A NOVEL SMALL-DIAMETER BIODEGRADABLE VASCULAR GRAFT BASED ON POLIOXYALCANOATES AND POLYCAPROLACTONE: PILOT STADY

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Abstract

Copolymer tubular scaffolds based on polyhydroxybutirate with hydroxyvalerate /polycaprolactone (5% PBHV: 10% PCL) were fabricated by the electrospinning setup. Physical and mechanical properties of the copolymer were assessed as well as their biocompatibility in vitro. According to the preliminary results, tubular copolymer scaffolds demonstrated improved stress-strain properties, compared to the native vessels used for coronary artery bypass grafting. High porosity of the internal surface allowed to create a cellular monolayer from bone marrow-derived multipotent mesenchymal stromal cells and human umbilical vein endothelial cells in a short-term period. There was no decrease in cellular viability in the cell-surface interaction. 2 mm tubular copolymer scaffolds were implanted in the abdominal rat aortas. After a 12-months follow-up period, good graft patency and the absence of organism reactions towards the implanted grafts have been observed.

Key words: *small-diameter vascular graft, biodegradable polymers, biocompatibility*

1. INTRODUCTION

Despite significant advances in reconstructive surgery, the search for new highly functional materials remains highly relevant to the needs of biomedical sciences, targeting to develop materials that can be used to renovate or restore function of diseased or traumatized tissues in the human body. Thus, an ideal small-diameter vascular graft has become a major focus of research (Chlupac et al, 2009). One starting point toward solving this problem is to manufacture a biodegradable vascular graft with improved hemocompatibility for further regeneration with capability to grow into a functional vessel. Long-term patency of grafts may be achieved by recreating neointima consisting of the patient's own cells, ideally - endothelial ones (McBaneaet al, 2012; Pawlowski et al, 2004). Therefore, material for an ideal vascular graft should possess excellent biocompatibility, long-term bioresorption and proper adhesive properties promoting successful culture of endothelial cells on the tubular scaffolds.

Aim: To develop and to assess in vitro and in vivo small-diameter biodegradable vascular grafts based on polyhydroxybutyrate / valerate and polycaprolactone.

2. MATERIALS AND METHODS

2 mm tubular scaffolds based on polyhydroxybutyrate / hydroxyvalerate, containing up to 10% of the valerate component (Sigma-Aldrich, USA) and polycaprolactone with the molecular weight of 80.000 (Sigma-Aldrich, USA) were fabricated using the electrospinning setup NANON (MECC, Japan) at the following processing parameters: the voltage - 20 kV, the flow rate of the polymer solution - 0.3 ml / hr, the distance between the capillary and collection screen - 150 mm, the diameter of the collection screen - 2 (for the subsequent implantation) and 4 mm (for cell culturing). The wall thickness and surface morphologies of tubular copolymer scaffolds were assessed by a scanning electron microscope S3400N (Hitachi, Japan). Physical and mechanical tests were carried out on a Zwick/Roell Universal Testing Machine (Zwick/Roell, Germany). The load was applied in the longitudinal direction to the samples (n = 10) fabricated according to the same protocol. Tensile and stress-strain properties, namely ultimate elongation and Young's modulus, were assessed. Native human veins for coronary artery bypass grafting were used as the control.

Cell adhesion and cytotoxicity of tubular scaffolds were tested on multipotent mesenchymal stromal cells derived from rat bone marrow (BM MMSCs) and human umbilical vein endothelial cells of (HUVEC). MMSCs were harvested from femurs of Wistar rats. Cells were grown using DMEM culture media (Gibco, USA) with 1% HEPES buffer, 10% fetal calf serum, 1% L-glutamine, 100 U/ml penicillin, 0.1 mcg/ml streptomycin, 0.1 .mcg / ml amphotericin B (Sigma Aldrich, USA) at a temperature of 37 ° C and 5% CO2.

Human umbilical vein endothelial cells were isolated using a technique adapted from Jaffe et all. (1973). The subsequent cell culturing was carried out using Endothelial Cell Cultur Medium Kit (BD Bioscience, USA). Culture medium was changed every 2 - 3 days.

