspension with the required concentration of cells was prepared.

A culture of human adipose-derived mesenchymal stem cells (hADMSCs) was obtained at the department of biomedical technologies and tissue engineering, Shumakov National Medical Research Center of Transplantology and Artificial Organs, Moscow according to the previously developed technique [32]. Prior to use, MSCs were stored in liquid nitrogen at –196 °C. After thawing, the hADMSCs were seeded into 75 cm^2 standard culture vials (CELLSTAR® Greiner Bio-One, Germany) and cultured in DMEM/F12 complete growth medium (CGM) (PanEco, Russia) with 10% fetal calf serum added (FCS, HyClone, USA), 10 μg/ml human basic fibroblast growth factor (FGF-2, Peprotech, AF-100-18B, USA), antibiotic and antimycotic Anti-Anti (Gibco® by Life Technologies™, UK), 1 mM HEPES (Gibco® by Life Technologies™, UK) and 2 mM alanyl-glutamine (PanEco, Russia) in CO_2 incubator under standard conditions: 37 °C, in a humid atmosphere containing (5 ± 1) % CO_2. Before the experiment, cells were removed from the surface of the culture plate using TrypLE™ Express Enzyme dissociating reagent (Gibco® by Life Technologies™, UK) and a suspension with the required concentration of cells was prepared. Cells of passages V–VI were used in the experiments. Various authors have shown that hADMSCs are used as an independent cellular component in the development of tissue-engineered small-diameter vascular grafts [33, 34]; also, hADMSCs maintain viability of ECs and promote vascularization of tissue-engineered small-diameter vascular constructs in vivo [35], which was the reason for using this cell culture in our work.

The initial number of cells in the suspension was determined on an automated cell counter (TC20™ Automated Cell Counter, BIORAD, Singapore) with simultaneous viability analysis by trypan blue dye exclusion (BIORAD, #145-0013, Singapore).

Hemolysis

The hemolytic action of the modified CPTB scaffolds was investigated on extracts from samples using rabbit red blood cell mass according to interstate standard GOST ISO 10993-4-2011 [36]. The extracts were prepared according to GOST ISO 10993-1-2011 guidelines [37]. A 0.9% sodium chloride solution was used as the extractant (model medium), the extraction time was 72 hours at 37 °C. The negative control sample was 0.9% sodium chloride solution, the positive control sample causing 100% hemolysis was distilled water. Blood from three rabbits was used to evaluate the hemolytic effect of one sample.

Blood containing 3.8% sodium citrate (1:9 ratio) obtained from chinchilla rabbits in compliance with the bioethical principles of animal handling approved by the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (2005) and in accordance with the Rules of Laboratory Practice, approved by Order #708 of the Russian Ministry of Health on 23 August 2010 on experimental animals, centrifuged at 900 rpm for 10 minutes to obtain erythrocytic mass. A 10% suspension of erythrocytes, obtained by dilution of the washed red blood cell mass three times (1:9 with 0.9% sodium chloride solution), was added to the test extracts from the modified CPTB scaffold samples, as well as to the negative control sample and positive control sample and incubated for 1 hour at (37 ± 2) °C, then centrifuged for 20 minutes at 2000 rpm.

The supernatant was separated, and the optical density was measured using a Stat Fax 4500 spectrophotometer (Awareness Technology, USA) at 540 nm wavelength. The percentage of hemolysis was calculated using formula (1):

$$\frac{E_{op} - E_c}{E_{100}} \times 100, \quad (1)$$

where E_{op} is the optical density of the test sample, E_c is the optical density of negative control sample, E_{100} is the optical density of the sample with 100% hemolysis.

Hemolysis in all blood samples should be less than 2%.

Cytotoxicity

The cytotoxicity of the fragments of modified CPTB in vitro was assessed according to the GOST ISO 10993-5-2011 interstate standard on NIH/3T3 mouse fibroblast cell line by direct contact method [38]. The surface of a culture plate (CP, CELLSTAR® Greiner Bio-One, Germany) served as a negative control sample, while a single-element aqueous zinc standard 10 mg/ml (Sigma-Aldrich, USA) served as the positive control sample. All procedures were performed under aseptic conditions.

To study the cytotoxic effect, fibroblasts were seeded in 24-well flat-bottom culture plates (CELLSTAR® Greiner Bio-One, Germany) at a concentration of 7–12 × 10^4 cells per well and incubated for 24 hours at 37 °C in a humid atmosphere containing (5 ± 1)% CO_2, until the formation of (80 ± 10)% monolayer, after which the studied fragments of matrices were placed directly on the fibroblast monolayer surface. The culture was visually assessed using a binocular fluorescence inverted microscope Nikon Eclipse TS100 (Japan).

The metabolic activity of fibroblasts after contact with matrix fragments was assessed after 24 hours using PrestoBlue™ HS Cell Viability Reagent (Invitrogen™ by Thermo Fisher Scientific, USA) according to the protocol recommended by the manufacturer. 10% PrestoBlue™ Viability Reagent was added to the wells containing the test samples, negative control and positive control samples, after which the plate was incubated for 3 hours at 37 °C in a humid atmosphere containing (5 ± 1) % CO_2. PrestoBlue™ is a vital dye containing redox indicator resazurin. Viable proliferating cells reduce resazurin with the participation of mitochondrial dehydrogenases, cytochromes and dehydrogenases located in the cell cytoplasm to resafurin [39], resulting in a color change from indigo to pink. Changes in medium absorbance were recorded using a Spark 10M microplate reader (Tecan, Austria) with Spark Control™ Magellan V1.2.20 software at 570 nm and 600 nm wavelengths. The percentage of reduced PrestoBlue™ characterizes the metabolic activity of the cells. The relative percentage difference between the metabolic activity of cells in the negative control sample and after contact with the test samples characterizes the cytotoxicity of the test sample and is calculated by formula (2):

$$\frac{117.216 \times A_{570\ Samp.} - 80.586 \times A_{600\ Samp.}}{117.216 \times A^0_{570} - 80.586 \times A^0_{600}} \times 100\%, \quad (2)$$

where 117.216 and 80.586 are molar extinction coefficients for the oxidized form of PrestoBlue™ Vital Reagent at 600 nm and 570 nm wavelengths, respectively; $A_{570\ Samp.}$ and $A_{600\ Samp.}$ are absorbance of the test sample at 570 nm and 600 nm wavelengths, respectively; A^0_{570} and A^0_{600} are absorbance of the negative control sample at 570 nm and 600 nm wavelengths, respectively.

