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A perivascular system releasing sirolimus prevented intimal hyperplasia in a rabbit model in a medium-term study

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ABSTRACT

The main complication of aortocoronary reconstruction with vein grafts is restenosis in the course of time. The aim was to assess the effect of a periadventitial polyester mesh releasing sirolimus on intimal hyperplasia of autologous grafts. We implanted *v. jugularis ext.* into *a. carotis communis* in rabbits. The vein graft was either intact, or was wrapped with a pure polyester mesh, or with a sirolimus-releasing mesh. Three and six weeks after surgery, the veins were subjected to standard histological staining and the thicknesses of the *tunica intima*, the media and the intima–media complex were measured. Wrapping the vein with a mesh releasing sirolimus or with a pure mesh decreased the thickness of the intima in comparison with a vein graft by 73 ± 11% or 73 ± 8% after 3 weeks, and by 73 ± 9% or 59 ± 12% after 6 weeks, respectively. Sirolimus-releasing meshes reduced the thickness of the media by 65 ± 9% and 20 ± 12% after 3 and 6 weeks. The thickness of the intima–media complex in grafts with sirolimus-releasing meshes decreased by 60 ± 6% and 30 ± 13% in comparison with pure PES meshes, after 3 and 6 weeks, respectively. A periadventitial polyester mesh releasing sirolimus has the potential to become an effective device in preventing vein graft restenosis.

1. Introduction

Occlusion of the graft lumen is considered as the main complication in the follow-up of patients after aortocoronary vein bypass grafting. This occurs almost in 15% within the first year, and increases up to 50% of occluded veins after 10 years (Motwani and Topol, 1998). In patent vein grafts, 7% show signs of degeneration after 1 year and 77% after 10 years (Fitzgibbon et al., 1996).

Due to a mechanical mismatch after implantation, an autologous vein graft in arterial circulation is prone to remodelling. This is accompanied by excessive proliferation of vascular smooth muscle cells (VSMC), which can migrate into the intima and produce extracellular matrix. Graft remodelling thus results in intimal hyperplasia and graft stenosis. In clinical practice, autologous graft stenosis is usually dealt with (after percutaneous transluminal angioplasty) by inserting stents, often loaded with antiproliferative drugs, e.g. sirolimus-eluting stents (Cypher™, Cordis J&J, NJ), or paclitaxel-eluting stents, into the graft lumen (Colombo and Iakovou, 2004). However, the use of stents is limited by the relatively complicated process for inserting them, by increased mechanical strain on the vessel wall, and by local damage to the endothelium and VSMC leading to reactivation of VSMC growth and restenosis of the vessel. In addition, the stents can be released and can move inside the vessel (Jeremy et al., 2004). Similar problems are associated with drug-releasing polymeric films covering the luminal surface of a vessel, e.g. hydrogel films loaded with paclitaxel (Livnat et al., 2005).

From this point of view, an external drug delivering system, i.e. placed on the adventitial surface of the vascular graft, seems to be more advantageous. Periadventitial delivery of heparin from matrices placed adjacent to rat carotid arteries was successfully

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used in the case of heparin, which is well known to attenuate proliferation of VSMC (Edelman et al., 1990). Other antiproliferative drugs have also been found to inhibit neointimal hyperplasia of vein grafts in animal experimental models after local extraluminal application, for example suramin, C-type natriuretic peptide, cilostazol, and sirolimus (Schachner et al., 2004a,b; Hu et al., 1999; Fujinaga et al., 2004). Especially sirolimus (Rapamycin) is a drug well known for its potent antiproliferative action. In addition to its application in drug-eluting stents (Colombo and Iakovou, 2004), this macrocyclic lactone has also been used clinically for immunosuppressive therapy after organ transplantation (Roque et al., 2001). The mechanism of its antiproliferative effect is very complex (Yakupoglu and Kahan, 2003; Regar et al., 2001), involving mainly blocking the transition from the G1 phase to the S phase of the cell cycle by interacting with a specific target protein (mTOR, mammalian target of sirolimus) and inhibiting its activation. Our earlier study focused on the kinetics of the release of sirolimus from polyester meshes *in vitro* (Filova et al., 2011). In these experiments, the release of sirolimus from polyester meshes coated with a degradable copolymer loaded with sirolimus was detected for several weeks. The proliferation of VSMC in culture plates was inhibited if sirolimus-releasing meshes were used. For the present study, we assumed that the graft wall of the vessel would be thin enough to enable diffusion of sirolimus released from a periadventitially placed mesh into the tunica media, so that VSMC proliferation could be inhibited.

