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HISTOLOGIC AND GENETIC FEATURES OF REMODELING OF TISSUE-ENGINEERED SMALL-DIAMETER VASCULAR GRAFTS: OUTCOMES OF SIX-MONTH IMPLANTATION IN A SHEEP MODEL

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Surface modification of polymeric scaffolds with drugs to avoid thrombus formation and infection is a promising area in tissue engineering, which also makes it possible to accelerate the remodeling of such scaffolds and improve long-term patency. **Objective:** to study the histologic and genetic features of remodeling of tissue-engineered small-diameter vascular grafts (SDVGs) with antithrombogenic drug-coated and reinforced external scaffolds, implanted into a sheep carotid artery. **Materials and methods**. Poly(ε-caprolactone) (PCL) matrices, ∅4 mm in diameter, were fabricated via electrospinning, followed by creation of a reinforcing spiral PCL scaffold on their outer surface by extrusion. To prevent thrombus formation and infection, the fabricated grafts were modified with iloprost and cationic amphiphile by complexation through polyvinylpyrrolidone (PVP). The work was carried out to evaluate, by infrared spectroscopy, the formation of PVP-based coating, to study the physical and mechanical properties of the grafts in longitudinal and transverse directions, and to implant the vascular grafts (VGs) into a sheep carotid artery. To assess and control the patency of the implanted grafts, Doppler ultrasound was performed at days 1 and 5, then at 1, 3 and 6 months. The explanted samples were studied via histological and immunofluorescent analyses; gene expression profile was evaluated. **Results.** Ultrasound on days 1 and 5 after implantation showed the patency of vascular grafts to be 100%. At 1 month, the patency decreased to 83.3%; patency was 50% by the end of the implantation period (6 months), without aneurysm formation and detachment of the reinforcing scaffold. Histological and immunofluorescence studies of patent grafts showed the formation of a newly formed three-layer vascular tissue structure on their basis, without signs of inflammation and calcification. However, despite the structural similarity between the newly formed vascular tissue and the native tissue of a sheep carotid artery, analysis of the gene expression profile revealed some differences in terms of genetic profile: *CNN* and *SNA12* expression levels in the neotissue decreased, and those of *CTSB*, *TNFa*, and *TGFb* increased. **Conclusion.** Modified polymeric vascular scaffolds showed good remodeling of the prosthetic wall, without aneurysm formation. The identified genetic differences between newly formed tissue and native tissue are logical in view of formation on the basis of the artificial polymeric scaffold. Further research on reinforced polymeric scaffolds will be aimed at improving the inner surface in order to improve their thromboresistance.

Keywords: vascular grafts; antithrombogenic treatment; antimicrobial treatment; iloprost; cationic amphiphiles.

INTRODUCTION

The increasing cases of cardiovascular diseases every year is the most common cause of death globally [1, 2]. Autologous grafts are the preferred material for restoring blood flow in the affected vascular area. However, accessibility of patients' arteries and veins is limited due to vascular quality associated with comorbidities (high blood pressure, diabetes, and others) [1].

An alternative to autografts are clinically approved synthetic vascular grafts (VGs) made from polytetrafluoroethylene (PTFE) and polyethylene terephthalate (PET, Dacron). They demonstrate high efficiency in replacing large vessels with an internal diameter of more than 6 mm. However, when replacing a vessel segment with a diameter of less than 5 mm, these prostheses become ineffective and have a propensity to cause thrombus formation, stenosis, occlusion of the vascular lumen and infection [2, 3]. Based on various tactics for handling biocompatible and biodegradable polymers, tissue engineering (TE) is a pertinent method for producing small-diameter vascular grafts (SDVGs). Artificial tissue-like matrices and tissues with the required structural and mechanical qualities can be designed using a variety of TE methods [4–6]. A vascular prosthesis made of such material can undergo synchronous biodegradation and remodeling of its wall, allowing body cells to participate in the development of a three-layer native blood vessel structure [7–9]. However, this is a lengthy process and is accompanied by such risks as thrombosis, aneurysm formation and microbial contamination [10–12].

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Highly porous tissue-engineered constructs in contact with blood can provoke thrombus formation. Therefore, when developing products for cardiovascular surgery, these features should be considered. Surface modification of prostheses with antithrombogenic drugs can prevent thrombosis of the lumen of implanted vascular prostheses both in the early postoperative period and in the process of long-term bioresorption of the main scaffold of the prosthesis [11, 13].

Although various approaches to modifying VGs to prevent their infection and thrombus formation have been developed over the years, none of them has been able to demonstrate any significant benefits [14–16].

Apart from the above challenges, if biodegradable VGs are used as prostheses, there should be a clear understanding of the level of synchronization of remodeling with biodegradation processes. There are already works that have analyzed in detail the outcomes of long-term implantation of biodegradable VGs using a large animal model [6, 8, 12, 17]. It has been demonstrated that tissue-engineered vascular prostheses are replaced by newly formed vascular tissue, becoming similar to autologous vessels. This shows the ability of biodegradable prostheses to adaptive growth. Preclinical tests for small-diameter biodegradable VGs on a large laboratory animal model revealed that, despite all encouraging developments and successful testing on a small animal model, the main outcome was aneurysm formation due to accelerated bioresorption of the main scaffold of the vascular prosthesis [6, 17].

Therefore, creation of an external reinforced scaffold would prevent aneurysm formation and other deformational changes in the prosthetic walls during its long-term functioning in the vascular bed. Furthermore, the use of drugs, for surface modification, that can prevent both thrombosis and infection will make highly porous tissue-engineered constructs developed for cardiovascular surgery more effective over the long run.

The **aim** of this study was to investigate the histologic and genetic features of remodeling of tissue-engineered SDVGs that were implanted into a sheep carotid artery and included antithrombogenic and antimicrobial drug-coated and reinforced external scaffolds.

MATERIALS AND METHODS

Fabrication of biodegradable vascular prostheses

Small (4 mm) diameter VGs were fabricated by electrospinning from 12% PCL (Sigma-Aldrich, USA) in trichloromethane. Electrospinning was performed on a Nanon-01A device (MECC, Japan) with the following parameters: voltage at the end of the needle, 22 kV; polymer solution feed rate, 0.5 ml/h; manifold rotation speed, 200 rpm; distance from the needle to the winding manifold, 150 mm; 22 G needle.

Creation of an external reinforcement layer of vascular prostheses

To prevent aneurysm formation on the outer surface of VGs, a reinforcement spiral scaffold was created, which was fabricated from PCL of molecular weight 90,000 Da (Sigma Aldrich, USA) using an original machine consisting of a carriage with an extruder and a rotating shaft, in the following mode: shaft rotation speed, 1 rev/s; carriage speed, 1 mm/s; plastic feed rate, 0.5 mm/s (extruder nozzle 0.5 mm); fiber feed temperature, 160 °C [13].

