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BIOMIMETIC APPROACH TO THE DESIGN OF ARTIFICIAL SMALL-DIAMETER BLOOD VESSELS

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Objective: to create 2-mm diameter multilayer porous tubular scaffolds (PTS) with characteristics that resemble small-diameter native blood vessels in terms of characteristics. **Materials and methods.** PTS made of polycaprolactone (PCL, MM 80000) with a PCL-made sealing coat/layer with gelatin addition (PCL-gelatin) with a diameter of 2 mm were created by electrospinning (NANON-01A). Bioactive coating was applied to the PTS surface by sequential incubation in solutions of bovine serum albumin, heparin (Hp), and platelet lysate (PL). Cytotoxicity was investigated under conditions of direct contact of PTS with a monolayer of NIH/3T3 mouse fibroblasts. Viability of human umbilical vein endothelial cells (EA.hy926) was evaluated using Live/Dead® Viability/Cytotoxicity Kit. Permeability and blood flow parameters of the PTS implanted in the infrarenal section of the rat aorta were recorded using Doppler imaging. **Results.** A three-layer PTS construct with an inner diameter of 2 mm was developed. Its inner and outer layers were formed from 0.2 mL of PCL solution, and the middle sealing coat/layer was from 0.5 mL of PCL with addition of 30% (by weight of polymer) gelatin. Introduction of the sealing coat/layer reduced surgical porosity (SP) from 56.2 ± 8.7 mL/(cm^2·min) for a single-layer PTS made of pure PCL to 8.9 ± 2.6 mL/(cm^2·min) for a three-layer PTS. The resulting PTS demonstrated physicomechanical characteristics similar to those of native blood vessels; it also showed no cytotoxicity. Application of a bioactive coating of Hp and PL allowed for increased *in vitro* adhesion and proliferation of endothelial cells. The technique of implantation of 10 mm long fragments of three-layer PTS into the infrarenal section of a rat aorta was corrected, thus minimizing blood loss and narrowing the anastomosis site. In an acute experiment, it was proven that the prostheses were patent and that blood flow parameters (systolic and diastolic velocity, resistivity index) were close to the corresponding indicators of native rat aorta. **Conclusion.** The developed three-layer PTS constructs have low SP and physicomechanical properties close to those of native blood vessels. Bioactive coating improves the *in vitro* matrix properties of PTS relative to human endothelial cells. At short-term implantation into the aorta of experimental animals, PTS showed no early thrombosis, while blood flow parameters were close to those of native rat aorta. Thus, three-layer PTS with bioactive coating can be used as a scaffold for creation of *in situ* tissue-engineered construct of a small-diameter blood vessel.

Keywords: vascular prosthesis, electrospinning, tubular porous scaffold, polycaprolactone, gelatin, heparin, platelet lysate, endothelial cells, cytotoxicity, implantation, rat aorta.

INTRODUCTION

Biomimetic materials, which are biocompatible composite materials that mimic the structure and basic characteristics of various human tissues and/or organs, are widely used in regenerative medicine [1]. Recently, prostheses – biomimetic vascular grafts, including those with a diameter of less than 5 mm – have been quite often developed using additive technologies, mainly electrospinning and bioprinting [2].

When developing blood vessel prostheses (BVPs), it is necessary to maintain a certain balance between sufficiently high biological porosity, which is provided by the presence of pores with a pore size large enough to allow tissue sprouting due to cell migration into the BVP, and low surgical porosity (SP), which is a criterion for blood loss through the walls of the vascular prosthesis after its incorporation into the bloodstream. A water permeability of BVPs of more than 50 mL/(cm^2·min) at a pressure of 120 mmHg is a criterion that determines the need for additional efforts to reduce SP [3, 4].

A study on textile BVPs made of polyethylene terephthalate showed that regardless of pore size (20 μm to 100 μm), there is a correlation ($R^2 > 0.9$) between water permeability and blood loss, with blood loss being

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about 10 times less than water permeability because of the higher viscosity and presence of formed elements [5].

Hydrogel coatings made of collagen or gelatin [6, 7], chondroitin sulfate [8], silk fibroin [9], sodium alginate [10], dextran derivatives [11], chitosan [12] and a number of others are actively used to reduce SP.

One of the significant problems of the sealing coat/layer for hydrogel-based vascular grafts is the insufficiently long functional life amidst contact with body media, which makes it necessary to apply additional crosslinking [13–15]. The crosslinking agents used in this approach, which belong to the class of aldehydes, epoxy compounds, isocyanates, etc. are toxic substances that are difficult to remove entirely from the coating volume. Their unpredictable behavior in the body makes it undesirable for them to enter the bloodstream.

