USE OF POLYCAPROLACTONE GRAFTS FOR SMALL-DIAMETER BLOOD VESSELS

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Current trends are toward actively developing approaches of tissue engineering, aimed at creating vascular grafts of small diameter. This is due to the existing in cardiovascular surgery demand for prostheses to be used in coronary artery bypass grafting. The present work was undertaken in order to assess possibilities of using small-diameter vascular grafts made of biodegradable polymer polycaprolactone by means of electrospinning. The authors studied physico-mechanical properties and structure of polycaprolactone grafts, as well as their thromboresistance and patency after implantation into the vascular bed of rats. The obtained results demonstrated optimal physico-mechanical properties of the vascular grafts, their biocompatibility, endothelialisation of the internal surface, and infiltration of the graft's wall by cells with the formation of new tissue, accompanied and followed by the development of an extensive intimal layer in the zones of the anastomoses.

Hence, the study showed possibilities of using polycaprolactone grafts as vascular prostheses, however requiring their further modification which would promote and contribute to a decrease in hyperplasia of connective tissue in the graft's lumen.

***Key words**: tissue engineering, vascular graft, polycaprolactone, electrospinning.*

INTRODUCTION

Cardiovascular diseases associated with obliteration of blood vessels are the leading cause of mortality and invalidization of the population in the developed countries of the world [1]. Treatment of such diseases is based on bypass operations. Surgical procedures aimed at restoring blood flow in ischemic zones are performed using autologous veins or arteries as bypass grafts. However, lack of the required veins or arteries resulting from their damage or carrying out re-operations in 30% of patients leads to the necessity of using alternative vascular grafts [2, 3]. To the latter belong homografts whose use is extremely limited due to small availability of the donor material, also synthetic grafts such as Dacron from polyethylenetetrafolate and Gore-Tex made of polytetrafluoroethylene, as well as biological prostheses made of xenomaterials [4]. Despite their usability and commercial availability synthetic and biological prostheses measuring less than 6 mm in diameter cannot be used as bypass grafts since there is high risk of their rapid obliteration resulting from extensive hyperplasia of the neointima or thrombus formation [5].

By now, there are several strategies aimed at avoiding such problems. One of them consists in creation of blood vessels by means of in vitro tissue engineering using a polymeric biodegradable matrix, patient's cells and biologically active molecules [6, 7]. Unfortunately, creation of an individual graft for a patient from his/her own cellular material is a complicated and laborious method which limits its clinical application. Of great interest is creation of polymeric grafts which would be inhabited by cells in vivo and simultaneously undergo biodegradation which should provide regeneration of the blood vessel. One of the promising materials for creating such grafts is a synthetic polymer polycaprolactone (poly-ε-caprolactone – PCL) known by its good mechanical properties. The mechanism of its in vivo degradation is conditioned by a slow hydrolytic process with the formation of non-toxic products [8].

The present work was aimed at evaluating possibilities of using grafts made of polycaprolactone as prostheses for small-diameter vessels.

MATERIALS AND METHODS

Manufacture of PCL grafts. Vascular grafts (an internal diameter of 2 mm and wall sickness measuring 100 μm) were manufactured by means of electrospinning from a biodegradable polymer PCL (M=80 000) (Sigma-Aldrich, USA). Electrospinning was carried out under the following conditions: a 10 % solution of PCL in

chloroform, voltage at the needle + 15 kV, solution flow rate 1 ml/h, distance between the needle and the collector 15 cm. A rotating pin measuring 2 mm in diameter was used as the collector.

Physico-mechanical properties of PCL grafts. Physico-mechanical tests were performed on versatile testing machine (Zwick/roell, Germany) under the conditions of uniaxial tension of the samples (n=15). Assessing the physico-mechanical properties of the biomaterial, we took into consideration the parameters of strength and elastic deformity. Strength was evaluated by the maximal tension stress, elastic-deformity properties were assessed by the modulus of elasticity (E_{mod}) and percent elongation until the integrity of the sample is broken. For better accuracy of measuring the relative elongation the sample was preliminarily loaded with 0.01 N. As the control, we used vascular bioprostheses "KemAngioprotez" («NeoCor» Closed Corporation, Russia) made of the bovine thoracic artery and treated with ethylene glycol diglycidyl ether. These prostheses are used for restoration of damaged small-to-medium-diameter arteries [9].