Formation of drug coating on the surface of vascular prostheses

The VG surface was modified with drugs to prevent thrombosis and infection. In the first step, a hydrogel coating was formed from 10% PVP (PanReac AppliChem, Spain) in 25% ethanol. Afterwards, the prostheses in glass tubes filled with argon were irradiated with ionizing radiation with a total absorbed dose of 15 kGy using pulsed linear gas pedal ILU-10 with a beam of 5 MeV 50 kW (manufacturer – Budker Institute of Nuclear Physics, Russia). This procedure grafted PVP onto the PCL surface through radiation-chemical crosslinking of polymers. Radical centers appear on the PCL surface and in the structure of the applied PVP layer because of ionizing effect, which leads to three-dimensional crosslinking of PVP and its binding to the surface of the polymer graft [18]. Alongside with PVP grafting, the tubular prosthetic scaffolds were sterilized.

At the second stage, using the method of complexation with PVP, antiplatelet agent iloprost (Ilo, Berlimed Sa, Spain) and cationic amphiphile 1,5-bis-(4-tetradecyl-1,4-diazoniabicyclo [2.2.2]octan-1-yl) pentane tetrabromide (A, Nanotech-s LLC, Novosibirsk), which has antimicrobial and antiviral properties, were introduced [5].

The quality of grafting was evaluated earlier by infrared spectroscopy, analyzing the attenuated total reflectance (ATR) spectra of the inner surface of modified prostheses on a Bruker Vertex 80v instrument (Germany) with an ATR attachment (Germany) in the spectral region of 4000–5000 cm^{-1} [19]. Studies have been conducted previously to confirm the formation of PVP coating on prosthetic surfaces and to assess the kinetics of drug release from compounds connected by complexation to the PVP layer [19].

Evaluation of physical and mechanical properties of biodegradable vascular prostheses

The physical and mechanical properties of PCL/PVP/Ilo/A vascular grafts with an external reinforcing scaffold were evaluated under uniaxial tensile conditions in accordance with GOST 270-75. Similar grafts without a reinforcing scaffold were used as a comparison group. The tests were performed on a Z-series universal testing

machine (Zwick/Roell, Germany), using a load cell with 50 N rated load with a permissible error limit of ±1%; a traverse lifting speed of 50 mm/min was used during testing, at an ambient temperature of 37 °C, maintained by a thermal chamber. The ultimate tensile strength of the material was evaluated as the maximum uniaxial tensile stress (MPa) before failure. The stress-strain properties of the material were evaluated using Young's modulus (MPa). The obtained data were exported as the ratio of relative elongation (mm) to stress (MPa) undergone by the sample.

Implantation of biodegradable vascular prostheses into a sheep carotid artery

The study was approved on June 10, 2020 by the local ethics committee of the Research Institute for Complex Issues of Cardiovascular Diseases via minutes No. 12. The handling of animals complied with the requirements of order No. 1179 of the USSR Ministry of Health dated October 10, 1983, order No. 267 of the Russian Ministry of Health dated June 19, 2003, "Rules for carrying out works using experimental animals", the principles of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 1986), the World Medical Association Declaration of Helsinki on humane treatment of animals (1996) and the Guide for the Care and Use of Laboratory Animals (1996).

PCL/PVP/Ilo/A vascular grafts (n = 12) with an external reinforcing scaffold were implanted into the carotid artery of Edilbay sheep (also known as Edilbaev sheep) using the "end-to-end" method according to the '1 animal – 1 prosthesis' scheme. The diameter and length of the prostheses were 4 mm and 40 mm, respectively. Implantation period was 6 months. Implantation of grafts and anesthetic therapy were carried out according to the scheme given below.

Large laboratory animals were premedicated with xylazine (Xylanite) 0.05–0.25 ml per 10 kg of animal weight and were intramuscularly injected with 1 mg of atropine. Injection anesthesia was performed with 5–7 mg propofol per 1 kg of animal weight, for 90 seconds, then atracurium besylate (Ridelat) was injected intravenously at a dosage of 0.5–0.6 mg per 1 kg of animal weight. Tracheal intubation was performed with a 9.0-diameter endotracheal tube. In the process of manipulations on implantation of VGs into the sheep carotid artery, anesthesia was maintained on sevoran 2–4 vol% and ridelat by continuous infusion at a rate of 0.3–0.6 mg/kg/h. Alongside with that, the following vital signs of the animal were monitored: blood pressure (BP), heart rate (HR), and blood oxygen level (SpO_2). The following parameters were recorded during artificial ventilation (ALV): respiratory rate (RR), 12–15/min; positive end-expiratory pressure (PEEP), 7–9 mbar; respiratory volume (RV), 6–8 ml/kg; fraction of inspired oxygen (FiO_2); 40–60%.

To access the sheep carotid artery, intravenous systemic heparinization was performed – 5000 units, with carotid artery clamping. Then a 40 mm longitudinal incision of the carotid artery was made. The VGs were immediately implanted by separate knotted sutures using Prolene 6/0 thread (Ethicon, USA). After the clamps were removed from the carotid artery, the standard air embolism prophylaxis protocol was followed with subsequent initiation of blood flow and suturing of the wound with Vicril 2.0 thread (Ethicon, USA). At the conclusion of the operation, the suture was treated with BF-6 glue, subcutaneous injection of enoxaparin sodium at a concentration of 4000 anti-Xa IU/0.4 ml was performed and the animal was extubated.

To assess the patency of the implanted prostheses, Doppler ultrasound was performed using an M 7 Premium apparatus (Mindray, China) on the following dates: day 1, day 5, and at 1, 3 and 6 months.

Histological examination

The explanted VG samples were fixed in 10% buffered formalin (BioVitrum, Russia) for 24 hours, then dehydrated in 6 portions of IsoPrep (BioVitrum, Russia) and impregnated with paraffin (3 portions) at 56 °C for 60 minutes in each portion. The impregnated samples were embedded in refractory paraffin HISTOMICS (BioVitrum, Russia). From the obtained samples, 8 μm thick slices were made using an HM 325 microtome (Thermo Scientific, USA). Then they were dried in a thermostat overnight at 37 °C, then dewaxed in 3 o-xylene portions for 1–2 minutes each and dehydrated in 3 portions of 96% alcohol for 1–2 minutes each. The dewaxed slices were then stained with hematoxylin-eosin, van Gieson, and alizarin red S in accordance with previously established staining protocols [19]. The resulting preparations of vascular graft slices were examined using light and fluorescence microscopy on an AXIO Imager A1 microscope (Carl Zeiss, Germany) with ×50 objective lens magnification.

Immunofluorescence test

Explanted VGs were frozen at –140 °C for immunofluorescence staining. Next, frozen samples were fixed in Tissue-Tek cryo-medium (Sakura, Japan) and 8 μm thick slices were made on a CryoStar NX50 cryostat (Thermo Scientific, USA). Then indirect fluorescence staining of the slices was performed using specific antibodies to CD31, von Willebrand factor (vWF), alpha-smooth muscle actin (α-SMA), collagen type III, IV (Abcam, England). Cell nuclei were contrasted with DAPI (Sigma, USA). Control samples were prepared similarly to experimental samples, but 1% bovine serum albumin was used instead of primary antibodies. The preparations were encapsulated in ProLong mounting medium (Life Technologies, USA) under a coverslip and analyzed using a laser scanning microscope LSM 700 (Zeiss, Germany).