In addition, a comparative study of BVPs fabricated using different techniques has shown that applying a coating that allows reducing SP is associated with undesirable changes in the physicomechanical properties of vascular grafts, in particular, an increase in Young's modulus [16].

Multilayer BVPs with highly porous outer and inner layers and a sealing layer located between them can be an alternative to hydrogel coatings. To form the sealing coat, they were dipped in aqueous solutions of gelatin [17] or a mixture of albumin and alginate [18]. The middle layer was also formed from densely packed fibers by electrospinning [19, 20].

Using the electrospinning method, we had previously developed a biocompatible highly porous scaffold made of polycaprolactone (PCL) in the form of tubes, with a diameter of 3 mm, which has physicomechanical properties that comparable to those of natural blood vessels [21]. Nonetheless, the minimal surgical porosity that was attained was close to the maximum permissible.

The aim of this work was to develop 2 mm diameter multilayer porous tubular scaffolds (PTSs) that would resemble small natural blood arteries in terms of characteristics.

MATERIALS AND METHODS

Fabrication of samples of porous tubular scaffolds

PTSs were obtained using a NANON-01A electrospinning machine (MECC CO, Japan) at 25 kV. The distance between the 18 G needle and the 2 mm diameter cylindrical electrode was 10 cm, the rod was rotated at 1000 rpm, and solutions were applied at a rate of 4 mL/h. The outer and inner PTS layers were formed with 0.2 mL of a 10% PCL solution (MM 80000, Sigma-Aldrich, USA) in methylene chloride (JSC ECOS-1, Russia), and the middle sealing coat/layer was formed with 0.5 mL of a 5% PCL-gelatin solution. The obtained scaffolds were dried at 37 °C for 2 hours and vacuumized at 20 mmHg and 37 °C for 24 hours.

Application of modifying coating

The PTS sample was sequentially treated with aqueous solutions of bovine serum albumin (1 mg/mL, 1.5–2 hours at 37 °C), heparin (Hp, 1 mg/mL, 1.5–2 hours at 37 °C), glutaraldehyde (1%, 18 hours at room temperature), then again with heparin (1 mg/mL, 1.5–2 hours, 37 °C). Between stages and at the end of coating, the PTS was washed three times in 100 mL of distilled water. The heparinized scaffold (PTS-Hp) was dried at 37 °C, vacuumized (10–20 mmHg at room temperature), and sterilized by gamma radiation at a 1.5 MRad dose.

Human platelet lysate (PL) solution was obtained by diluting a lyophilized preparation (Renam, Russia) with Hanks' Balanced Salt Solution (HBSS) containing no Ca^{2+} and Mg^{2+} ions (HBSS, Gibco® by Life Technologies™, SC) in the ratio of 1:9 followed by sterilization by filtration through a filter with a pore diameter of 0.22 μm.

Sterile PTS-Hp samples were treated with PL solution under aseptic conditions for 1 hour at 37 °C (PTS-PL) immediately before the experiment.

Surface morphology of blood vessel prostheses

A scanning electron microscope (SEM) JSM-6360LA (JEOL, Japan) was used to analyze the surface morphology of PTSs. The accelerating voltage was 5 kV. The conductive coating on the surface of the studied samples was formed by gold sputtering on the JFC-1600 unit (JEOL, Japan) for 40 seconds at a constant current of 5–7 mA.

Physicomechanical properties of blood vessel prostheses

To investigate the physical and mechanical properties of PTSs, tensile tester Shimadzu EZ Test EZ-SX (Shimadzu Corporation, Japan) was used at a tensile speed of 5 mm/min. The results obtained were processed in the TrapeziumX program, version 1.2.6, which allows calculating the elongation at break (%), tensile strength (N) and Young's modulus (MPa).

Cytotoxicity study

The cytotoxic effect of laboratory PTS samples was evaluated in accordance with the requirements of the

interstate standard GOST ISO 10993-5-2023 "Studies on cytotoxicity" [22].