The results were analyzed using a grading scale of the degree of cell response after direct contact with the samples according to GOST ISO 10993-5-2011 (Table 1).

Table 1

Cell response rate

Cytotoxicity grading scale	Interpretation of result
0	No cytotoxicity
1	Mild cytotoxicity
2	Moderate cytotoxicity
3	Severe cytotoxicity

The negative control sample should correspond to response grade 0 (no cytotoxicity), while the positive control sample should correspond to response grade 3 (severe cytotoxicity, almost completely destroyed monolayer). The degree of response of the test specimen should correspond to response grade 0.

Cultivation of EA.hy926 and hADMSCs on the surface of CPTB scaffolds

Sterile samples of modified CPTB scaffolds were precut lengthwise, straightened out, placed on the bottom of a flat-bottom 24-well culture plate (CELLSTAR® Greiner Bio-One, Germany) with the appropriate side (inner or outer), and fixed with sterile silicone rings. EA.hy926 and hADMSCs were seeded under aseptic conditions on the inner and outer sides of the samples, respectively. The initial seeding density on the test samples and controls was 4×10^4 kL/cm^2 for EA.hy926 culture and 3×10^4 kL/cm^2 for hADMSCs culture. Since the test samples were obtained by electrospinning and are three-dimensional, it would be incorrect to use a two-dimensional CP surface as a control sample. In these experiments, the CP surface was used to control the morphology and adequate growth of the cell cultures under study. After seeding, 24-well

plates with the samples were cultured in a CO_2 incubator under standard conditions for 24 hours and 96 hours or 24 hours, 96 hours, and 168 hours, after which appropriate studies were performed.

Viability assessment

The Live/Dead® Viability/Cytotoxicity Kit (Molecular Probes® by Life Technologies™, USA) was used to assess the viability and visualize the number of EA.hy926 and hADMSCs on the surface of modified CPTB scaffolds according to the protocol recommended by the manufacturer. The method consists of double immunofluorescence staining, with simultaneous determination of live and dead cells by binding dyes: ethidium bromide homodimer (EthD-1) and calcein acetoxymethyl (calcein AM). EthD-1 is a marker of dead cells, penetrating the damaged plasma membrane and binding to nucleic acids, giving a bright red fluorescent glow (excitation/emission ~495 nm/~635 nm). Calcein AM penetrating into viable cells is exposed to intracellular esterases, transforming into calcein, which gives a bright green homogeneous fluorescent glow (excitation/emission ~495 nm/~515 nm). Dulbecco's phosphate-buffered saline containing 2 μM calcein AM and 4 μM EthD-1 was added to the test wells, and after 15 minutes, the staining results were visualized using a Nikon Eclipse TS100 binocular inverted fluorescence microscope (Japan) equipped with a Digital Sight DS-Vil (Nikon, Japan). Photographs of the test and control samples during cultivation of EA.hy926 and hADMSCs were taken 24 hours and 96 hours after seeding.

Assessment of metabolic activity and cell count

The metabolic activity of EA.hy926 and hADMSCs on the surface of matrix samples was assessed after 24, 96 and 168 hours using PrestoBlue™ HS Cell Viability Reagent (Invitrogen™ by Thermo Fisher Scientific, USA) according to the protocol recommended by the manufacturer. 10% of PrestoBlue™ viability reagent was added to wells containing the test samples and a cell-free control sample (CGM containing no cells), after which the plate was incubated for hours at 37 °C in a humid atmosphere containing (5 ± 1) % CO_2. Changes in media absorbance were recorded using a microplate reader as previously, described at 570 nm and 600 nm