Determination of mRNA level

To obtain endothelial lysate from explanted sections of native arteries and patent VGs, the inner endothelial layer was washed with lysing reagent TRIzol (Invitrogen, USA). Samples were placed in TRIzol reagent followed by homogenization (FastPrep-24 Instrument and Lysing Matrix S, MP Biomedicals, USA). The quality and quantity of isolated RNA was determined on a Qubit 4 instrument (Invitrogen, USA) by measuring the RNA Integrity and Quality (RIQ) index using the Qubit RNA IQ Assay Kit (Invitrogen, USA). Based on the isolated RNA, cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA). Gene expression was determined by quantitative PCR (qPCR) using BioMaster UDG HS-qPCR Lo-ROX SYBR (2×) master mix (MHR033-2040, Biolabmix, Russia) and primers synthesized by Eurogen on a CFX96 amplifier (BioRad, USA). Characteristics of the genes included in the study are presented in Table 1. The results of qPCR were normalized using three reference genes ACTB, GAPDH, B2M according to available guidelines. Expression of the studied genes was calcula-

Table 1

Characteristics of primers

Gene	Forward primer	Reverse primer
IL1B	5’-TGCTGAAGGCTCTCCACCTC-3’	5’-ACCCAAGGCCACAGGAATCTT-3’
IL6	5’-TGTCATGGAGTTGCAGAGCAGT-3’	5’-CCAGCATGTCAGTGTGTGTGG-3’
IL10	5’-ATGCCACAGGCTGAGAACCA-3’	5’-TCGCAGGGCAGAAAACGATG-3’
IL12A	5’-GCAGAAGGCCAGACAAACCC-3’	5’-TGGAAGCCAGGCAACTCTCA-3’
IL12B	5’-AGAGCCTGCCCATTGAGGTC-3’	5’-GGTTCTTGGGTGGGTCTGGT-3’
CXCR4	5’-CTGGAGAGCAAGCGGTTACCA-3’	5’-ACAGTGGGCAGGAAGATCCG-3’
CXCL8	5’-CTTCCAAGCTGGCTGTTGCTC-3’	5’-ATTTGGGGTGGAAAGGTGTGG-3’
IFNG	5’-TGAACGGCAGCTCTGAGAAAC-3’	5’-TGGCGACAGGTCATTCATCA-3’
TNF	5’-CTTCTGCCTGCTGCACTTCG-3’	5’-TGGCTACAACGTGGGCTACC-3’
ICAM1	5’-GTCACGGGGAACAGATTGTAGC-3’	5’-TGAGTTCTTCACCCACAGGCT-3’
NOS3	5’-CTTCCGTGGTTGGGCAAAGG-3’	5’-CGTTTCCAGCTCCGTTTGGG-3’
FGF2	5’-AGAGCGACCCTCACATCAAACT-3’	5’-TCAGTGCCACATACCAACTGGA-3’
VEGFA	5’-GCTTCTGCCGTCCCATTGAG-3’	5’-ATGTGCTGGCTTTGGTGAGG-3’
TGFB1	5’-TGAGCCAGAGGCGGACTACT-3’	5’-ACACAGGTTCAGGCACTGCT-3’
KDR	5’-ACAGAACCAAGTTAGCCCCATC-3’	5’-TCGCTGGAGTACACAGTGGTG-3’
MMP2	5’-ACCCCGCTACGGTTTTCTCG-3’	5’-ATGAGCCAGGAGCCCGTCTT-3’
NR2F2	5’-GCAAGCGGTTTGGGACCTT-3’	5’-GGACAGGTAGGAGTGGCAGTTG-3’
SNAI2	5’-ACCCTGGTTACTGCAAGGACA-3’	5’-GAGCCCTCAGATTGGACCTG-3’
YAP1	5’-TGCTTCGGCAGGAATTAGCTCT-3’	5’-GCTCATGCTCAGTCCGCTGT-3’
CXCL1	5’-AACATGCAGAGCGTGAAGGTG-3’	5’-CGGGGTTGAGACACACTTCCT-3’
CD14	5’-AATCAAGGCTCTGCGCGTTC-3’	5’-CGTTGGGCCAGTTACCTCCA-3’
CD40LD	5’-ACTGAGAGCTGCAAACACCCA-3’	5’-AAACACCGAAGCACCCTGTT-3’
CNN	5’-CCAACCACACGCAAGTGCAG-3’	5’-TCCTGCTTCTCCGCGTATTTCA-3’
CTSB	5’-AGTGTGGGGACGGCTGTAAC-3’	5’-AGGGAGGGATGGAGTACGGT-3’
CXCL1	5’-AACATGCAGAGCGTGAAGGTG-3’	5’-CGGGGTTGAGACACACTTCCT-3’
CXCL10	5’-AGTACCTTCAGTTGCAGCACCA-3’	5’-TGGGCAGGATTGACTTGCAG-3’
EDN1	5’-GCGACAGTCCACAGGAAGAGA-3’	5’-GGTTGTCCCAGGCTTTCTCC-3’
EDNRA	5’-AGGAACGGCAGCCTGAGAAT-3’	5’-AGGGAACCAGCACAGAGCAA-3’
IL1A	5’-TGACCTGGAAGCCATTGCCA-3’	5’-TGAGGGCGTCGTTCAGGATG-3’
IL4	5’-GGCGTATCTACAGGAGCCACA-3’	5’-ACTCGTCTTGGCTTCATTCACA-3’
IL18	5’-AGGAAGCTATTGAGCACAGGCAT-3’	5’-CTGATTCCAGGTCTTCGCCAT-3’
KLF4	5’-AGGACGGCCACTCACACTTG-3’	5’-ACTTCCACCCACAGCCATCC-3’
MIF	5’-TGCCGATGTTCGTGGTGAAC-3’	5’-GGTCATGAGCTGGTCTGGGA-3’
SELE	5’-CACTGGACCCCAGCACTTACA-3’	5’-GCTGATGGCTGCACAGGTTAC-3’
SELP	5’-TTCCACTGCGCTGAAGGGTA-3’	5’-TGGACTGGTGCTGGAATGCT-3’
PAI	5’-GCAGTGGCAGCAGGAACAAA-3’	5’-TGGTGCTGGTAGGAGGCAGA-3’
SMAD4	5’-TCTGGAGGAGATCGCTTTTGCT-3’	5’-TTCCAACTGCACACCTTTGCC-3’
B2M	5’-CCTTCTGTCCCACGCTGAGT-3’	5’-TGGTGCTGCTTAGAGGTCTCG-3’
ACTB	5’-AGCAAGAGAGGCATCCTGACC-3’	5’-GGCAGGGGTGTTGAAGGTCT-3’
GAPDH	5’-TGGTGAAGGTCGGAGTGAACG-3’	5’-AGGGGTCATTGATGGCAACG-3’

ted using the $2^{-\Delta\Delta Ct}$ method and expressed as a multiple change relative to native carotid arteries.