Mouse fibroblast cell line NIH3T3 (American Type Culture Collection) was cultured in a CO_2 incubator at 37 °C, in a humidified atmosphere containing (5 ± 1) % CO_2 in culture vials (CELLSTAR® Greiner Bio-One, Germany) with complete growth medium DMEM containing high glucose (PanEco, Russia) and 10% fetal bovine serum (Biosera, Germany), antibiotic and antimycotic Anti-Anti (Gibco® by Life Technologies™, USA) and 2 mM glutamine (PanEco, Russia). Cells were suspended using TrypLE™ Express Enzyme dissociation reagent (Gibco® by Life Technologies™, UK). Cell count in the suspension was determined using a Goryaev chamber (MiniMed®, Russia). Fibroblasts were then seeded into flat-bottomed 24-well culture plates (CELLSTAR® Greiner Bio-One, Germany) at a seeding density of 7–12 × 10^4 cells per well and incubated for 24 hours at 37 °C in a humidified atmosphere containing (5 ± 1) % CO_2 until formation of (80 ± 10) % monolayer. The test PTS samples were placed directly on the surface of the fibroblast monolayer. At the end of incubation, the test samples were removed from the wells, washed with phosphate buffered saline (PBS), and 0.1% trypan blue solution (PanEco, Russia), which stains lysed cells and cells with damaged cell membranes, was added for 1–2 minutes. After removal of the dye and washing with PBS, the culture was assessed for morphological changes and decreased cell density using a fluorescence microscope.

To analyze the results, the following scale of the degree of cell reaction to direct contact with PTS was used:

0 means no lysis (no reaction);

1 means ≤20% of cells lysed (minor reaction);

2 means ≤50% of cells lysed (mild reaction);

3 means ≤70% of cells lysed (moderate reaction);

4 means >70% of cells lysed (strong reaction).

A non-cytotoxic material should have a reaction grade of "0".

Study of PTS interaction with endothelial cells

Immortalized human umbilical vein cell line, EA.hy926 (ATCC®CRL-2922™), from the American Type Culture Collection (ATCC), which have similarity with primary cultures [23, 24], were selected for *in vitro* study.

Sterile samples of unmodified and modified PTSs were placed on the bottom of a flat-bottomed 24-well culture plate (CELLSTAR® Greiner Bio-One, Germany), human endothelial cells (ECs) were plated at a seeding density of 5 × 10^4 cells/cm^2 under aseptic conditions and cultured in a CO_2 incubator under standard conditions for a selected time interval.

Human EC viability was assessed using Live/Dead® Viability/Cytotoxicity Kit (Molecular Probes® by Life Technologies™, USA): Dulbecco′s PBS containing 2 μM calcein AM and 4 μM EthD-1 was added to the wells of the plate after 15 minutes. The staining results were visualized using a Nikon Eclipse TS100 inverted fluorescence microscope (Japan), equipped with a Digital Sight DS-Vil digital camera (Nikon, Japan).

Metabolic activity of human ECs on the surface of PTS was evaluated using PrestoBlue™ HS Cell Viability Reagent (Invitrogen™ by Thermo Fisher Scientific, USA): 10% PrestoBlue™ Cell Viability Reagent was added to wells with the test samples and control (cell-free medium), after which the plate was incubated for 3 hours at 37 °C in a humidified atmosphere containing (5 ± 1) % CO_2. Changes in absorbance of the medium were recorded using a microplate reader as previously described at 570 nm and 600 nm wavelengths. The percentage of reduced PrestoBlue™ was calculated using formula (1):

$$\frac{117{,}216 \cdot A_{570\ Samp} - 80{,}586 \cdot A_{600\ Samp}}{155{,}677 \cdot A'_{600} - 14{,}652 \cdot A'_{570}} \times 100\%, \quad (1)$$

where 117,216 and 80,586 are the molar extinction coefficients for the oxidized form of PrestoBlue™ Vital Reagent at λ = 600 nm and λ = 570 nm, respectively; 155,677 and 14,652 are the molar extinction coefficients for the reduced form of the reagent at λ = 600 nm and λ = 570 nm, respectively; $A_{570\ Samp}$ and $A_{600\ Samp}$ are the absorbance of the test sample at λ = 600 nm and λ = 570 nm, respectively; A'_{570} and A'_{600} are the absorbance of the cell-free control sample at λ = 600 nm and λ = 570 nm, respectively.

Human EC count on the PTS surface was estimated using a calibration curve, linear in semi-logarithmic coordinates up to a concentration of human ECs of 0.8 × 10^5.

Implantation of a PTS fragment into the infrarenal aorta of a rat

Three-layer PTS with a diameter of 2 mm and length of 10 mm were implanted into the abdominal aorta of rats using the end-to-end technique. The anesthetized animal was fixed on the operating table in the supine position. Hair was removed from the anterior abdominal wall with a razor, the surgical field was treated with an antiseptic. Access to the abdominal aorta was performed by midline laparotomy. Next, the infrarenal aorta was isolated and the proximal part of the vessel was clamped below the renal arteries. The distal part was clamped above the trifurcation of the abdominal aorta. After clamping, the

infrarenal aorta was resected, proximal and then distal anastomoses with PTS were formed using atraumatic polypropylene suture 8-0. After initiating blood flow, hemostasis was monitored. At the final stage, layer-by-layer suturing of the surgical wound was performed with subsequent treatment of the sutures with antiseptic agent and anesthetic solution. After the surgery, the animals were kept in vivarium conditions with free access to food and water.