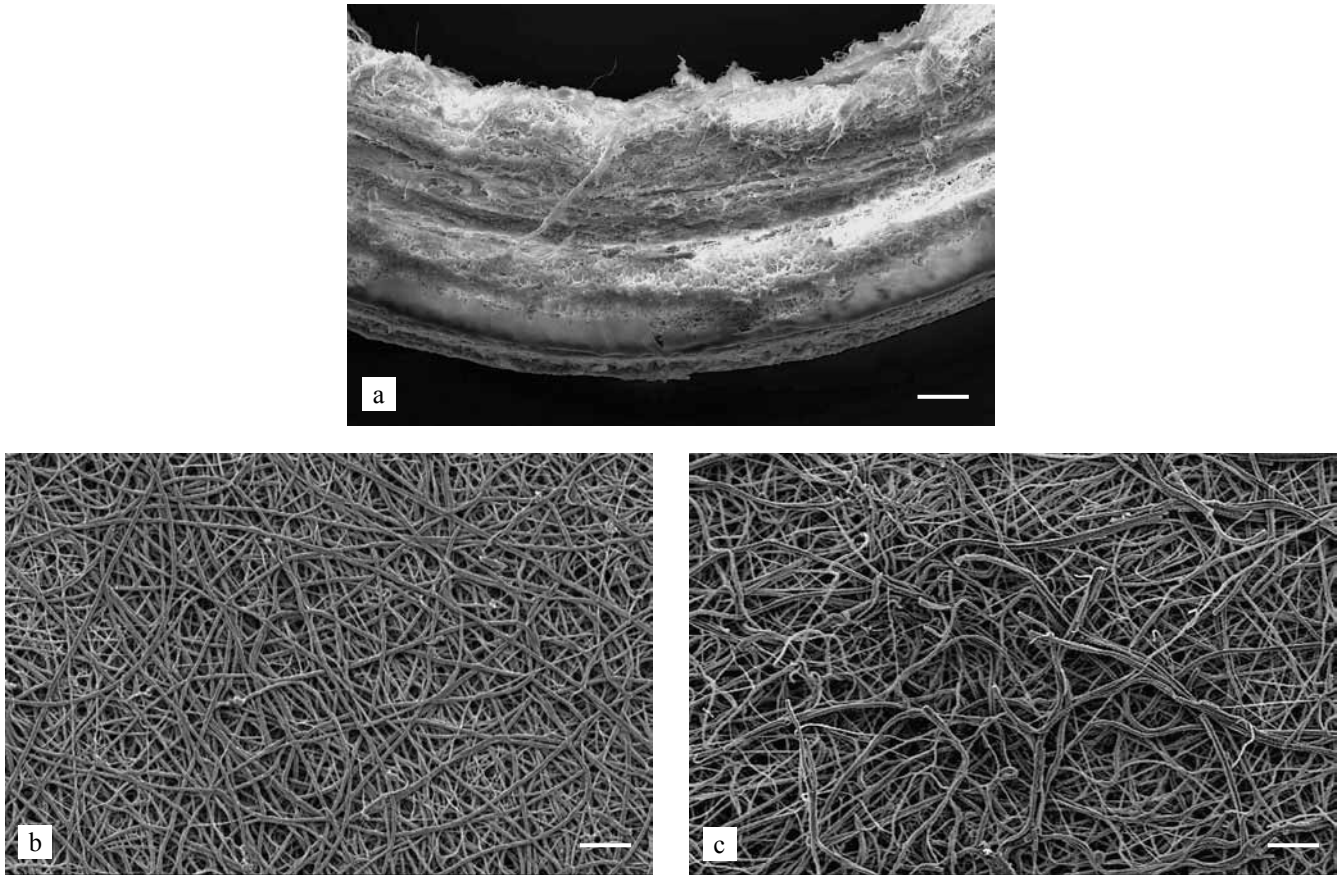

Fig. 1. Structure of CPTB scaffold modified with double-layered coating, d = 4 mm; a, cross-section (100× magnification, Bar 100 μm); b, inner surface (1000× magnification, Bar 10 μm); c, outer surface (1000× magnification, Bar 10 μm)

Table 2

Percentage of viable NIH/3T3 fibroblast cell line relative to a negative control sample after contact with the modified samples of CPTB scaffolds

Sample	% of viable cells relative to negative control sample	Cell response rate
Modified (HP)	95.55 ± 2.06	0
Modified (double-layer bioactive coating HP + hPL)	97.83 ± 1.67	0
Positive control sample	9.87 ± 0.73	3