RESULTS

Physical and mechanical properties of prostheses

A comparative evaluation of physical and mechanical properties of the PCL/PVP/Ilo/A vascular grafts was carried out before and after formation of an outer reinforcing layer in longitudinal and transverse directions. Indices obtained in the longitudinal direction were also compared with similar indices of the sheep carotid artery. It has been proved that the formation of a reinforcing layer in the form of a polycaprolactone spiral on the outer surface of PCL/PVP/Ilo/A grafts helped to increase the strength of VGs in longitudinal and transverse directions, but at the same time the stiffness of grafts in transverse direction increased 2 times, which is an undesirable moment for such products (Table 2). Nevertheless, the critical difference between polymeric VGs and the parameters of the sheep carotid artery was only in Young's modulus, which in PCL/PVP/Iloo/A vascular grafts with and without a reinforcing external spiral exceeded the Young's modulus values of the sheep carotid artery (in the longitudinal direction) by 13.3 and 17.4 times, respectively, $p < 0.05$.

In our previous studies, it was shown that in contrast to unmodified PCL-based VGs, the spectrum of modified PVP-based grafts contained a band at 1654 cm^{-1} corresponding to the $–CONH_2$ group of the pyrrolidine ring, which confirms PVP grafting onto the surface of vascular prosthesis [19].

The kinetics of drug release combined with complexation with grafted PVP provided indirect proof of the stability of PVP grafted to the surface upon its interaction with the aqueous phase. In particular, iloprost has been shown to be able to be released into phosphate-buffered saline within three months. Cationic amphiphile in the form of phosphate salt of molecular ion C41H84Br3N4+ was also co-preserved on the surface of prostheses incubated for 3 months in phosphate-buffered saline [19]. At the same time, cationic amphiphile was determined unchanged on the surface of prostheses stored for 6 months at –20 °C [19].

Results of implantation of PCL/PVP/Ilo/A prostheses

The original protocol of layer-by-layer fusion method used to form the PCL external reinforcement scaffold prevented the vascular graft's visual and surgical properties from deteriorating. As shown in Fig. 1, a, the surrounding polymer fiber did not deform the product wall and did not peel off from it, allowing the outermost reinforcing threads to be captured in a continuous microsurgical suture during prosthesis implantation into the sheep carotid artery using the end-to-end method. This ensured the robustness of the "prosthesis + artery" complex and prevented the reinforcing scaffold from peeling off the base of the product (Fig. 1, b).

Ultrasound results

According to ultrasound results, 100% patency was noted on days 1 and 5 after implantation of PCL/PVP/Ilo/A grafts. Blood flow velocity during this period ranged from 120.35 cm/s to 153.43 cm/s. At 1 month after implantation, patency was 83.3%, but by the third month, it decreased to 50.0% with this figure remaining after 6 months of implantation, until the 6-month follow-up deadline. Ultrasound did not reveal any aneurysm formation and stenosis in the prosthetic walls, neither did it detect any detachment of the external reinforcing scaffold (Fig. 2).

Results of the study of explanted vascular prostheses

Upon examining the implanted prosthesis site after 6 months of the experiment, it was observed that each of them had developed a moderately vascularized connective tissue capsule that clearly showed an intact exterior PCL scaffold. There were no stenoses, aneurysms, or any

Table 2

Mechanical properties of PCL/PVP/Ilo/A tubular polymeric scaffolds before and after creation of an outer reinforcing layer (longitudinal and transverse direction)

	n	Stress (MPa)	Relative elongation (%)	Young's modulus (MPa)
		Longitudinal direction (Me (25–75%))		
PCL/PVP/Ilo/A	6	0.98 (0.79–1.13)	285.0 (166.2–392.9)**	8.54 (6.04–15.87)**
PCL/PVP/Ilo/A with an outer reinforcing layer	6	1.35 (1.29–1.39)*	201.2 (128.1–232.3)*	6.5 (5.54–11.09)**
Sheep carotid artery		1.2 (1.06–1.9)	158.5 126.0–169.5)	0.49 (0.39–0.66)
		Transverse direction (Me (25–75%))		
PCL/PVP/Ilo/A	6	1.24 (1.13–1.31)	28.43 (20.64–38.45)	9.62 (8.84–10.49)
PCL/PVP/Ilo/A with an outer reinforcing layer	6	2.43 (2.2–2.87)*	30.83 (28.66–34.60)	20.62 (17.58–23.04)*

Note: *, $p < 0.05$ relative to PCL/PVP/Ilo/A vascular grafts with no outer reinforcing layer; **, $p < 0.05$ relative to sheep carotid artery (longitudinal direction).

significant prosthetic deformities found. There were no foci of inflammation (Fig. 3).

Based on the outcomes of explantation and further transverse dissection of the PCL/PVP/Ilo/A grafts, it was determined that 50% of the explanted prosthesis had no thrombus in the lumen. Occluded prostheses contained grayish thrombus over the entire area and length of the product (Fig. 3, b). Patent prostheses were elastic, no parietal thrombi were found, and the anastomoses were a smooth connection between the prosthesis and the artery, maintaining the blood vessel diameter (Fig. 3, c). After six months of functioning as a component of the vascular bed, visually patent prostheses matched the native sheep artery: the middle of the prosthesis and the "prosthesis + artery" complex maintained a round vascular lumen;

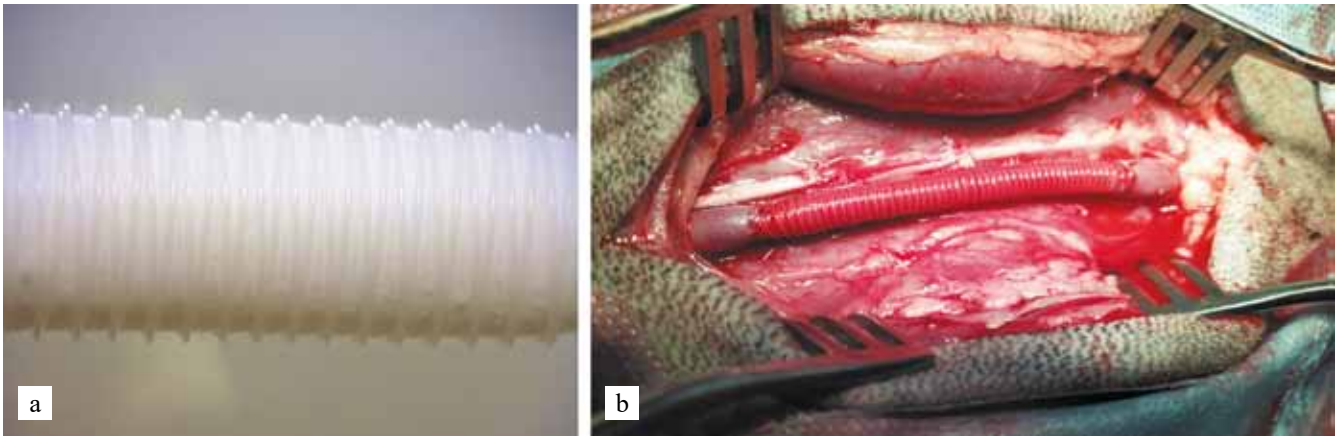

Fig. 1. PCL/PVP/Ilo/A vascular graft. a, prosthesis stereomicroscopy with an anti-aneurysmal scaffold, magnification 10×; b, prosthesis implanted in a sheep carotid artery