To confirm the patency of the implanted PTS, the study was performed on a Vivid E9 ultrasound machine (General Electric, USA) with WindowBlinds™ software (M OCX© Stardock) using sector (1.5–5 mHz) and linear (2.5–12 mHz) transducers and conductive gel Mediagel (Geltek, Russia). Zoletil (Virbac Sante Animale, France) was used for sedation of the test animals before dopplerometry, followed by shaving of the abdominal region with a veterinary trimmer. Aortic lumen diameter or PTS (d, cm), peak systolic velocity (Vps, m/s) and maximum end diastolic velocity (Ved, m/s) were measured, after which resistivity index was calculated according to formula (2).

$$RI = (Vps - Ved)/Vps \quad (2)$$

Statistical processing

Quantitative and statistical processing of data obtained was performed using Microsoft Excel 2007 application. All results are presented as mean value ± standard deviation. Differences were considered significant at $p < 0.05$ when the number of samples (n) ranged from 3 to 5.

RESULTS AND DISCUSSION

The lowest water permeability of our previously developed 3 mm diameter PCL-based PTS was quite high at 30.4 ± 1.5 mL/(cm^2·min) [20], which was still below the maximum permissible value of 50 mL/(cm^2·min). When switching to 2 mm diameter PTS, the water permeability of PCL-based PTS could not be reduced to satisfactory values, which determined the need for additional efforts to reduce SP. To address this problem, we proposed to form multilayer PTSs incorporating an inner sealing coat/layer made of PCL with gelatin addition.

Fig. 1 summarizes the results of the study of the effect of gelatin concentration in the sealing coat/layer on the water permeability of a 2 mm diameter three-layer PTS.

As can be seen from Fig. 1, water permeability decreased with increasing gelatin concentration, reaching values less than 10 mL/(cm^2·min) at 30% protein concentration. Attempts to increase the gelatin level above 30% resulted in a jump in the risk of delamination of the multilayer structure upon contact with water. As a result, a gelatin level of 30% (by weight relative to PCL) was chosen as the optimal concentration for formation of the sealing coat/layer.

Both surfaces of the triple-layer PTS exhibit a highly porous structure formed by fibers, several micrometers in diameter, with pores large enough to allow cell migration into the matrix (Fig. 2).

Table summarizes the physicomechanical characterization of small-diameter three-layer PTS with bioactive coating.

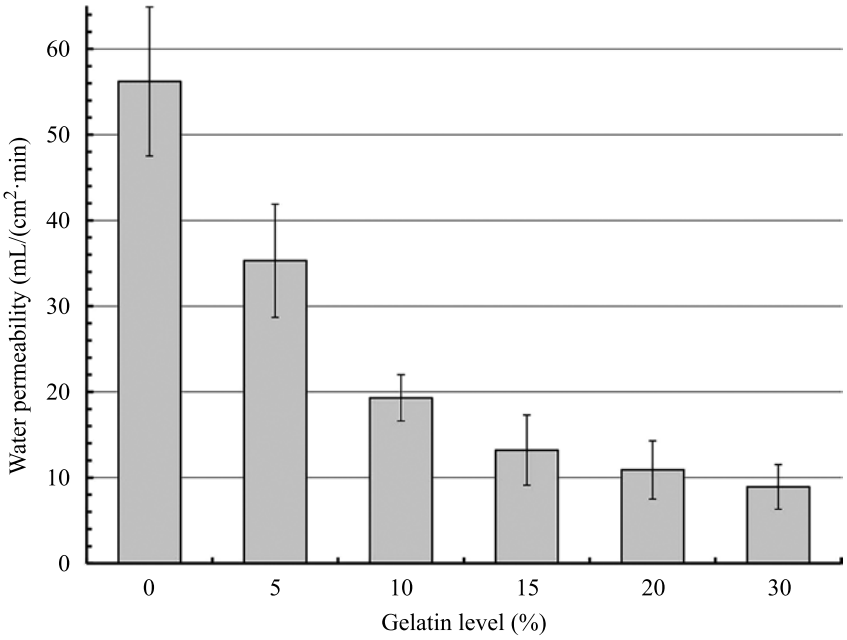


Fig. 1. Effect of gelatin levels in the sealing coat/layer on the water permeability of a 2 mm diameter three-layer PTS. Inner and outer layers are made from 0.2 mL of PCL, the middle sealing coat/layer is made from 0.5 mL of PCL-gelatin solution

As can be seen from Table, the three-layer PTS has physicomechanical properties comparable to the mechanical properties of blood vessels of similar diameter.