The third-passage of BM MMSCs and the second passage HUVEC were cultivated on the scaffolds. The percent of viable and apoptotic cells was detected before cultivation by flow cytometric analysis with co-staining of cell suspension with Annexin V labeled with PE in conjunction with 7-AAD. The cells were seeded at a density of 2.5 $\times 10^5$ per 1.8 mm^2 onto the scaffold surface and cultivated for 4 days. BM MMSCs, seeded in tissue culture plastic, were used as controls. After cultivation, the cells were stained with PKH 26 (Sigma, USA) and assessed by fluorescence microscopy. Other cells were detached from the surface of scaffolds with 0.5% trypsin-EDTA solution (Sigma Aldrich, USA), incubated for 10 minutes at 37 °C. After the incubation, the trypsin activity was quenched with warm growth medium (10% of the volume of trypsin solution). The cells were centrifuged, washed and subjected to flow cytometric analysis to determine the percentage of viable and apoptotic cells (staining with Annexin V, labeled with PE in conjunction with 7-AAD).

To assess the long-term patency, 2 mm tubular copolymer scaffolds were implanted into the abdominal aorta of 6-month-old Wistar male rats weighing about 300 - 350 g. (n = 12). Serial Doppler ultrasound was performed to assess graft patency within a 12-months follow-up period.

The samples were stained with hematoxylin and eosin using the standard technique to perform histological assessment by the light microscope AXIO Imager A1 (Carl Zeiss, Germany). PECAM-1 antibodies (SantaCruze, USA) were used to perform immunohistochemical studies according to the standard methods.

To assess immune and inflammatory responses towards the vascular graft, serum levels of proinflammatory cytokines, namely TNF-α, IL-1β, growth factors, VEGF, TGF-β1, and adhesion molecule, sICAM-1, were measured in rats, euthanatized after a 12-months follow-up period. Intact rats were used as controls (n = 12). Serum levels of the markers were determined by ELISA using kits purchased from eBioscience, R & D Systems, Hycult biotech, Life technologies (USA) according to the manufacturers' instructions.

The data were processed using the standard software package Statistica (version 6.0). The non-parametric Wilcoxon test was used to determine significant differences. Data are presented as median and 25^{th} and 75^{th} quartiles (Me (25%, 75%). The confidence interval was 95%, $p < 0.05$.

3. RESULTS

The studied polymers and the ratio of polymers in their common solution, used to fabricate a small-diameter vascular graft, have been previously discussed in (Antonova et al, 2013).