Assessment of blood platelet adhesion on PCL grafts. Blood platelet adhesion on the PCL grafts and "KemAngioprostez" biografts was assessed in in vitro experiments by means of the multichannel peristaltic pump 2054U/CA24 (Watson-Marlow, Great Britain). The lines containing fixed samples 3 cm long were filled with fresh citrated donor blood, with a blood: citrate ratio of 9:1. The velocity of blood circulation amounted to 0.04 l/min, at t=37 degrees Centigrade. Forty minutes after contact with blood the grafts' segments (n=10) were fixed in 2% solution of glutaric aldehyde in phosphate buffer (pH=7.4), then were placed into a thermostat at t=37°C until dried completely. Adhesion and morphological alterations of blood platelets on the surface of the material were assessed by means of scanning electron microscopy.

Scanning electron microscopy. While assessing the structure of the graft's surface and platelet adhesion on the polymeric grafts and bioprostheses "KemAngioprostez", the samples were covered with gold current-conducting sputtered 30-nm coating to be further examined using the scanning electron microscope S3400N (Hitachi, Japan).

Implantation of PCL grafts into the abdominal portion of the aorta of rats. The vascular grafts with an internal diameter of 2 mm and wall thickness of 100 μm were implanted into the abdominal portion of the aorta of rats. The animals were kept in the conditions of a vivarium with ad libitum access to food and water on the nutrition ration. The experiment was carried out at the Laboratory of the Cleveland VA Medical Center according to the protocol approved by the IACUC (Institutional Animal Care and Use Committee, Cleveland VA Medical Center). The Wistar male rats weighing 400–450 g (n=5) were narcotized by an intra-abdominal injection of 400 mg/ml of sodium thiopental. Prior to operation 10 mg/kg cefazolin was injected to the rats. During surgical intervention ECG was performed and body temperature measured. All animals were operated on under inhalation anaesthesia with 1% isoflurane. Median laparotomy was followed by opening of the retroperitoneal space and exposing of the aorta which was then clamped below the renal artery and above the level of bifurcation. The proximal anastomosis was performed using the suture material 9–0. The graft was washed out and the aorta re-clamped. The distal anastomosis was established in a similar manner. Once the clamps removed, the presence of blood flow through the graft was confirmed intraoperatively by means of vascular Doppler. After six weeks the animals were withdrawn from the experiment. The anastomosis zone and the PCL graft itself were assessed for the presence of haemorrhage and thrombus formation. Neointimal hyperplasia and the degree of colonization of the graft with cells were studied by means of light microscopy, staining the preparations with haematoxylin-eosin, as well as according to the Mallory and van Gieson techniques.

Statistical methods. The obtained findings were processed using the Applied Programs Package Statistica 6.0 (StatSoft Inc., USA). Normalcy of distribution was evaluated by means of the Kolmogorov–Smirnov criterion. Statistical significance was determined by means of the Mann–Witney non-parametric criterion. The differences were regarded statistically significant if $p<0.01$. The data were represented as the mean ± standard error or as a median and the 25th and 75th percentiles (25‰<M>75‰).

RESULTS

Studying the polycaprolactone grafts by means of scanning electron microscopy demonstrated that the surface had a high-porous structure, formed by polymeric fibres 3.340±0.510 μm thick. The fibres were uniform in thickness with no visible defects (Fig. 1).