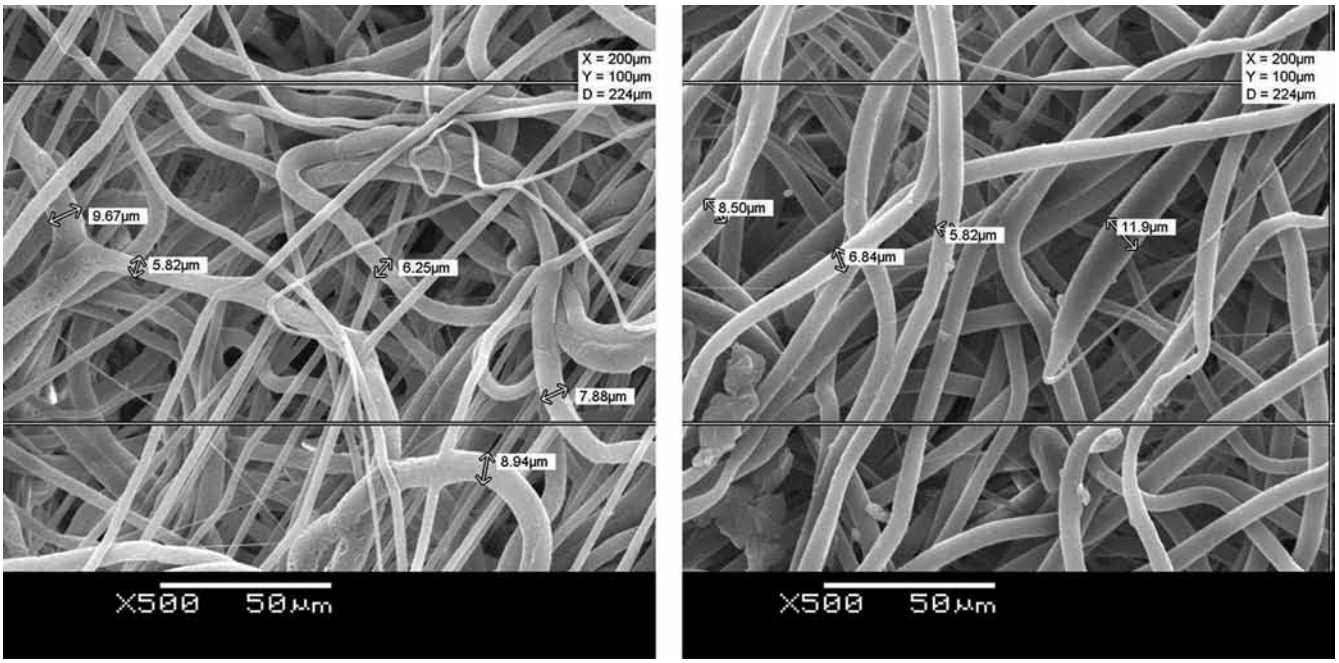
In the cytotoxicity test, a three-layer PTS with bioactive coating showed a level of "0" – no cytotoxicity.

Fig. 3 shows photographs of human EC culture of the EA.hy926 line 24 hours and 168 hours after seeding on the surface of culture plastic (CP) and the inner surface of PTS without modifying coating, modified with heparin only (PTS-Hp), and with bioactive coating from Hp and PL (PTS-PL). The CP surface was used to control the morphology and adequate growth of the investigated cell cultures.

At 24 hours after seeding, the inner surface of PTS-PL samples showed the highest number of adhered and spread ECs, compared to samples from unmodified PCL and PTS-Hp, which had substantially less number of adhered endothelial cells. It is important to note that a large number of non-spread and dead cells was observed on the inner surface of unmodified PTS; this finding appears to be connected to PCL's hydrophobic nature [13, 25, 26]. After 168 hours of culturing, an EC monolayer was formed on the CP surface and on the PTS-PL sample. In contrast, no EC monolayer was formed on the surface of either the heparinized sample or the unmodified control, no proliferation occurred, and there was a significant number of non-viable cells. Note that, despite hydrophilization of the surface of PCL-based PTS under the conditions of this experiment, heparin modification does not promote scaffold endothelialization *in vitro*.