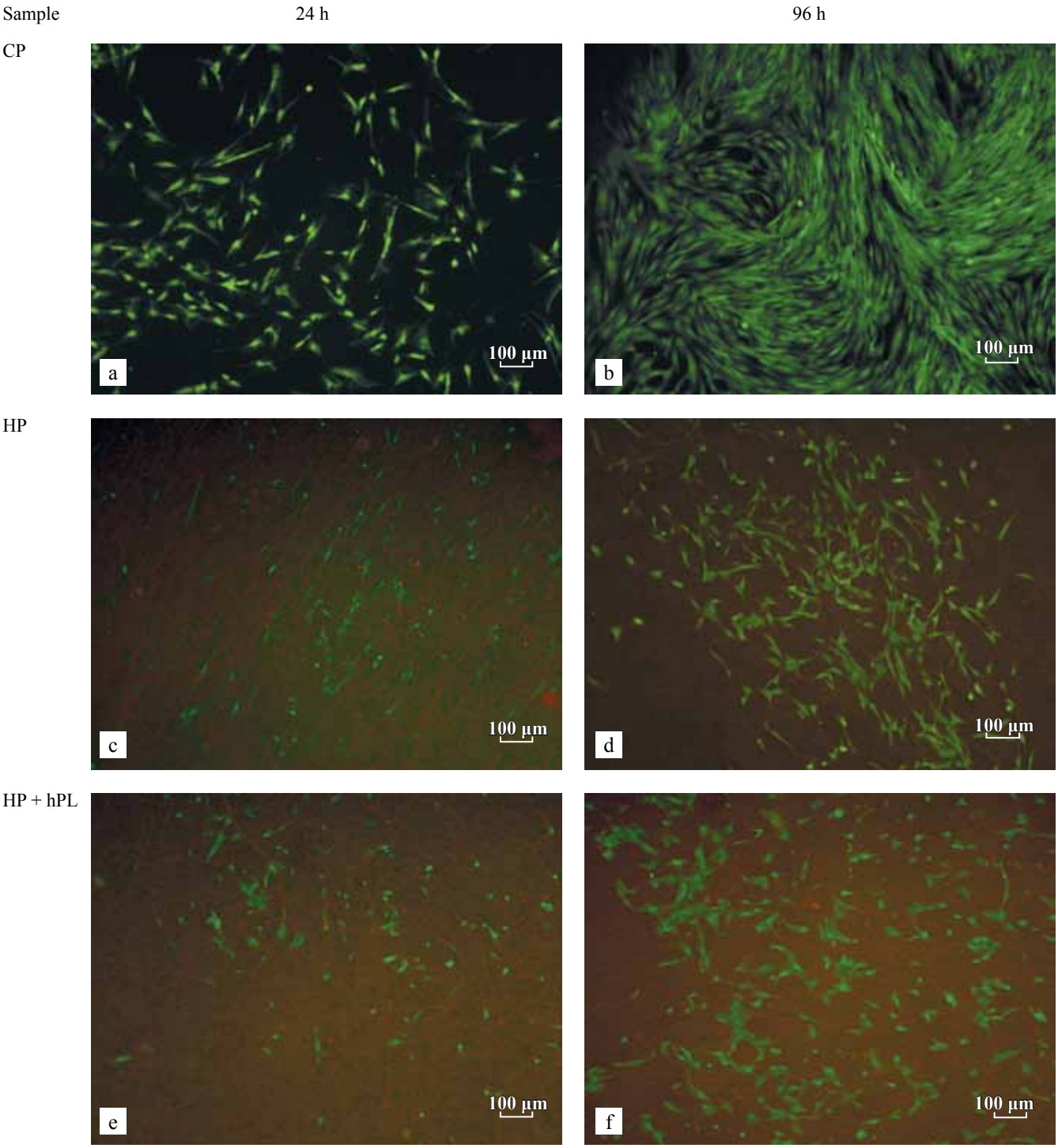

Fig. 2. hADMSCs culture (passage 6) on the outer surface of modified CPTB scaffolds; a, b, culture plate, 24 h and 96 h, respectively; c, d, scaffolds with modifying covalently immobilized HP coating, 24 h and 96 h, respectively; e, f, scaffolds with modifying human platelet lysate coating, 24 h and 96 h, respectively. Live/Dead® Viability/Cytotoxicity Kit staining. 10× magnification, Bar 100 µm

wavelengths. The percentage of reduced PrestoBlue™ characterizes the metabolic activity of cells and is calculated by formula (3):

$$\frac{117.216 \times A_{570\ Samp.} - 80.586 \times A_{600\ Samp.}}{155.677 \times A'_{600} - 14.652 \times A'_{570}} \times 100\%, \quad (3)$$

where 117.216 and 80.586 are molar extinction coefficients for the oxidized form of PrestoBlue™ Vital Reagent at 600 nm and 570 nm wavelengths, respectively; 155.677 and 14.652 are molar extinction coefficients for the reduced form of PrestoBlue™ Vital Reagent at 570 nm and 600 nm wavelengths, respectively; $A_{570\ Samp.}$ and $A_{600\ Samp.}$ are absorbance of the test sample at 570 nm and 600 nm wavelengths, respectively; A'_{570} and A'_{600} are absorbance of the cell-free control sample at 570 nm and 600 nm wavelengths, respectively.

The number of EA.hy926 and hADMSCs on the surface of scaffold samples was estimated using calibration curves, linear in semi-logarithmic coordinates to a 0.8×10^5 cell concentration in the case of EA.hy926 and 1×10^5 in the case of hADMSCs (VI passage). To construct a calibration curve, the selected cell type was seeded into flat-bottomed 24-well culture plates (CELLSTAR® Greiner Bio-One, Germany) at a seeding density of $1–20 \times 10^4$ kL/cm^2. After 24 hours, PrestoBlue™ vital reagent was added to the wells containing the required number of cells and a cell-free control sample, the plate was incubated for 3 hours at 37 °C in a humid atmosphere containing (5 ± 1) % CO_2 and changes in medium uptake were recorded. The percentage of reduced PrestoBlue™ determined by formula (3) was plotted on the graph on the Y axis, and the corresponding number of cells was plotted on the X axis.

Statistical processing

Quantitative and statistical processing of the obtained data was done using Microsoft Excel 2007. All results were presented as mean ± standard deviation. Differences were considered reliable at $p < 0.05$ with the number of samples (n) from 3 to 5.

RESULTS AND DISCUSSION

Fig. 1 shows the structure of the CPTB scaffold. The cross section (Fig. 1, a) shows a pronounced highly porous structure. The wall thickness is ~500 μm. The inner and outer layers of the scaffold (see Figs. 2, b, c) are similarly organized from disordered fibers with predominantly 0.8 to 2.0 μm thickness, 10–25 μm pore size.

Data from the performed hemolytic activity study allow us to conclude that the tested samples of modified bioactive HP and hPL coating for CPTB scaffolds have no hemolytic effect (hemolysis percentage 0.07%) and correspond to acceptable values (hemolysis percentage less than 2%), satisfying the requirements for medical products according to the GOST ISO 10993-4-2011 standard.

Table 2 shows the values characterizing the metabolic activity of the NIH-3T3 mouse fibroblasts after direct contact with samples of the modified CPTB scaffold relative to the negative (non-cytotoxic) control sample (CP). The sample has no cytotoxic effect if the metabolic activity of the fibroblasts relative to the negative control sample remains above 90%. As can be seen from Table 3, after cells come into contact with samples modified with HP or HP + hPL, the relative metabolic activity of fibroblasts is above 90%, indicating the absence of cytotoxic action. We have previously shown that PHBV- and gelatin-based CPTB scaffolds stabilized in GA vapor without additional treatment exhibit significant cytotoxicity (response rate 3) [31].

Fig. 2 shows photographs of hADMSC culture (passage VI) on the surface of CP and the outer surface of the modified CPTB scaffolds 24 and 96 hours after seeding.