It has been suggested that thickening of the intima of the vein grafts increased in the distended regions, where the grafts were subjected to low flow velocity (Dobrin, 1995). Thus, we expected that in addition to the antiproliferative effects of sirolimus, our meshes would also reduce the risk of graft restenosis by minimizing distension of the graft. From this point of view, encouraging results were obtained in an *in vivo* study performed on sheep. Sheathing the implanted vein grafts with a pressure-resistant polyester (torlen/dacron) mesh significantly reduced intima thickening in these grafts compared to control untreated vein grafts within 12 weeks after implantation (Krejca et al., 2002). Taken together, the mesh placed around the graft should serve as a mechanical support for the graft wall, which increases the resistance of the graft against high pressure, decreases tangential stress and retards graft degeneration (Jeremy et al., 2004; Krejca et al., 2002).

We therefore developed a novel combined device composed of two synergistically effective components: a mechanically supportive polyester mesh, and an antiproliferative drug (sirolimus). Our work extended previous preliminary study about early intimal changes in the autologous vein grafts (Skalsky et al., 2011). We evaluated the dynamics of the vascular wall changes during a medium-term examination in vein grafts in rabbits, wrapped with a sirolimus-releasing polyester mesh. Sustained release of sirolimus within a period of several weeks suppressed VSMC proliferation for the time necessary for re-endothelialization of the graft. These effects reduced autologous graft remodelling and the need for subsequent treatment.

2. Materials and methods

2.1. Materials

Polyester mesh (CHS 50, PES mesh) was obtained from VUP Joint-Stock Co., Brno, CR. Purasorb PLC 7015, and a grade copolymer of L-lactide and ε-caprolactone (70/30 molar ratio, inherent viscosity midpoint of 1.5 dl/g) was purchased from PURAC biomaterials. Sirolimus (Rapamycin from Streptomyces hygroscopicus, Cat. No. R0395) was obtained from Sigma–Aldrich (Germany).

2.2. Mesh impregnation

Polyester mesh impregnation was described in a previous paper (Filova et al., 2011). Briefly, the mesh was coated with a solution containing 5.2 mg of sirolimus and 36.4 mg of purasorb in 1 ml of chlorobenzene–ethanol (1.75:1, v/v). It was dried, and then coated for a second time with the same solution and dried again. Finally, the impregnated mesh contained 0.14 mg of sirolimus per cm^2. The mesh was dried out in a vacuum oven for 3 weeks, and was then sterilized with ethylene oxide (sirolimus-releasing PES mesh).

2.3. Implantation procedures

The experiments on laboratory animals were approved by the Authorization No. 48/2009 issued by the Chief Hygienist of the Czech Republic, the Ministry of Health of the Czech Republic according to the law No. 246/1992 of the Collection and in compliance with further regulations, for the protection of animals against suffering, and in accordance with the Project of Experiments and the statement of the Ethical Committee. Male Giant Chinchilla rabbits (3.0–3.5 kg; $n = 65$; Table 1) were anaesthetized using an intramuscular injection of ketamine hydrochloride (30.0 mg/kg). Anaesthesia was maintained with isoflurane (2.5–3.0%), inhaled through a mask. Heparin (300 IU/kg) was given intravenously to the animals. The operative procedure was performed with an aseptic technique with operating glasses (magnification 2.5×). The right external jugular vein and the right common carotid artery were exposed through a vertical midline cervical incision. The vein bypass grafts were constructed using an anastomotic cuff technique (Jiang et al., 2004) (Fig. 1).