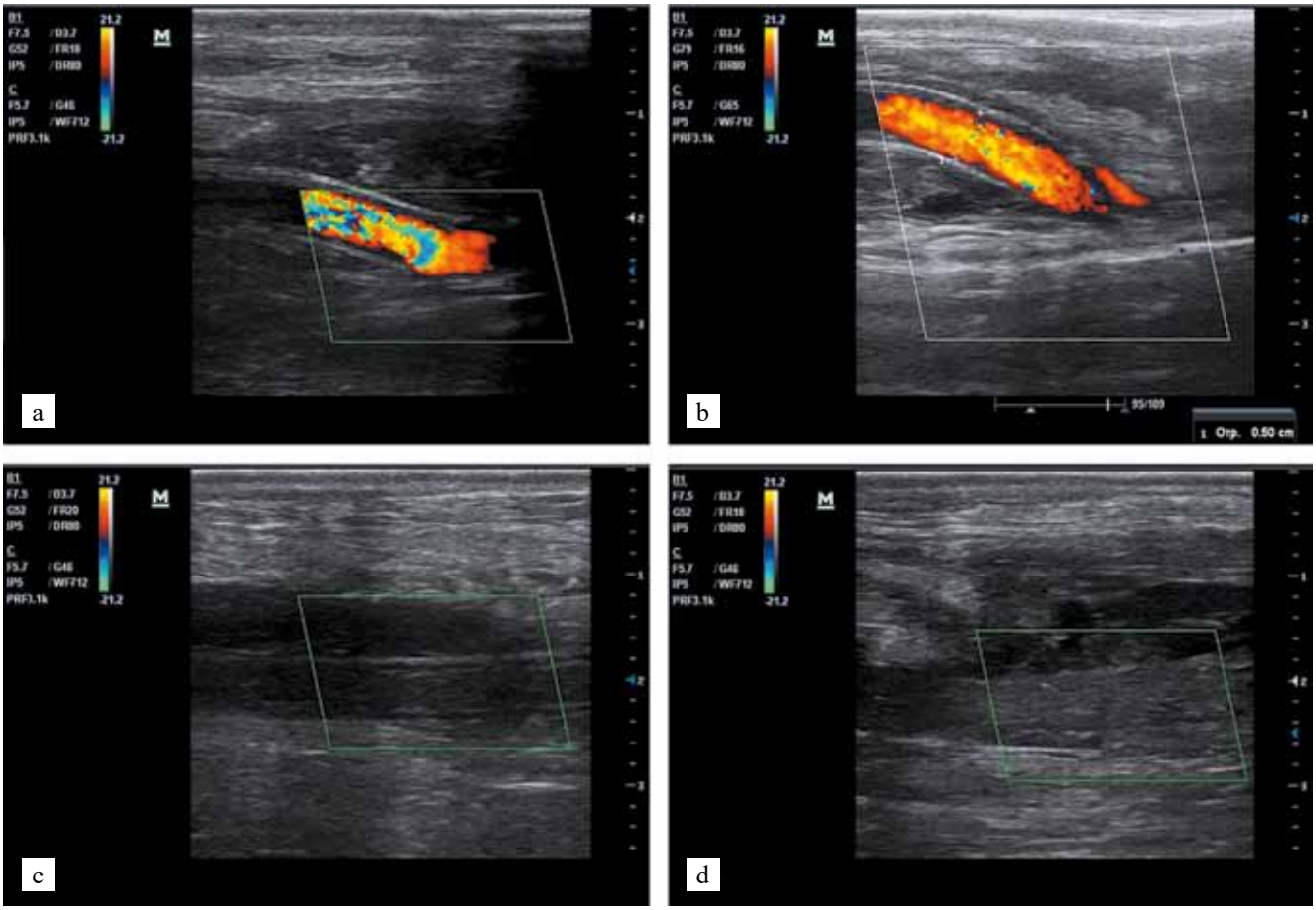

Fig. 2. Results of ultrasound monitoring of the patency of PCL/PVP/Ilo/A vascular grafts at different periods of implantation in a sheep carotid artery. Patent grafts: a, 5 days; b, 6 months. Thrombosed grafts: c, 5 days; d, 6 months

the thickness of the remodeled wall of the product was comparable to that of the artery wall (Fig. 3, c, d).

Results of histological examination

Based on the results of histological study of patent PCL/PVP/Ilo/A vascular grafts after 6 months of implantation into a sheep carotid artery, it was revealed that the tubular polymer base of the product almost completely biodegraded with the formation of newly formed three-layer vascular tissue on its base (Fig. 4). The newly created vascular wall consisted of a neointima with a thickness of 81 to 146 μm, neomedia with a thickness of 198 to 232 μm, and neoadventitia with a thickness of 144 to 217 μm. In the combined measurements, the wall of the remodeled vessel was 423 to 595 μm, which is comparable to thickness values for native sheep carotid artery (320 to 430 μm). On the side of the vascular lumen, the neointima was covered by a monolayer of elongated endothelium-like cells. The structural basis of neomedia was represented by cells similar in morphology to smooth muscle cells (see Fig. 4). The cell clusters were framed by collagen fibers. Scattered small clusters of structureless polymeric masses surrounded by single multinucleated foreign body giant cells, macrophages and fibroblast-like cells were also found in this layer. Vasa vasorum was predominantly detected along the border with the neoadventitia. The latter was the outer layer of the newly formed tissue, which resembled the structure of sheep artery adventitia and was formed by collagen bundles with partial filling with fibroblast-like and multinucleated giant cells (see Fig. 4). There were no signs of inflammation and calcification of the wall of the patent prosthesis.

The polymer base of the thrombosed PCL/PVP/Ilo/A grafts was also almost completely degraded 6 months after implantation without calcium deposits and inflammatory infiltration. These prostheses were distinguished by the presence of a thickened vascularized connective tissue capsule (see Fig. 4).

It is important to note the high preservation of the external wraparound scaffold in both occluded and patent prostheses: non-fibered round-shaped PCL filaments, surrounded by a collagen capsule without signs of calcification, were visualized in the transverse projection (see Fig. 4).