After 24 hours, comparable numbers of adhered and melted hADMSCs were observed on the surface of all modified CPTB scaffolds. After 96 hours of cultivation, hADMSCs form a monolayer on the CP surface. On the test samples, the number of hADMSCs increases markedly, but does not reach the monolayer; all cells are viable and fused, which also confirms the previously obtained data that the modified samples have no cytotoxicity. Fig. 3 shows the results of hADMSCs proliferation on the outer surface of the modified CPTB scaffolds. After 24 hours, the number of hADMSCs on the outer surface of HP-modified and HP + hPL-coated samples differed only slightly ($11.8 \pm 0.6 \times 10^3$ kL and 15.1 ±

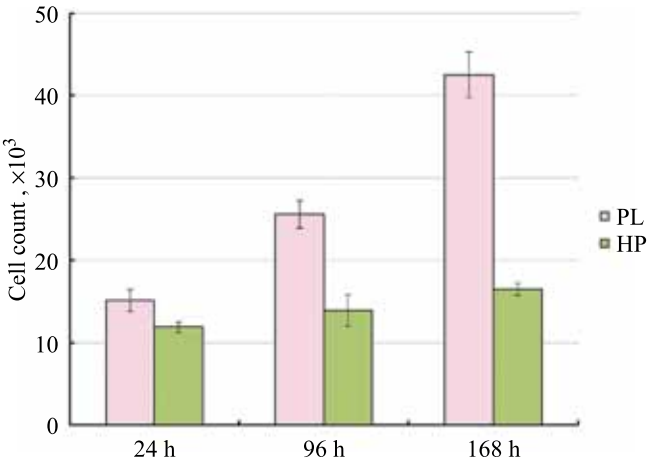

Fig. 3. hADMSCs proliferation (passage 6) on the outer surface of CPTB scaffolds modified with covalently immobilized heparin and heparin with human platelet lysate coatings

1.3×10^3 kL, respectively). By the end of the cultivation period, after 168 hours, the number of hADMSCs on the external surface of the test samples with the modifying HP + hPL-based coating increases 3-fold ($42.5 \pm 2.8 \times 10^3$ kL), while the number of hADMSCs on the external surface of heparinized matrices increases only 1.3-fold ($16.5 \pm 0.7 \times 10^3$ kL). Thus, the modifying platelet lysate-based coating promotes adhesion and proliferation of hADMSCs on the external surface of CPTB scaffolds. A modifying coating based on covalently immobilized HP alone in relation to the culture of hADMSCs promotes adhesion rather than proliferation of this cell type.

Figs. 4 and 5 show the results of the interaction of the modified inner surface of CPTB scaffolds with a

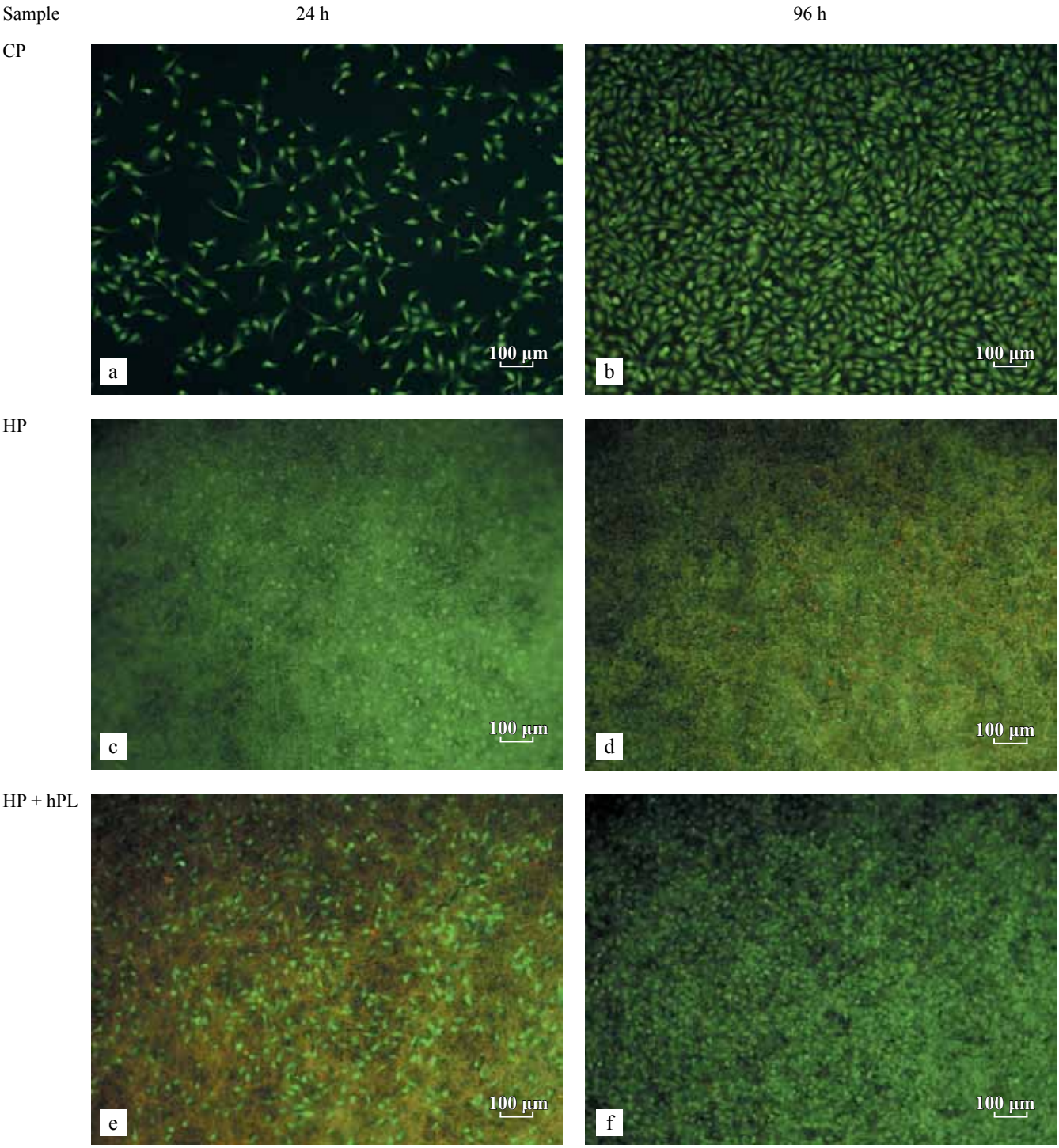

Fig. 4. EA.hy926 culture on the inner surface of modified CPTB scaffolds; a, b, culture plate, 24 h and 96 h, respectively; c, d, scaffolds with modifying covalently immobilized HP coating, 24 h and 96 h, respectively; e, f, scaffolds with modifying heparin and human platelet lysate coating, 24 h and 96 h, respectively. Live/Dead® Viability/Cytotoxicity Kit staining. 10× magnification, Bar 100 µm