Fig. 4 presents the results of the quantitative study of EC proliferation on the inner surface of the original and modified PTS.

As shown in Fig. 4, after 24 hours, EC count on the inner surface of the original, modified scaffolds differs slightly ($10.1 \pm 1.3 \times 10^3$ kL, $13.2 \pm 1.4 \times 10^3$ kL, and $16.7 \pm 1.1 \times 10^3$ kL for PCL, PCL-Hp, and PCL-PL, respectively). After 96 hours of cultivation, cell count on each scaffold variant uniformly increased approximately 2-fold. By the end of the period, PCL-PL increases 3.6-fold (to $60.4 \pm 1.8 \times 10^3$ kL), while EC count on the outer surface of the original and PCL-Hp increases 2.9-fold (to $29.1 \pm 1.9 \times 10^3$ kL and $32.0 \pm 1.4 \times 10^3$ kL, respectively). The difference in cell count on the inner



Inner surface **Outer surface**

Fig. 2. Surface microstructure of a three-layer PTS. Inner and outer layers are made from 0.2 mL of PCL, the middle sealing coat/layer is from 0.5 mL of PCL-gelatin solution

Table

Physicomechanical characteristics of three-layer scaffolds

	Young's modulus (MPa)	Tensile strength (N)	Elongation at break (%)
PTS, diameter 2 mm	6.6 ± 0.3	12.6 ± 3.2	412 ± 30
Rat aorta, diameter 2 mm	8.5 ± 2.2	2.0 ± 0.3	93 ± 16

surface of unmodified PCL and PCL-Hp is insignificant despite hydrophilization of the surface due to heparin immobilization.

Thus, the presence of PL in the modifying coating under the *in vitro* conditions of the experiment is a key factor in promoting adhesion, spreading and stimulating proliferation of endothelial cells (EA.hy926) on the inner surface of the scaffolds, increasing their matrix properties.

An *in vivo* study of the functional properties of small-diameter vascular grafts was conducted by prosthetizing the infrarenal section of the rat abdominal aorta with three-layer PTS that had a 2 mm inner diameter and a 10 mm length.