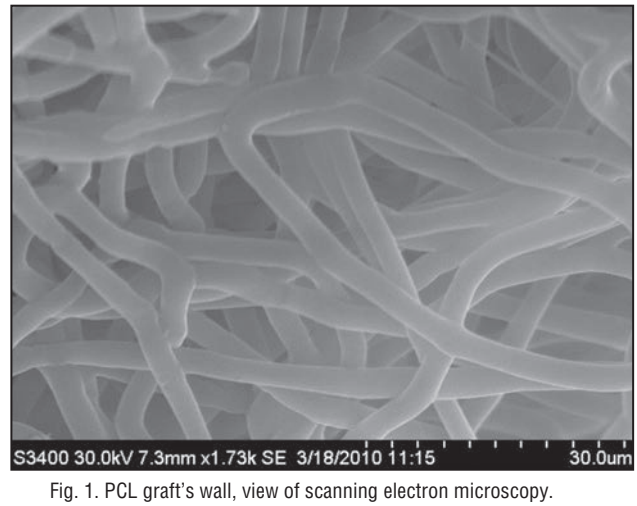

Fig. 1. PCL graft's wall, view of scanning electron microscopy.

Table

Physico-mechanical properties of polycaprolactone grafts and bioprostheses "KemAngioprotez"

Vascular prostheses	Strength, MPa (25‰<M<75‰)	Elastic deformation	
		Percent elongation, % (25‰<M<75‰)	$E_{мод}$ (25‰<M<75‰)
Bioprosthesis "KemAngioprotez"	0.85<1.26<1.42	79.8<95.4<100.6	0.24<0.28<0.34
PCL graft	1.51<1.88*<1.98	202.2<232.1*<441.4	3.27<3.89*<5.57

**p<0.01 as compared to bioprostheses "KemAngioprotez".*

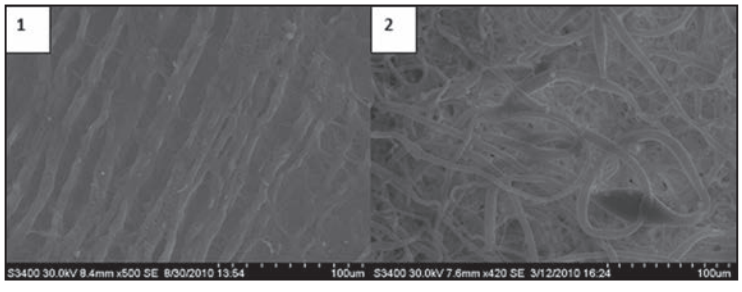

Fig. 2. Scanning electron microscopy of the surface of the bioprosthesis "KemAngioprotez" (1) and PCL graft (2) 40 minutes after contact with blood.

Assessing the mechanical strength and elasticity of vascular grafts we used biological prostheses of blood vessels "KemAngioprotez" as control, since the physico-mechanical properties of these prostheses do not differ from those of the native arteries. The results of physico-mechanical tests of vascular grafts showed that the indices of strength of the PCL grafts are higher than those of bioprostheses "KemAngioprotez" (Table). The indices of elastic deformation of the PCL grafts also exceed those of bioprostheses "KemAngioprotez": relative elongation – by a factor of 2.4 (p=0.00001), elasticity modulus (E_{mod}) by a factor of 14 (p=0.00001), thus strongly suggesting their greater elasticity as compared to the control samples. As a whole, the results of physico-mechanical tests are indicative of sufficient strength and satisfactory properties of elastic deformation of PCL grafts.

Haemocompatibility is known to be an important property of bioprostheses interacting with blood. For assessment of medical-purpose items contacting with blood, one of the main tests recommended by ISO 10993:2009 is the study of activation of blood platelets. Examining the internal surface of the grafts after contact with blood by means of scanning electron microscopy revealed a considerable number of adhered thrombocytes, while the samples of bioprostheses "KemAngioprotez" contained no blood platelets (Fig. 2). Despite the fact that the grafts' surface showed a considerable number of adhered thrombocytes there were neither changed nor spread out forms of cells, which may be indicative of haemocompatibility of the tested samples.