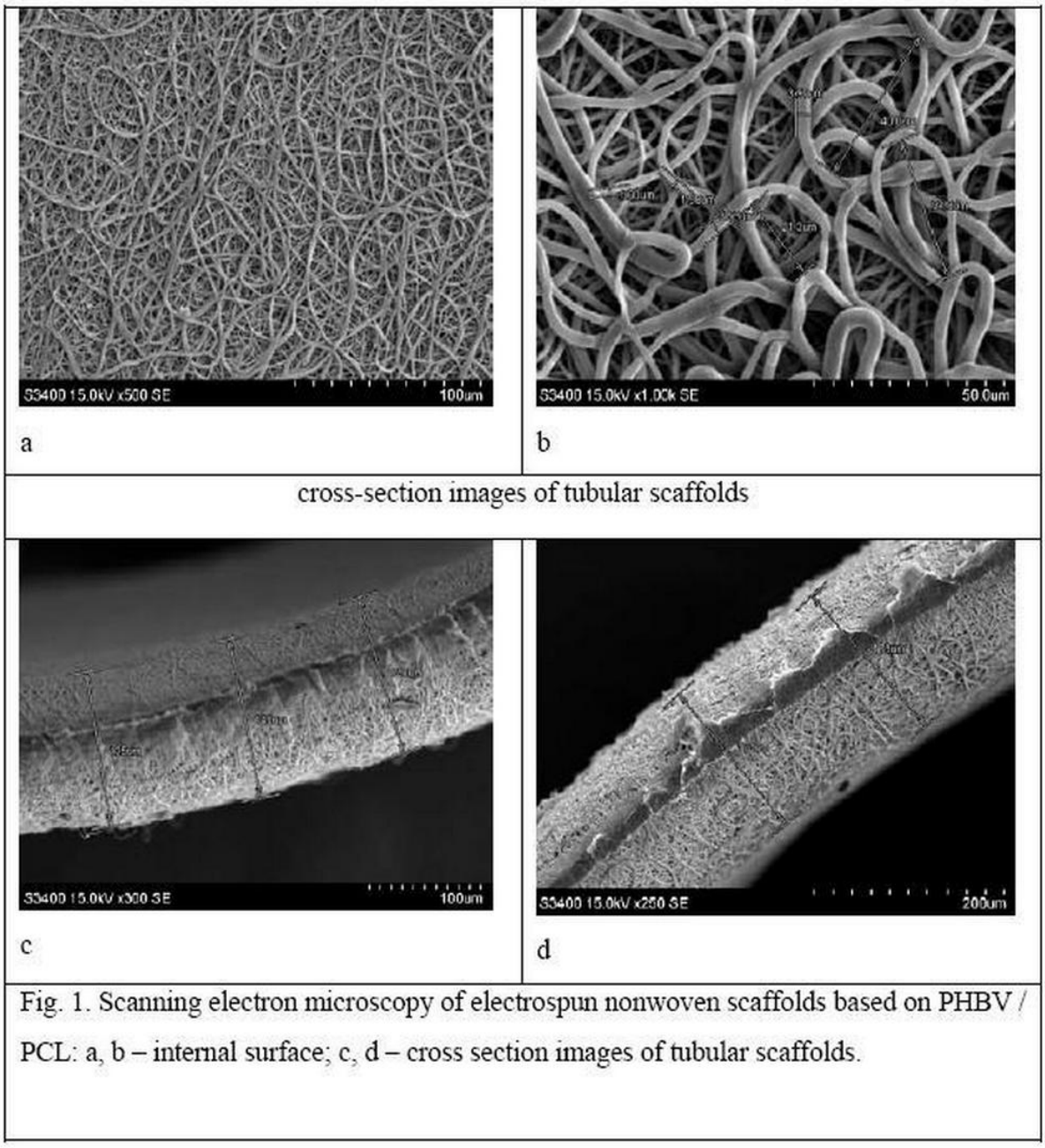


Fig. 1. Scanning electron microscopy of electrospun nonwoven scaffolds based on PHBV / PCL: a, b – internal surface; c, d – cross section images of tubular scaffolds.

The most suitable composition comprises polyhydroxybutyrate with hydroxyvalerate / polycaprolactone (PHBV / PCL). Moreover, the optimal ratio of PHBV:PCL in the polymer solution is 5%:10%. The scaffolds produced from this co-polymer solution has long-term resorption, necessary for successful tissue reconstitution de novo in the implant sites.

Tubular scaffolds, with a high surface area to volume ratio, based on PHBV / PCL solution, were fabricated using the electrospinning setup (Fig. 1).

The tubular co-polymer scaffolds have highly porous internal surface. The thickness of the polymer fibers ranges from 1.44 micron to 3.97 micron. The fibers are randomly arranged, promoting the formation of pores ranging in size from 21.3 mm to 40.0 mm.

Tubular scaffolds possess improved stress-strain properties compared to the native vessels (Table 1).