A segment of the external jugular vein approximately 2 cm in length was harvested for an autologous reversed-vein graft; the segments were also used as the graft_0 control group. The polymer cuffs were prepared from a 4F endovascular catheter (Terumo Medical Corp, Elkton, MD). The jugular vein ends were passed through a cuff, everted, and fixed using 8-0 monofilament polypropylene silk. The common carotid artery was clamped distally and proximally, and the lumen was then exposed using a small arteriotomy, and the cuffed, reversed vein ends were inserted. A second 8-0 polypropylene silk was used to ligature (i.e. secure) the artery around the cuff on both sides. The back wall of the carotid artery between the cuffs was excised to allow a vein graft extension (an untreated autologous graft). Finally, a pure PES mesh or a sirolimus-releasing PES mesh was put on around the vein graft and fixed by polypropylene 8/0 on the side (autologous graft wrapped with a PES mesh, an autologous graft wrapped with sirolimus-releasing PES mesh) (Jiang et al., 2004).

Groups of animals were euthanized 3 and 6 weeks after implantation. The specimens were equally divided in the middle of the vein graft (without the cuff segment) to obtain equal parts for histology and for immunohistochemistry.

2.4. Histology and immunohistochemistry

The venous grafts were divided into two parts. One part was fixed in 10% formalin and then it was embedded in paraffin. The second part was embedded in Sakura Finetek Tissue Tek© Cryomold holders and Sakura Finetek Tissue Tek© OCT Compound (both from Sakura Finetek, Tokyo, Japan). The samples were subsequently frozen in 2-methylbutane (Fluka Chemika, Buchs, Switzerland) cooled by liquid nitrogen, and then stored at −80 °C.

2.5. Histological analysis

The samples embedded in paraffin were cut into 3–4 μm sections and stained with haematoxylin-eosin, Van Gieson with

Table 1
Number of CD4+ cells, CD8+ cells, plasmocytes, macrophages and Fe particles in transverse histological sections through untreated autologous grafts (Graft), grafts wrapped with a pure PES mesh (PES mesh) or with a sirolimus-releasing PES mesh (Sirolimus) 3 and 6 weeks (3w, 6w) after implantation. Mean ± SEM from 7 to 10 rabbits (10 microscopic fields per animal). Olympus BX41 microscope, magnification 600×, numbers of rabbits with any kind of thrombus, with oblitering or recanalized thrombi are also given. Non-parametric Kruskal–Wallis one-way analysis of variance (ANOVA) was used.

	1. Graft 0w	2. Graft 3w	3. PES mesh 3w	4. Sirolimus 3w	5. Graft 6w	6. PES mesh 6w	7. Sirolimus 6w
No of animals	12	7	9	9	10	8	10
CD4+	[#] vs. 2 [**] vs. 3 1.1 ± 0.4	[#] vs. 1 [#] vs. 4 50.7 ± 19.9	[**] vs. 1 [*] vs. 4 30.0 ± 2.6	[#] vs.2 [*] vs. 3 0.6 ± 0.4	10.7 ± 1.1	12.8 ± 4.5	12.8 ± 3.8
CD8+	[**] vs. 3 [#] vs. 2, 6 0.6 ± 0.2	[#] vs. 1 27.5 ± 17.3	[#] vs. 5, 7 [**] vs. 1, 4 17.0 ± 2.4	[**] vs.3 0.8 ± 0.4	[#] vs. 3 2.9 ± 0.3	[#] vs. 1 10.0 ± 3.4	[#] vs. 3 1.8 ± 0.5
Plasmocytes	[**] vs. 5 0 ± 0	4.6 ± 1.8	3.2 ± 1.6	[*] vs. 5 0 ± 0	[*] vs. 4, [#] vs.7 [**] vs. 1 6.9 ± 2.0	2.5 ± 0.7	[#] vs. 5 0.4 ± 0.4
Macrophages	[**] vs. 7 0 ± 0	1.6 ± 1.1	5.7 ± 4.0	[*] vs. 7 0 ± 0	2.8 ± 1.3	1.2 ± 0.6	[**] vs. 1 [*] vs. 4 13.2 ± 9.6
Fe particles	[*] vs. 5 0 ± 0	[#] vs. 5 0 ± 0	[*] vs. 5 0 ± 0	[*] vs. 5 0 ± 0	[*] vs. 1, 3, 4, 7[#] vs. 2 120.8 ± 79.1	5.1 ± 5.1	[*]vs. 5 0 ± 0
Rabbits with any kind/oblitering/ recanalized thrombus	0/0/0	3/0/1	5/1/3	5/0/1	5/3/0	5/1/2	3/0/0