According to the results of similar studies on implantation of synthetic grafts into the blood channel of small laboratory animals complete endothelialisation of the prosthesis occurs by the end of week 6 [10]. In this connection, the term of 6 weeks was chosen as critical for termination of the experiment and withdrawal of animals. The implanted graft was exposed with the adjacent portions of the native aorta. Histological studies showed that in the lumen of the graft there was a continuous layer of the neointima (Fig. 3). The inner surface of the graft was covered with endothelial cells the majority of which had increased hyperchromatic nuclei and decreased nuclear-cytoplasmatic index as compared to the endothelial cells of the native aorta. The graft was infiltrated by cells with morphological properties of myofibroblasts and macrophages. Portions of accumulation of collagen rich with glycosaminoglycanes, laminin and fibronectin were revealed in the whole thickness and all long the length of the graft. Besides, there were no histological signs of the graft's degradation, thus suggesting a slow rate of the polymer destruction.

DISCUSSION

The fact of currently lacking small-diameter vascular conduits possessing good patency and durability calls for attempts aimed at creating new vascular grafts to be infiltrated with cells in vivo on the basis of biodegradable polymeric matrices.

On the one hand, an ideal graft should possess mechanical properties corresponding to native arteries. On the other hand, it should also imitate morphology of the extracellular matrix which may be provided by

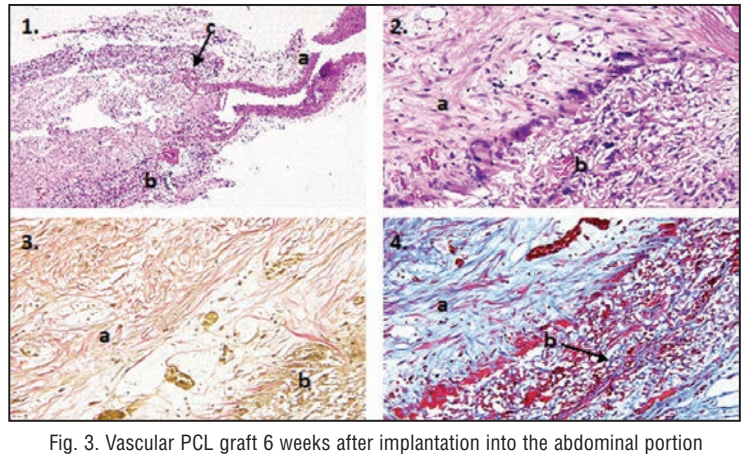

Fig. 3. Vascular PCL graft 6 weeks after implantation into the abdominal portion of the aorta of the rat: 1. Haematoxylin and eosin stain, magnification × 40: a – intact aorta, b – PCL graft, c – anastomosis zone; 2. Haematoxylin and eosin stain, magnification × 400: a – neointima, b – graft's wall infiltrated with cells. 3. Haematoxylin and eosin stain, magnification × 400: a – neointima, b – graft's wall; 4. Mallory's stain, magnification × 400: a – neointima, b – portions of collagen in the graft's wall.

highly porous surface consisting of nanofibers measuring 5–500 nm in diameter and 5–500-μm pores [11]. In its term, mechanical properties, as well as micro and nano-structure of the final product would depend not only on the polymer used but also on the method of its manufacturing. One of the methods of creating polymeric grafts, attracting ever increasing attention during recent years, is electrospinning of polymeric solutions. This method makes it possible to create tissue-engineering matrices consisting of the thinnest fibres.

The findings of scanning electron microscopy of the graft made by the method of electrospinning demonstrated that its wall consisted of intertwisted between each other fine fibres forming pores. Such structure of the surface is capable of imitating the cellular matrix suitable for formation of new tissue on its base. Thanks to a high coefficient of the ratio of the matrix's surface to its volume there occurs an increase in cell adhesion of cells on the tissue-engineering graft, cell migration, as well as cellular proliferation and differentiation. Besides, porosity of the material promotes transport of nutrient substances to the newly forming tissue.