Table 1. Mechanical properties of electrospun tubular polymeric scaffolds based on PHBV / PCL and native veins

	Shear stress, MPa	Ultimate elongation, %	E_{mod}, MPa
PHBV / PCL grafts, n=10	7.05 (5.58-8.02)*	431.33 (397.3-574.55)*	28 (22-3.9)*
Autoveins, n=10	10.09 (8.05-12.13)	73.1 (62.8-81.8)	1.37 (0.89-1.75)

* - significant differences between vascular graft and autoveins, $p<0.001$

Tubular PHBV / PCL scaffolds demonstrated a 5.9-fold increase in tensile strength compared to the native veins. Thus, vascular graft stiffness increased 20.4-fold in comparison with the native veins, whereas critical stress decreased 1.4-fold, but Young's modulus reported a 20.9-fold increase compared to the native veins ($p <0.001$). Thus, tubular scaffolds based on PHBV / PCL are more stiff and rigid, but they may stretch more than the native vein. However, increased stiffness of the grafts may affect their hemodynamic properties and their durability, requiring further investigation and improvement.

Advanced architectonics of the internal surface (high porosity) accelerates the formation of a MMSCs and HUVEC monolayer after a 4 days culture in the scaffolds (Fig. 2).

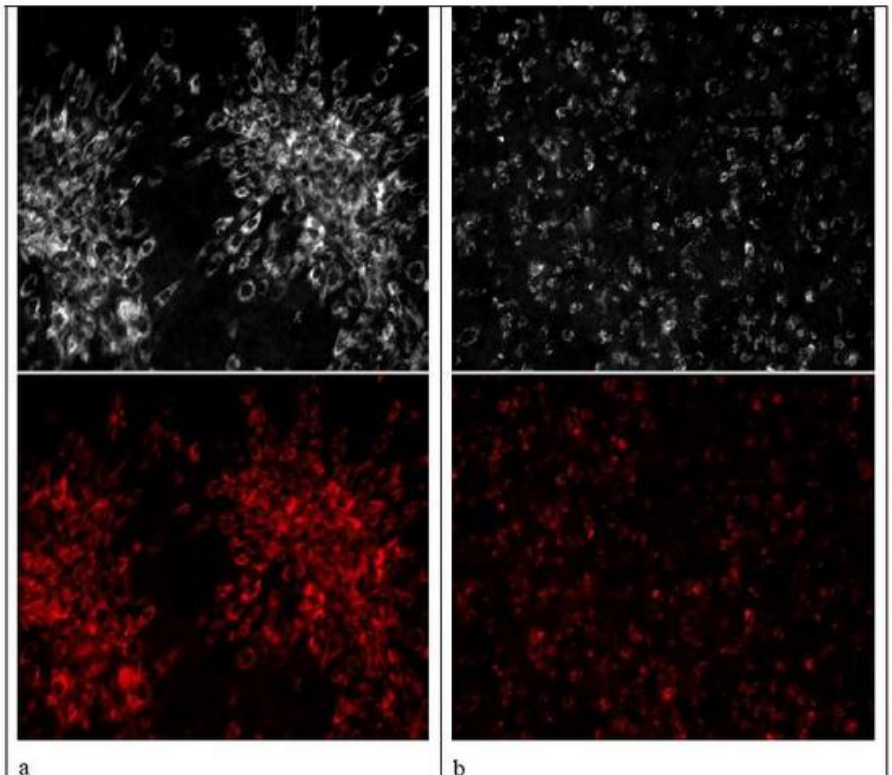


Fig. 2. Fluorescence microscopy of cells, seeded onto polymer scaffolds based on PHBV / PCL: a – BM MMSCs, b - HUVEC. x 200.

However, there was no significant reduction in cell viability after culturing of HUVEC and MMSCs in the scaffolds (Table 2).

Table 2. The assessment of cytotoxicity of the scaffolds based on PHBV / PCL

Cell type	Viability, %	Early apoptosis, %	Late apoptosis, %	Necrosis, %
HUVEC on the scaffolds	83.7	6.0	9.1	1.1
BM MMSCs on the scaffolds	80.4	4.2	13.5	1.9
BM MMSCs in tissue culture plastic	94.1	1.26	4.1	0.54

Serial Doppler ultrasound within a 12-months follow-up period after the implantation of 2 mm co-polymer tubular scaffolds into rat abdominal aortas reported good patency. There were no visible signs of injuries and aneurysms on the surface of the grafts (Fig. 3).