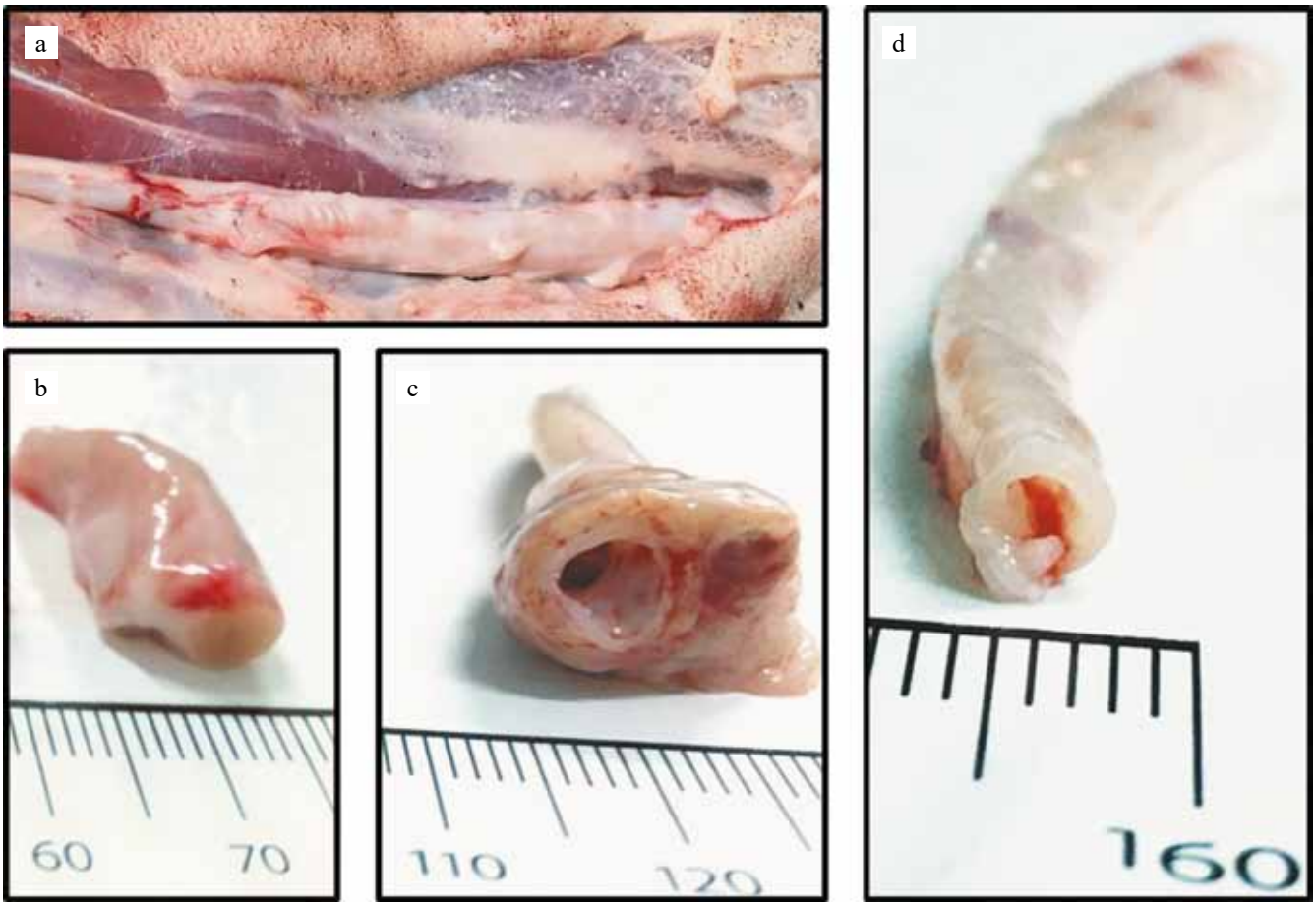

Fig. 3. Macrophotograph of explanted PCL/PVP/Ilo/A vascular grafts: a, macrophotograph of a PCL/PVP/Ilo/A graft at the moment of explantation; b, thrombosed graft; c, patent graft; d, intact sheep carotid artery

Immunofluorescence test results

The results of immunofluorescence study of explanted patent PCL/PVP/Ilo/A prostheses are consistent with those of histological study, confirming the formation of a continuous endothelial layer on the inner surface of the prosthesis (Fig. 5). The functionality of endothelial cells was demonstrated by the presence of vWF. A neointima of typical structure was formed on the graft wall, which consisted of α-SMA-positive cells and contained types III and IV collagens. A large amount of collagen types III and IV was also detected in the subendothelial layer. The prosthesis wall was actively populated with cells. Small complexes of $CD31^+$, α-$actin^+$, and vWF^+ cells were present in the wall thickness, which may indicate the formation of capillaries in the prosthesis wall. Also, large amounts of type III and IV collagens were found in the prosthesis wall (see Fig. 5).

Results of gene expression profiling

A comparative analysis of mRNA levels in the endothelial lysate and homogenate of patent remodeled PCL/PVP/Ilo/A grafts after 6 months of implantation showed that the newly formed vascular tissue at the site of the biodegradable vascular graft had differences from the genetic characteristics of native sheep carotid artery tissue. Genes found in the endothelial lysate were categorized into three groups according to the level of changes in expression: increased level (*IL6* (4.93-fold), *TNFA* (5.52-fold), *CTSB* (2.40-fold), *SMAD4* (2.63-fold)); decreased level (*ICAM1, FGF2, TGFB, MIF1, IL18, CNN, CXCL1, CXCL10, IL4, SELP, KLF4*); unchanged level (*IL1B, IL10, IL8, IL12A, VEGF, CXCR4, NR2F2, SNAI2, YAP1, KDR, MMP2, CD14*, *CD40L, EDN, PAI, SELE*).

For newly formed tissue, overexpression of the following genes in the homogenate was noted: *IL10* (2.83-