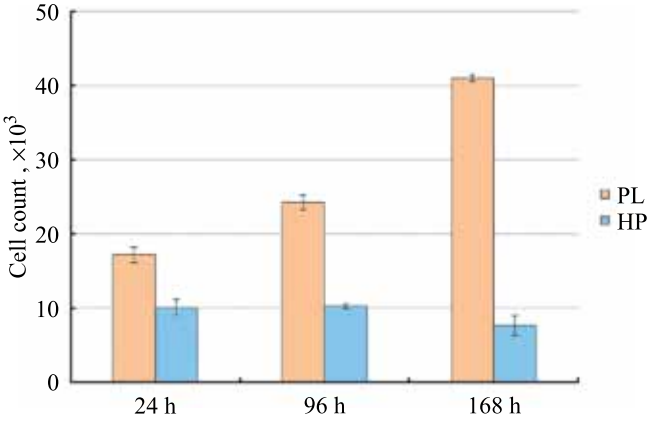

Fig. 5. EA.hy926 proliferation on the inner surface of modified CPTB scaffolds

culture of human endothelial cell line EA.hy926 24 and 96 hours after seeding. After 24 hours, a greater number of adherent and molten endothelial cells were observed on the surface of HP + hPL-modified scaffolds compared to samples modified with HP alone. After 96 hours of cultivation, no proliferation and a significant number of nonviable cells were observed in the HP-modified sample. At the same time, a monolayer of viable cells was formed on the samples modified with a two-layer coating. Note that a similar effect is observed for the CP. Fig. 5 shows that the modifying bioactive coating based on immobilized HP and hPL promotes adhesion, spreading and stimulates proliferation of EA.hy926 in vitro on the inner surface of CPTB scaffolds, due to a fairly high content of growth factors contained in platelet lysate, including VEGF: the number of ECs after 168 hours increases 2.4-fold. Our results are indirectly confirmed by data available in the literature. SM Oliveira et al. showed that PL-based nanocoating activates endothelial cells and promotes angiogenesis (microtubule formation) in the presence of sulfated polysaccharides in a three-dimensional matrix [29].

Probably, surface heparinization, under the conditions of this experiment, does not contribute to endothelialization in vitro. This, in our opinion, is due to the fact that although heparin is able to bind growth factors present in the serum component of the medium, their concentration is clearly insufficient to support endothelial cell proliferation. Despite the fact that HP plays an important role in endothelial cell adhesion and homeostasis [1], RJ Smith Jr et al. showed that when implanting cell-free prosthetic blood vessels modified with HP and with HP + VEGF into murine aorta, the samples although were completely endothelialized after 4 weeks of implantation, the composition of the prosthetic wall for samples with different coating differed significantly [40]. In the grafts modified only by HP, the endothelial and smooth muscle cell layers were not pronounced and were not formed typically for native vessels. But in the HP + VEGF-modified samples, there were clearly separated inner and middle layers, similar in structure to native vessels. and there was also a functioning endothelium. It means that modifying the prosthesis with HP alone is not sufficient to obtain an adequately functioning tissue-engineered graft.

CONCLUSION

So, for the surface of small-diameter CPTB scaffolds obtained by electrospinning from a PHBV and gelatin mixture, we developed a method of modification involving a two-layer bioactive coating based on covalently immobilized HP and hPL, whose components form polyionic complexes with HP. The modified CPTB scaffold samples were shown to have no cytotoxicity and hemolytic activity in vitro. It was also demonstrated that the developed bioactive coating promotes adhesion and proliferation of human adipose-derived mesenchymal stem cells on the outer surface of CPTB scaffolds and human umbilical vein endothelial cell on the inner surface of CPTB scaffolds in vitro.

The results obtained show that the developed coating can be used to form in vivo tissue-engineered constructs of small-diameter vascular grafts.

The authors declare no conflict of interest.

REFERENCES

1. *Matsuzaki Y, Ulziibayar A, Shoji T, Shinoka T.* Heparin-eluting tissue-engineered bioabsorbable vascular grafts. *Appl Sci.* 2021; 11: 4563–4575. https://doi.org/10.3390/app11104563.
2. *Henry JJD, Yu J, Wang A, Lee R, Fang J, Li S.* Engineering the mechanical and biological properties of nanofibrous vascular grafts for *in situ* vascular tissue engineering. *Biofabrication*. 2017; 9 (3): 035007. doi: 10.1088/1758-5090/aa834b. PMID: 28817384; PMCID: PMC5847368.
3. *Pashneh-Tala S, MacNeil S, Claeyssens F.* The Tissue-Engineered Vascular Graft-Past, Present, and Future. *Tissue Eng Part B Rev*. 2016; 22 (1): 68–100. doi: 10.1089/ten.teb.2015.0100. Epub 2015 Oct 8. PMID: 26447530; PMCID: PMC4753638.
4. *Ren X, Feng Y, Guo J, Wang H, Li Q, Yang J et al.* Surface modification and endothelialization of biomaterials as potential scaffolds for vascular tissue engineering applications. *Chem Soc Rev.* 2015; 44 (15): 5680–5742. doi: 10.1039/c4cs00483c. PMID: 26023741.
5. *Cai Q, Liao W, Xue F, Wang X, Zhou W, Li Y et al.* Selection of different endothelialization modes and different seed cells for tissue-engineered vascular graft.

Bioact Mater. 2021; 6 (8): 2557–2568. doi: 10.1016/j.bioactmat.2020.12.021. PMID: 33665496; PMCID: PMC7887299.