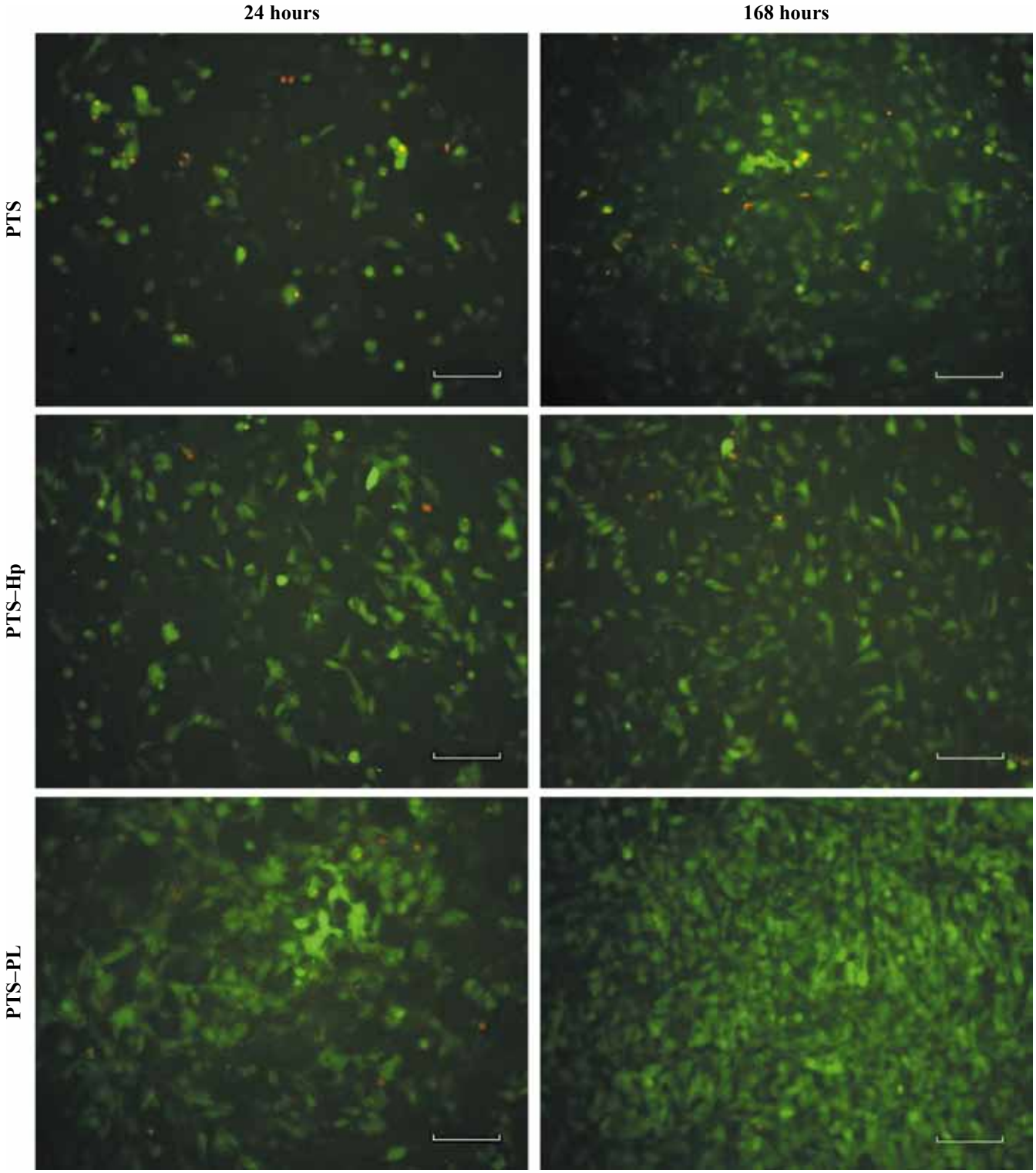


Fig. 3. Endothelial cell growth on the inner surface of PTS. Seeding density of 5×10^4 cells/cm^2. Live/Dead® Viability/Cytotoxicity Kit staining. Magnification 20×. Scale bar: 100 μm

The implantation technique was corrected. Initially, the artery was transected after clamping, and 6–8 interrupted sutures were used to produce direct anastomoses with the PTS. In order to minimize blood loss and narrow the anastomosis area, we tried to create oblique slices of the proximal and distal ends of the aorta with the formation of anastomoses by continuous locking sutures. As a result, we were able to optimize hemostasis and maintain ideal anastomosis diameter.

Doppler ultrasound was carried out to confirm PTS patency shortly after implantation (Fig. 5).

Fig. 6 shows Doppler ultrasound results for the implanted PTS. Implanted modified and unmodified PTS demonstrated similar blood flow parameters that were no different from those obtained for rat aorta.

Future *in vivo* studies are to be continued for up to 1 year with a detailed study of the neointima formation process involving histologic methods.

CONCLUSION

The three-layer PCL-based PTS constructs that were created, with a PCL-gelatin sealing coat/layer, have low SP and demonstrate physicomechanical properties that are close to those of native blood vessels. Application of bioactive coating based on Hp and PL improves the *in vitro* interaction of PTS with ECs. When implanted into the aorta of an experimental animal, PTS demonstrated