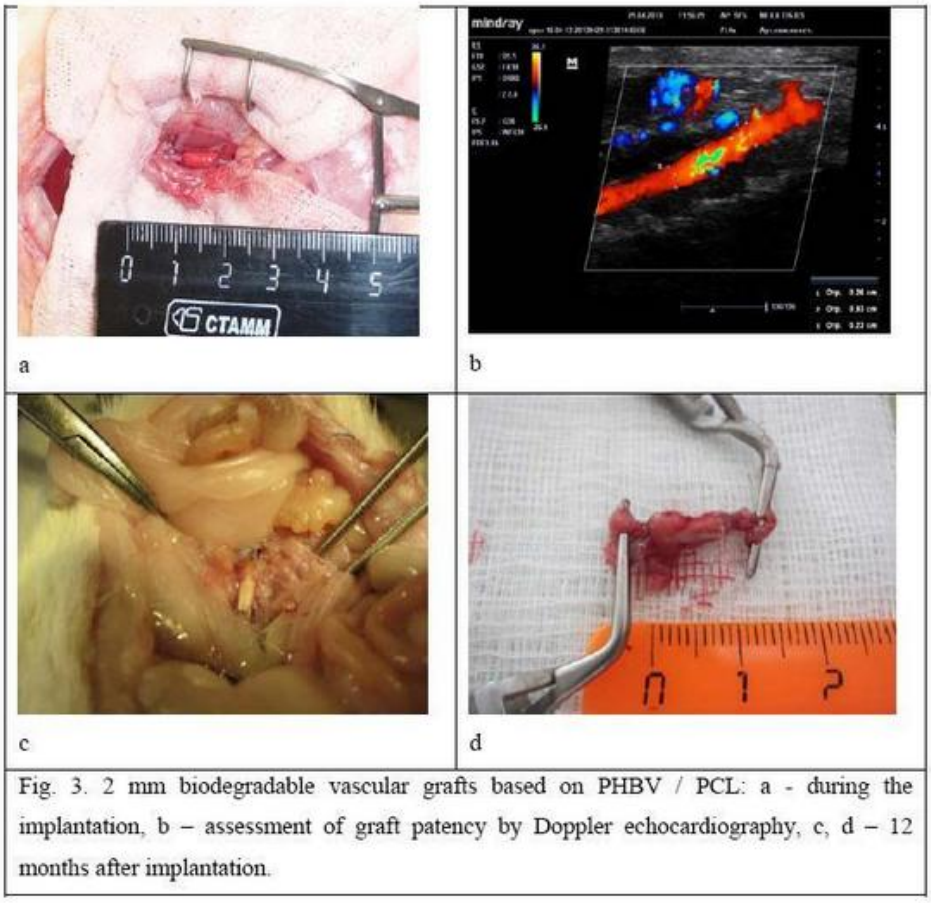


Fig. 3. 2 mm biodegradable vascular grafts based on PHBV / PCL: a - during the implantation, b – assessment of graft patency by Doppler echocardiography, c, d – 12 months after implantation.

However, the histological assessment suggested the implantation of unloaded and unmodified tubular co-polymer scaffolds into vascular beds of laboratory animals to provoke complications, namely thrombus formation at the site of the distal anastomosis (16.7%), chronic inflammation (16.7%) and neointimal hyperplasia (16.6%). These specific device-related complications are associated with incompleteness of graft design. Importantly, 50% of vascular grafts with endothelial cell monolayer did not demonstrate the complications mentioned above (Fig. 4). Moreover, light microscopy images clearly shows the presence of cellular elements localized inside the walls of the implanted grafts and proves that the bioresorption of tubular scaffold s is accompanied with its full regeneration by the living organism's own cells. Thus, it confirms high biocompatibility of polymeric materials used to produce these scaffolds, emphasizing their suitability for tissue-engineering.