6. *Hasan A, Memic A, Annabi N, Hossain M, Paul A, Dokmeci MR et al.* Electrospun Scaffolds for Tissue Engineering of Vascular Grafts. *Acta Biomater.* 2014; 10 (1): 11–25. doi: 10.1016/j.actbio.2013.08.022.
7. *Smith RJ, Nasiri B, Kann J, Yergeau D, Bard JE, Swartz DD et al.* Endothelialization of arterial vascular grafts by circulating monocytes. *Nat Commun.* 2020; 11: 1622–1638. https://doi.org/10.1038/s41467-020-15361-2.
8. *Neufurth M, Wang X, Tolba E, Dorweiler B, Schröder HC, Link T et al.* Modular Small Diameter Vascular Grafts with Bioactive Functionalities. *PLoS One.* 2015; 10 (7): e0133632. doi: 10.1371/journal.pone.0133632. PMID: 26204529; PMCID: PMC4512703.
9. *Matsuzaki Y, John K, Shoji T, Shinoka T*. The evolution of tissue engineered vascular graft technologies: from preclinical trials to advancing patient care. Appl Sci (Basel). 2019; 9 (7): 1274–1295. doi: 10.3390/app9071274.
10. *Ardila DC, Liou JJ, Maestas D, Slepian MJ, Badowski M, Wagner WR et al.* Surface Modification of Electrospun Scaffolds for Endothelialization of Tissue-Engineered Vascular Grafts Using Human Cord Blood-Derived Endothelial Cells. *J Clin Med.* 2019; 8 (2): 185. doi: 10.3390/jcm8020185. PMID: 30720769; PMCID: PMC6416564.
11. *Melchiorri AJ, Hibino N, Fisher JP.* Strategies and techniques to enhance the *in situ* endothelialization of small-diameter biodegradable polymeric vascular grafts. *Tissue Eng Part B Rev.* 2013; 19 (4): 292–307. doi: 10.1089/ten.TEB.2012.0577. PMID: 23252992; PMCID: PMC3690089.
12. *Chen FM, Liu X.* Advancing biomaterials of human origin for tissue engineering. *Prog Polym Sci.* 2016 Feb 1; 53: 86–168. doi: 10.1016/j.progpolymsci.2015.02.004. PMID: 27022202; PMCID: PMC4808059.
13. *Mota C, Puppi D, Chiellini F, Chiellini E.* Additive manufacturing techniques for the production of tissue engineering constructs. *J Tissue Eng Regen Med.* 2015; 9 (3): 174–190. doi: 10.1002/term.1635. PMID: 23172792.
14. *Schmedlen RH, Elnjeirami WM, Gobin AS, West JL.* Tissue engineered vascular grafts. *Tissue engineering: principles and practices.* ed. by JP Fisher, AG Mikos, JD Bronzino, DR Peterson. 1st ed. USA: CRC Press Taylor & Francis Group; 2013: 1–9.
15. *Mallis P, Kostakis A, Stavropoulos-Giokas C, Michalopoulos E*. Future Perspectives in Small-Diameter Vascular Graft Engineering. *Bioengineering*. 2020; 7 (4): 160–200. https://doi.org/10.3390/bioengineering7040160.
16. *Han F, Jia X, Dai D, Yang X, Zhao J, Zhao Y et al.* Performance of a multilayered small-diameter vascular scaffold dual-loaded with VEGF and PDGF. *Biomaterials*. 2013; 34 (30): 7302–7313. doi: 10.1016/j.biomaterials.2013.06.006. PMID: 23830580.
17. *Kuwabara F, Narita Y, Yamawaki-Ogata A, Kanie K, Kato R, Satake M et al.* Novel small-caliber vascular grafts with trimeric peptide for acceleration of endothelialization. *Ann Thorac Surg.* 2012; 93 (1): 156–163; doi: 10.1016/j.athoracsur.2011.07.055. PMID: 22054652.
18. *Antonova LV, Silnikov VN, Khanova MYu, Koroleva LS, Serpokrilova IYu, Velikanova EA et al.* Adhesion, proliferation and viability of human umbilical vein endothelial cells cultured on the surface of biodegradable non-woven matrices modified with RGD peptides. *Russian Journal of Transplantology and Artificial Organs*. 2019; 21 (1): 142–152. (In Russ.). https://doi.org/10.15825/1995-1191-2018-1-96-109.
19. *Smith RJ, Koobatian MT, Shahini A, Swartz DD, Andreadis ST.* Capture of endothelial cells under flow using immobilized vascular endothelial growth factor. *Biomaterials*. 2015 May; 51: 303–312. doi: 10.1016/j.biomaterials.2015.02.025.
20. *Wang K, Chen X, Pan Y, Cui Y, Zhou X, Kong D et al.* Enhanced vascularization in hybrid PCL/gelatin fibrous scaffolds with sustained release of VEGF. *Biomed Res Int.* 2015; 2015: 865076. doi: 10.1155/2015/865076. PMID: 25883978; PMCID: PMC4390103.
21. *Lee KW, Johnson NR, Gao J, Wang Y.* Human progenitor cell recruitment via SDF-1α coacervate-laden PGS vascular grafts. *Biomaterials.* 2013; 34 (38): 9877–9885. doi: 10.1016/j.biomaterials.2013.08.082. PMID: 24060423; PMCID: PMC3882008.
22. *Antonova LV, Sevostyanova VV, Kutikhin AG, Velikanova EA, Matveeva VG et al.* Influence of bFGF, SDF-1α, or VEGF incorporated into tubular polymer scaffolds on the formation of small-diameter tissue-engineered blood vessel *in vivo*. *Russian Journal of Transplantology and Artificial Organs.* 2018; 20 (1): 96–109. (In Russ.). https://doi.org/10.15825/1995-1191-2018-1-96-109.
23. *Wan X, Li P, Jin X, Su F, Shen J, Yuan J.* Poly(ε-caprolactone)/keratin/heparin/VEGF biocomposite mats for vascular tissue engineering. *J Biomed Mater Res A.* 2020; 108 (2): 292–300. doi: 10.1002/jbm.a.36815. PMID: 31606923.
24. *Emechebe GA, Obiweluozor FO, Jeong IS, Park J-K, Park CH et al.* Merging 3D printing with electrospun biodegradable small-caliber vascular grafts immobilized with VEGF. *Nanomedicine: NBM* 2020; 30: 102306, https://doi.org/10.1016/j.nano.2020.102306.
25. *Spadaccio C, Nappi F, De Marco F, Sedati P, Sutherland FW, Chello M et al.* Preliminary *in vivo* evaluation of a hybrid armored vascular graft combining electrospinning and additive manufacturing techniques. *Drug Target Insights.* 2016; 10 (Suppl 1): 1–7. doi: 10.4137/DTI.S35202. PMID: 26949333; PMCID: PMC4772909.