[#] $p < 0.05$ in comparison with the sample labelled with the same number (statistical significance).
[*] $p < 0.01$ in comparison with the sample labelled with the same number (statistical significance).
[**] $p < 0.001$ in comparison with the sample labelled with the same number (statistical significance).

elastica, von Kossa and Pearl's stains. The sections were taken from the midportion of the graft to avoid tissue that may have reacted to the suture material. Each section stained with Van Gieson with elastica was photographed using an Olympus IX 51 microscope and a DP 70 digital camera.

2.6. Immunohistochemical staining

Information concerning the primary antibodies used for the immunohistochemical analyses is shown in Table 2.

2.6.1. Formol fixed, paraffin embedded tissues

Immunohistochemistry was performed on 4 μm-thick paraffin sections using a three-step indirect method. The slides were deparaffinized in xylene and rehydrated in graded ethanol. After deparaffinization and rehydration, the slides were cooked in a microwave oven using 0.01 M citrate buffer pH 6.0 (detection of smooth muscle actin), or EDTA buffer pH 8.0 (detection of PCNA) for target retrieval, or proteinase K was applied (detection of macrophages and CD31). Endogenous peroxidase was blocked by 0.3% H_2O_2 in 70% methanol for 30 min. Endogenous biotin was blocked with the biotin-blocking system (Dako, Glostrup, Denmark). The tissues were then preincubated with a 10% horse serum (Vector laboratories, Burlingame, CA) for 20 min to prevent nonspecific binding and FcR binding. The primary antibody was applied for 30 min RT or incubated overnight at 4 °C (CD31). Detection of the monoclonal antibody was performed using biotinylated horse anti-mouse IgG (Vector laboratories, Burlingame, CA, USA) diluted 200× for 30 min. The specimens were then incubated with RTU Vectastain Elite ABC Reagent for 30 min. Finally, the specimens

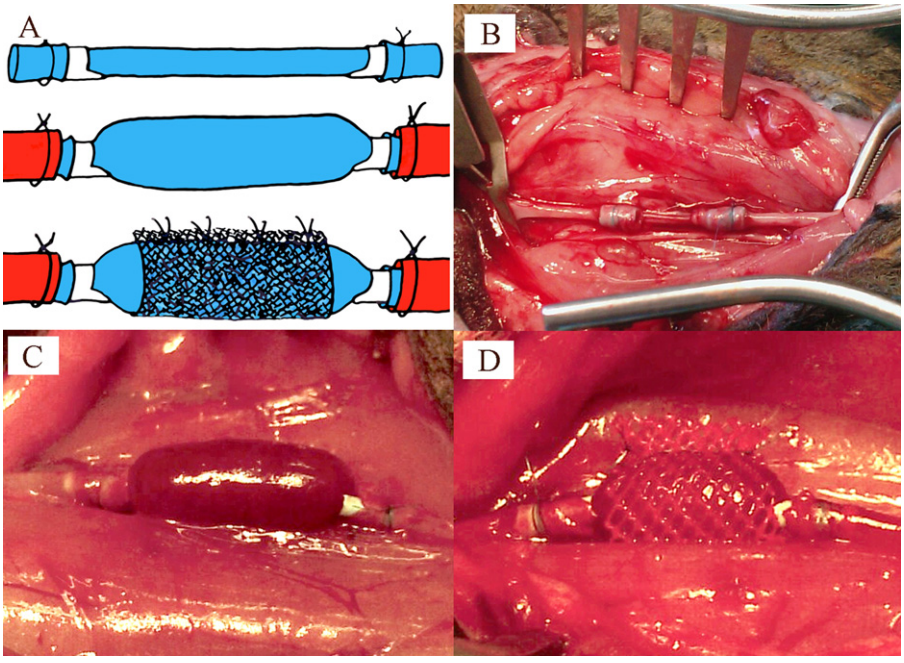

Fig. 1. Scheme of the surgical technique for graft implantation (A), the autologous vein before a clip release (B), the dilated vein graft (C) and the dilated vein graft wrapped with a PES mesh (D).