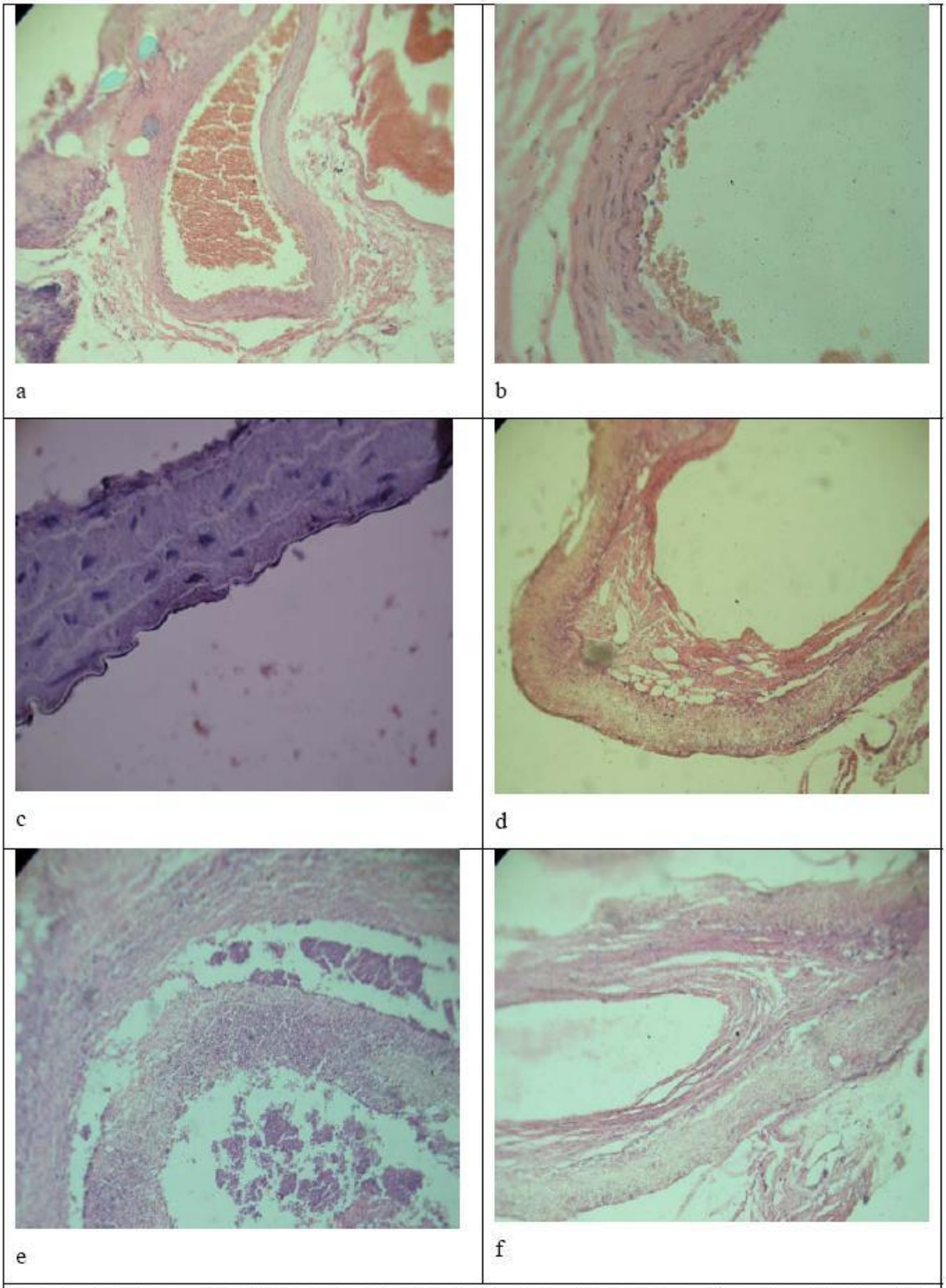


Fig. 4. Histological and immunohistochemical study of the vascular grafts based on PHBV / PCL after 12 months of implantation: a – graft without signs of pathology (hematoxylin-eosin staining, × 50); b – an endothelial cell monolayer on the internal surface of the graft (hematoxylin-eosin staining, × 200); c - immunohistochemical staining of endotheliocytes (staining with antibodies to PECAM-1, x 400); d – recanalization of the thrombus in the graft lumen (hematoxylin-eosin staining, x 100); e - inflammatory infiltration of the graft wall (hematoxylin-eosin staining, x 100); f - neointimal hyperplasia, narrowing the graft lumen (hematoxylin-eosin staining, x 100).