26. *Wight TN.* Cell biology of arterial proteoglycans. *Arteriosclerosis.* 1989; 9 (1): 1–20. doi: 10.1161/01.atv.9.1.1. PMID: 2643420.
27. *Santos SCNDS, Sigurjonsson ÓE, Custódio CA, Mano JFCDL.* Blood plasma derivatives for tissue engineering and regenerative medicine therapies. *Tissue Eng Part B Rev.* 2018; 24 (6): 454–462. doi: 10.1089/ten.TEB.2018.0008. PMID: 29737237; PMCID: PMC6443031.
28. *Giusti I, D'Ascenzo S, Macchiarelli G, Dolo V. In vitro* evidence supporting applications of platelet derivatives in regenerative medicine. *Blood Transfus.* 2020; 18 (2): 117–129. doi: 10.2450/2019.0164-19. PMID: 31657710; PMCID: PMC7141937.
29. *Oliveira SM, Pirraco RP, Marques AP, Santo VE, Gomes ME, Reis RL et al.* Platelet lysate-based pro-angiogenic nanocoatings. *Acta Biomater.* 2016; 32: 129–137. doi: 10.1016/j.actbio.2015.12.028. PMID: 26708711.
30. *Shanskij YD, Sergeeva NS, Sviridova IK, Kirakozov MS, Kirsanova VA, Ahmedova SA et al.* Issledovanie lizata trombocitov cheloveka kak perspektivnoj rostovoj dobavki dlja kul'tivirovanija stvolovyh i drugih tipov kletok. *Kletochnye tehnologii v biologii i medicine.* 2013; 3: 153–158. (In Russ).
31. *Nemets EA, Belov VYu, Ilina TS, Surguchenko VA, Pankina AP, Sevastianov VI.* Composite porous tubular biopolymer matrix of small diameter. *Perspektivnye materialy.* 2018; 9: 49–59. (In Russ.). doi: 10.30791/1028-978X-2018-9-49-59.
32. *Egorova VA, Ponomareva AS, Bogdanova NB, Abramov VYu, Sevastianov VI.* Characterization of human adipose-derived stem cells phenotype by flow cytometry method. *Tehnologii zhivyh system.* 2009; 6 (5): 40–46. (In Russ.).
33. *Heydarkhan-Hagvall S, Schenke-Layland K, Yang JQ, Heydarkhan S, Xu Y, Zuk PA et al.* Human adipose stem cells: a potential cell source for cardiovascular tissue engineering. *Cells Tissues Organs.* 2008; 187 (4): 263–274. doi: 10.1159/000113407. PMID: 18196894.
34. *Zhang X, Simmons CA, Paul Santerre J.* Paracrine signalling from monocytes enables desirable extracellular matrix accumulation and temporally appropriate phenotype of vascular smooth muscle cell-like cells derived from adipose stromal cells. *Acta Biomater.* 2020; 103: 129–141. doi: 10.1016/j.actbio.2019.12.006. PMID: 31821896.
35. *Ciucurel EC, Sefton MV.* Del-1 overexpression in endothelial cells increases vascular density in tissue-engineered implants containing endothelial cells and adipose-derived mesenchymal stromal cells. *Tissue Eng Part A.* 2014; 20 (7–8): 1235–1252. doi: 10.1089/ten.TEA.2013.0242. PMID: 24151812; PMCID: PMC3993021.
36. GOST ISO 10993-4-2020 Izdelija medicinskie. Ocenka biologicheskogo dejstvija medicinskih izdelij. Chast' 4. Issledovanija izdelij, vzaimodejstvujushhih s krov'ju. Data vvedenija 2021-03-01. M.: Standartinform, 2020.
37. GOST ISO 10993-12-2011 Izdelija medicinskie. Ocenka biologicheskogo dejstvija medicinskih izdelij. Chast' 12. Prigotovlenie prob i kontrol'nye obrazcy. Data vvedenija 2013-01-01. M.: Standartinform, 2014.
38. GOST ISO 10993-5-2011 Izdelija medicinskie. Ocenka biologicheskogo dejstvija medicinskih izdelij. Chast' 5. Issledovanija na citotoksichnost': metody *in vitro*. Data vvedenija 2013-01-01. M.: Standartinform, 2014.
39. *Rampersad SN.* Multiple applications of alamar blue as an indicator of metabolic function and cellular health in cell viability bioassays. *Sensors.* 2012; 12 (9): 12347–12360. doi: 10.3390/s120912347.
40. *Smith RJ Jr, Yi T, Nasiri B, Breuer CK, Andreadis ST.* Implantation of VEGF-functionalized cell-free vascular grafts: regenerative and immunological response. *FASEB J.* 2019; 33 (4): 5089–5100. doi: 10.1096/fj.201801856R. PMID: 30629890; PMCID: PMC6436645.

The article was submitted to the journal on 8.10.2021