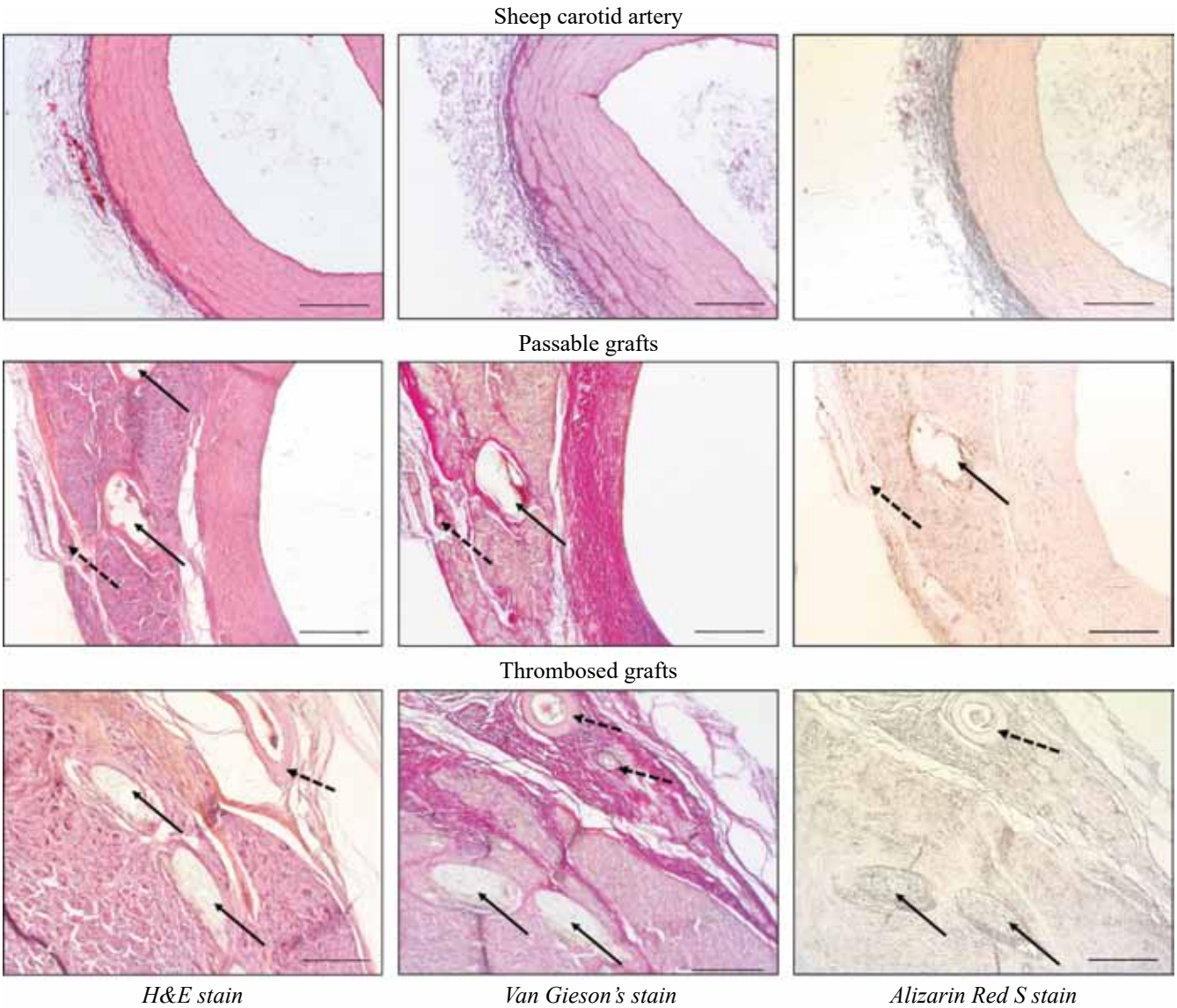

Fig. 4. Results of histological examination of explanted samples of PCL/PVP/Ilo/A vascular grafts at 6 months of implantation: solid arrow, PCL-strands of reinforced scaffold; dotted arrow, vasa vasorum. Scale bar 500 μm

fold), *CXCL 8* (4.56-fold), *TNF* (17.95-fold), *CXCR4* (13.42-fold), *TGFB* (6.06-fold), and *CTSB* (2.05-fold). Decreased mRNA levels compared to native sheep carotid artery tissue were noted for the following genes: *IL6, VEGF, NR2F2, SNAI2, ICAM1, YAP1, FGF2, MIF1, IL18, CD14, CD40L, EDN, EDN, IL4, SELE, SELP, SMAD4, KLF4, EDNRA* (Table 3).

Remodeling of patent PCL/PVP/Ilo/A grafts over 6 months of implantation was characterized by expression of genes with significant changes in mRNA levels, which are key markers from the position of endothelial biology processes such as inflammation (*IL6, IL4, CXCL8, IL10, TNFa, CD40L, CXCL1, CXCL10, MIF1*), endothelial to mesenchymal transition (*SNAI2*), endothelial differentiation (*VEGF*) and endothelial mechanotransduction (*YAP1, KLF4*), and leukocyte adhesion (*SELE, SELP*). It is worth noting that some important tissue remodeling markers were also associated with pronounced changes in mRNA levels: intercellular adhesion molecule (*ICAM*), smooth muscle cell marker (*CNN*), *TGFb* signaling pathway molecule (*SMAD4, TGFb*), and fibroblast growth factor (*FGF2*); (see Table 3).

DISCUSSION

High clinical demand is directing research efforts towards development of alternative small-caliber vascular shunts. The design of such artificial functionally active vascular prostheses should be based on the characteristics of the target native blood vessels in terms of implantation locus. The design of such a product should support the required blood flow, withstanding blood current pressure without experiencing mechanical damage. In addition, the structure of the inner surface of the prosthesis must prevent thrombus formation. Many functions of blood vessels are conditioned by structural components of different vascular layers at the biomolecular level. Therefore, it is necessary to ensure proper remodeling of the artificial scaffold of the vascular graft to fully reproduce the vascular tissue on its basis. Particular attention should be directed towards solving the problem of microbial contamination of the porous structure of a vascular graft. So, inclusion of an effective local antibacterial agent is necessary to prevent infection.

Formation of anti-aneurysmatic protection on the outer surface of polycaprolactone VGs contributed to higher prosthesis strength in longitudinal and transverse directions but caused a twofold increase in stiffness in the transverse direction, which is a standard but an undesirable moment for polymeric products.

The results of our own study of PCL/PVP/Ilo/A vascular grafts with antimicrobial coating and external reinforcing spiral implanted into a sheep carotid artery demonstrated 50% patency 6 months after implantation. When explanted, these prostheses were visually similar to the native sheep artery; There were no aneurysms, inflammation or other deformities. The exterior PCL spiral was highly preserved, indicating that its scaffolding function to prevent aneurysm formation at even