However, there are several disadvantages, which need further investigations to improve antithrombotic protection, sterilization method and additional modification of the internal surface according to the graft model.

The assessment of the organism response towards long-term implantation of vascular grafts reported the absence of significant differences in the serum levels of proinflammatory cytokines, TNF-α, IL-1β, between the experimental and control groups (Table 3).

Table 3. Serum levels of pro-inflammatory cytokines, growth factors and cell adhesion molecules in the experimental group and control group

Serum markers	Experimental group, n=12	Control group, n=12
IL-1β, pg/ml	5.631 (4.023; 7.992)	7.2 (7.1; 7.3)
TNF-α, pg/ml	0	0
VEGF, pg/ml	0.551 (0; 4.599)*	0
TGF-β1, pg/ml	28.11 (21.458; 35.293)*	24.25 (21.0; 25.698)
sICAM-1, ng/ml	28.808 (27.273; 30.508)*	25.95 (24.795; 27.658)

* - $p<0.05$

Vascular endothelial growth factor (VEGF) is known to be one of the key molecules for angiogenesis and for the survival of the endothelium cells. It is a specific endothelial cell mitogen and a vascular permeability factor. Serum VEGF levels (0.551 (0.0; 4.599) pg/ml) were detected only in rats with implanted small-diameter vascular grafts (Table 3). This fact can be explained by either the formation of the endothelial monolayer on the internal surface of vascular grafts, confirmed by the histological and immunohistochemical studies, or the origination of vasa vasorum (vascular vessels) at the site of implanted grafts. However, the last is a standard host reaction to the implantation of prosthesis at different sites of vascular system.

Transforming growth factor TGF-β1 is involved in various physiological and pathophysiological processes, including immunological, inflammatory and neoplastic. TGF-β1 is synthesized and secreted by various cells, including lymphocytes and macrophages, and is involved in angiogenesis and tissue repair. In our experiments, TGF-β1 levels was detected in both groups. Thus, its levels were 1.2 times higher in the experiment group than in the control group (Table 3). This may be explained by the fact that after a 12-months period of the graft implantation, repair and bioresorption process continued over time.

Cell adhesion molecule, sICAM-1, is expressed on non-hematopoietic cells, namely vascular endothelial cells, fibroblasts, and on hematopoietic cells, namely tissue macrophages. Its expression is correlated with the infiltration of lymphocytes into the inflammation region. sICAM-1 is known to be a marker, triggering inflammatory reactions. In our experiment, serum levels of sICAM-1 increased 1.2-fold in rats with implanted grafts to compared to the intact animals . This may be associated with the constant attraction of cells of the monocyte-macrophage area to the grafts sites as the primary cellular pool involved in bioresorption of biodegradable polymers.

4. CONCLUSION

The results of the comprehensive assessment in vitro and in vivo of small-diameter biodegradable vascular grafts (2 mm) demonstrated high biocompatibility of PCL and PHBV polymers, used to fabricate tubular scaffolds. The patency of the grafts, implanted in the vascular beds of laboratory animals for 12 months, and the absence of immune response to the implanted graft have confirmed the possibility to use these polymers as a basis for creating a hybrid small-diameter vascular graft. Some structural rigidity, signs of thrombosis in the area of the distal anastomosis, inflammatory infiltration into the graft wall and neointimal hyperplasia require further improvement of physical, mechanical and surface properties of the grafts in order to ensure their functional viability and long-term patency.

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