Since a vascular graft is implanted directly into the blood flow, it analogously to arteries should withstand pressure of blood. The results of physico-mechanical tests demonstrated that PCL grafts are superior to bioprostheses "KemAngioprotez" by the indices of strength and elasticity. The porous structure of PCL grafts contributes to an increase in their relative elongation without decreasing the modulus of elasticity. In its turn, according to earlier studies it is known that bioprostheses "KemAngioprotez" possess satisfactory physico-mechanical properties, which makes it possible to use them in surgical practice for implantation during operations on replacement of affected medium-to-small diameter arteries [9]. It may be supposed that PCL grafts would not be inferior by strength to prostheses from biological material, as well as native vessels, with the greater elasticity should positively tell on their haemodynamic characteristics. In its turn, a considerable number of polymeric portions revealed on histological examination of the grafts six months after implantation into the abdominal portion of the aorta of rats strongly suggest a slow rate of prosthesis's degradation. This makes it is possible to maintain optimal strength of the graft till the moment of formation of a new blood vessel.

Adhesion and morphological changes of blood platelets on the surface of the material on contact with blood are amongst indices of haemocompatibility. The results of scanning electron microscopy of the inner surface of the PCL grafts and bioprostheses after contact with blood demonstrated a higher level of adhesion of blood platelets to the polymeric conduits as compared to biological prostheses. Although a large number of thrombocytes were adhered on the PCL grafts, no activated forms thereof were revealed. Mention should be made that in other works dedicated to studying tissue-engineering matrices from polycaprolactone showed that this material is not cytotoxic [12]. In its turn based on the results obtained in the present work one could also draw a preliminary conclusion that polycaprolactone-based vascular grafts are sufficiently biocompatible in vitro. However for more complete assessment it is necessary to study their properties in vivo in experiments on animals.

In order to assess possibilities of surgical application, functionality and biocompatibility, vascular PCL grafts with an internal diameter of 2 mm were implanted into the abdominal portion of the aorta of rats. Six week after graft's implantation the zones of anastomosis showed an extensive neointimal layer extending along the whole internal inner surface of the graft. The presence of neointimal hyperplasia in the graft's lumen is characteristic of the majority of synthetic vascular prostheses and appears as a process of remodelling of the blood vessel after its damage during 2–24 months [5]. In the normal course of the process of the blood vessel remodelling the formation of the neointima does not lead to considerable stenos [13] but there is a series of causes which may result in active synthesis and accumulation of a great number of the extracellular matrix and hence to neointimal hyperplasia. To such factors belong difference in the diameters of the vessel and prosthesis, lack of endothelial cells, as well as migration and proliferation of smooth muscle cells. Damage of the vascular wall stimulates a change of the phenotype of smooth muscle cells. In its turn activated smooth muscle cells migrate from the media to the intima of the vessel where they actively secrete the extracellular matrix as well as a series of cytokines and growth factors, including IL-1, bFGF, FGF-β1, TNF-α, stimulating metaplasia of connective tissue [14]. Besides, one of the causes may be low shearing stress on the walls of the vessel in the places of anastomoses. At low shearing stress on the inner surface of the graft, the cells and secreted extracellular matrix are not oriented in one direction but are situated haphazardly, which also provokes neointimal growth. Present-day approaches aimed at suppression of neointimal hyperplasia are sufficiently diversified and include delivery into the perivascular zones of medicinal agents, genes, growth factors, as well as adsorption of endothelial cells on vascular prostheses. Despite the fact that these methods of influencing neointimal growth demonstrate positive results on experimental models the problem concerning neointimal hyperplasia in small-diameter vascular prostheses remains unsolved as yet [15, 16].

At the same time, formation of the endothelial level on the inner surface of the graft, infiltration of its wall with cells, as well as formation of the extracellular matrix replacing the polymeric material of the graft, discovered

6 months after implantation are the main characteristics of the conduit's healing process.

Hence, while studying properties of PCL grafts manufactured by electrospinning revealed that these prostheses possess the necessary physico-mechanical properties, are sufficiently hemocompatible, and may serve as a carcass for formation of the own vessel after implantation into the blood channel of the mammals. Despite this, polymeric grafts require further modification which would promote inhibition of neointimal hyperplasia and preservation of patency of the blood vessel.

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