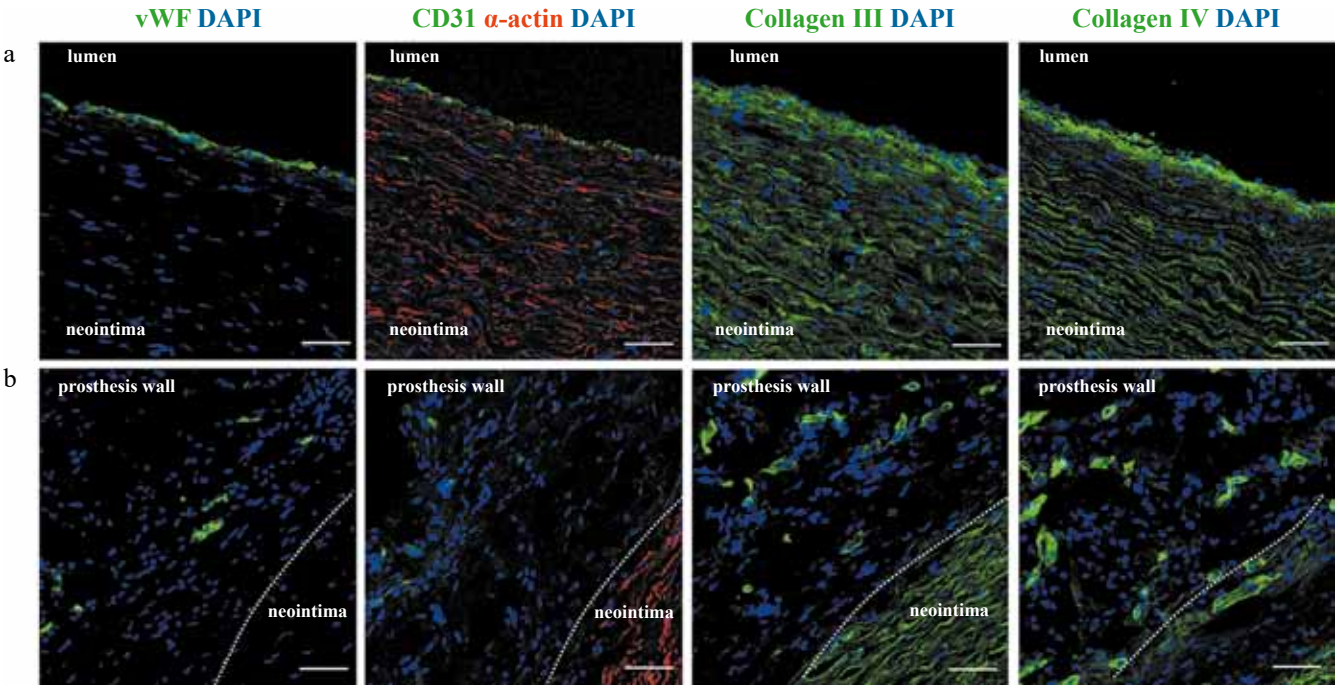

Fig. 5. Confocal microscopy of PCL/PVP/Ilo/A vascular grafts with anti-aneurysmal winding at 6 months of implantation: staining with specific fluorescent antibodies for von Willebrand factor (vWF, green), CD31 (endothelial cells, green), α-actin of smooth muscle cells (α-actin, red), collagen type III (green), collagen type IV (green). Cell nuclei were contrasted with DAPI (blue); a, prosthesis neointima and lumen; b, prosthesis wall. Scale bar 50 μm

longer implantation durations was successfully fulfilled. After implanting a nanocomposite vascular graft into a sheep carotid artery for 9 months, Ahmed et al. observed similar outcomes in terms of patency and development of prosthetic wall failure [20].

We were able to determine the features of the surrounding tissues and the degree to which the wall of the patent VGs had been successfully remodeled using histological methods. The explanted specimens had a neointima on their inner surface, which was covered in a continuous layer of mature, functionally active endothelial cells. Collagen fibers, smooth muscle-like cells, and vasa vasorum made up the middle layer. Neoadventitia was a representation of the outer layer. The newly formed tissue showed no signs of inflammation and calcification. These prostheses had morphological synchronization of the processes of biodegradation and remodeling of their wall.

Despite structural similarity between the newly formed vascular and native tissue, which was revealed by histological and immunofluorescent staining, a comparative analysis of gene expression profile showed some differences in their genetic profile. The CNN gene, which encodes the calponin protein and is a marker of smooth muscle cells, is of particular note in the results obtained. We found a significant decrease in gene expression for this gene in the neointima of remodeled prostheses, which is consistent with the proteomic profiling data we had previously obtained [19]. Furthermore, there was elevated expression of the CTSB gene encoding the lysosomal protein cathepsin B, in both endothelium lysate and homogenate. This finding could suggest chronic inflammation [21].

The process of reticulo-histiocytic cells biodegrading the polymer scaffold may be linked to an increase in inflammatory transcripts (TNFa) and transforming growth factor (TGFb) in homogenates of remodeled grafts. This

Table 3

Multiplicity of gene expression changes in endothelial lysate and homogenate of patent vascular grafts relative to intact sheep carotid arteries

Gene	Encoded protein	Homogenate	Endothelial cell lysate
IL1B	Interleukin-1 beta	0.58	0.65
IL4	Interleukin-4	0.11	0.37
IL6	Interleukin-6	0.27	4.93
IL10	Interleukin-10	2.83	0.71
CXCL8	Interleukin-8	4.56	1.99
IL12A	Interleukin-12A	0.97	1.57
IL18	Interleukin-18	0.2	0.5
TNF	Tumor necrosis factor	17.95	5.52
CXCL1	Growth-regulated alpha protein	0.88	0.06
CXCL10	C-X-C motif chemokine	0.08	0.16
VEGF	Vascular endothelial growth factor A	0.27	1.17
CXCR4	C-X-C chemokine receptor type 4	13.42	1.41
NR2F2	Nuclear receptor subfamily 2 group F member 2	0.14	0.75
SNAI2	Snai2	0.22	1.71
ICAM1	Intercellular adhesion molecule 1	0.36	0.35
YAP1	Yes-associated protein 1	0.005	0.68
KDR	Vascular endothelial growth factor receptor 2	0.84	1.06
FGF2	Fibroblast growth factor 2	0.014	0.18
MMP2	Matrix metallopeptidase 2	1.71	1.82
TGFB	Transforming growth factor beta-1	6.06	0.45
MIF1	Macrophage migration inhibitory factor	0.43	0.27
CTSB	Cathepsin B	2.05	2.40
CD14	Monocyte differentiation antigen CD14	0.41	1.35
CD40L	CD40 ligand	0.21	1.24
CNN	Calponin-1	0.03	0.42
EDN1	Endothelin-1	0.24	1.12
PAI1	Plasminogen activator inhibitor type 1	0.56	0.64
SELE	Selectin E	0.33	0.55
SELP	P-selectin	0.45	0.29
SMAD4	Mothers against decapentaplegic homolog 4	0.30	2.63
KLF4	Krueppel-like factor 4	0.40	0.09

is a known mechanism for resorption of biodegradable polymers. In addition, it is important to acknowledge the significant decrease in SNAI2 gene expression. This gene encodes a particular transcription factor that guarantees a modification in endothelial phenotype.

CONCLUSION

After being implanted into a sheep's carotid artery for six months, tissue-engineered SDVGs with an antithrombogenic drug-coated and reinforced external scaffold showed harmonious remodeling of the graft wall. Given that the newly formed vascular tissue was created entirely from scratch using an artificial matrix, the genetic discrepancies between it and the native one should not be surprising. This histological similarity indicates that the resulting graft wall remodeling rate is appropriate for the developed product to successfully function as part of the bloodstream. To enhance thromboresistance, more research is needed to improve the inner surface of the vascular graft.

The study was performed within the framework of the fundamental theme (No 0419-2022-0001) of the Research Institute for Complex Issues of Cardiovascular Diseases – "Molecular, Cellular and Biomechanical Mechanisms of the Pathogenesis of Cardiovascular Diseases in the Development of New Methods of Treatment of Cardiovascular Diseases based on personalized pharmacotherapy, introduction of Minimally Invasive Medical Devices, Biomaterials and Tissue-Engineered Implants".

The authors declare no conflict of interest.

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