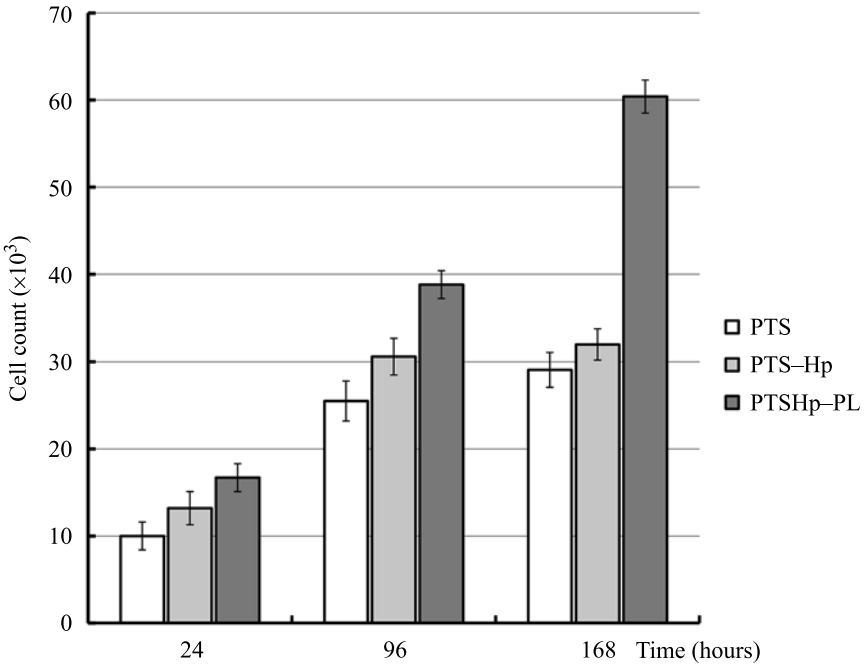
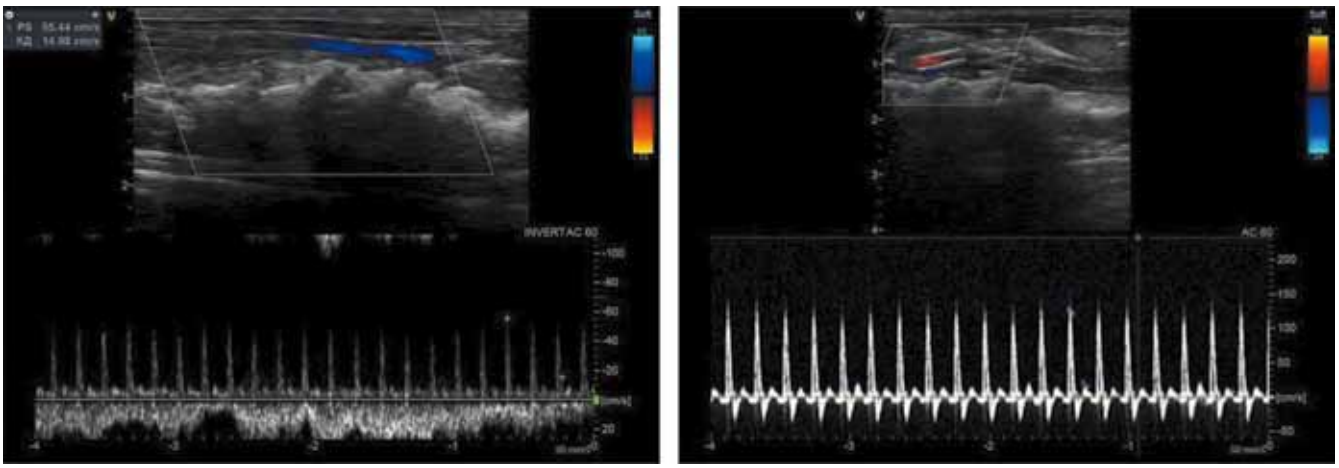


Fig. 4. Endothelial cell proliferation in the inner surface of PTS. Seeding density 5×10^4 cells/cm^2



Unoperated rat (control) **PTS, 4 weeks after implantation**

Fig. 5. Doppler ultrasound of implanted coated PTS 4 weeks after implantation (typical measurement)

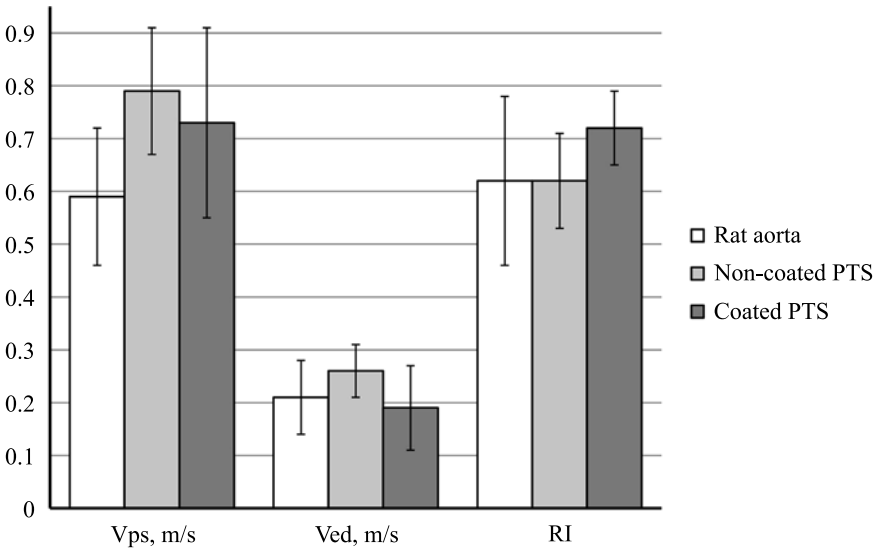


Fig. 6. Peak systolic velocity (Vps), diastolic velocity (Ved) and resistive index (RI) 4 weeks after PTS implantation (n = 6)

no early thrombosis, while blood flow parameters were close to those of the rat aorta. Thus, three-layer PTS with bioactive coating can be used as a scaffold to produce *in situ* tissue-engineered constructs of small-diameter blood vessels.

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The authors declare no conflict of interest.

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