Table 2
The list of monoclonal antibodies used for immunohistochemical staining.

Specificity	Origin	Company	Dilution	Clone
CD4+ cells	Mouse	Novus Biological, CO, USA	50×	KEN-4
CD8+ cells	Mouse	LifeSpan Biosciences, WA, USA	100×	NA
α-Smooth muscle actin	Mouse	Sigma, MO, USA	900×	1A4
Macrophage	Mouse	Abcam, UK	200×	MAC387
CD31	Mouse	Abcam, UK	20×	JC/70A
PCNA	Mouse	Dako, Denmark	6000×	PC 10

were stained with Dako Liquid DAB+ Substrate-Chromogen System (Dako, Glostrup, Denmark) for 5 min, and were counterstained with Harris's haematoxylin before they were embedded in Entellan (both from Merck, Germany).

2.6.2. Snap-frozen tissue samples

Immunohistochemistry was performed on sections 8 μm in thickness, using a three-step indirect method. The sections were fixed for 10 min in cold acetone. Following this, the sections were rinsed in 0.2% Triton X-100 and phosphate-buffered saline, and endogenous biotin was blocked with a biotin-blocking system (Dako, Glostrup, Denmark). The tissues were then incubated in 10% horse serum, after which a primary antibody (anti-CD4/CD8) was applied for 60 min. In addition, endogenous peroxidase was blocked in 0.3% H_2O_2 and 70% methanol for 30 min. The specimen was incubated with a secondary biotinylated horse anti-mouse antibody (Vector Lab, Burlingame, CA, USA), followed by incubation with RTU Vectastain Elite ABC Reagent (Vector Lab, Burlingame, CA, USA). Finally, the specimens were incubated for 5 min with the Dako Liquid DAB+ Substrate-Chromogen System (Dako, Glostrup, Denmark), counterstained with Harris's haematoxylin and embedded in Entellan (both from Merck, Germany).

The total numbers of CD4+ cells, CD8+ cells, neutrophils, plasmocytes, eosinophils, macrophages, foreign body giant cells, Fe and Ca particles were counted in the entire cross-section of the vein grafts in 10 microscopic fields per rabbit at magnification 600×.

2.7. Thickness of intima, media, media–intima complex, media–intima ratio

The following parameters were measured: the thickness of the intima from the endothelial surface to the inner border of the tunica media, the thickness of the media from the inner border of tunica media to the border between tunica media and adventitia, and the thickness of the media–intima complex from the endothelial surface to the media–adventitia border. Data was collected from 32 to 84 measurements per rabbit in 12–28 microscopic fields, using the MeasureStackLines plug-in module of Ellipse Software (ViDiTo Systems, Slovakia). The media/intima ratio was calculated from the measured values. The mean value and the Standard Error of Mean (mean ± SEM) were calculated for each vein and for each animal group. Grafts with thrombosis were not measured.

2.8. Statistical methods

Non-parametrical one-way Kruskal–Wallis ANOVA was used for a statistical evaluation of the histology and histomorphometry. Two-way ANOVA and the Tukey studentized range method were used for the data analyses of the thickness of the intima, media, intima/media complex and the media/intima ratio. The variables were tested for normality by Shapiro and Wilk's statistics. Since normality was rejected, the logarithmic transformation was applied. The effects of time, drug, and the interaction between time and drug were tested, and $p < 0.05$ was considered to be significant.

3. Results

3.1. Histology and immunohistochemical staining

3.1.1. Tunica intima

In all experimental groups of animals, the luminal surface of the venous graft was covered by a layer of endothelial cells (Figs. 2 and 3). In some grafts, thrombi were observed on the luminal surface of tunica intima (Table 1). Some thrombi entirely obliterated the graft lumen, while others were only parietal or recanalized, but no significant differences were found among the rabbit groups. However, there was a significant difference in the thickness of the intima among the samples (see also below in Section 3.3). Focally, smooth muscle cells were observed in the neointima, especially in samples from animals without the application of sirolimus.

3.1.2. Tunica media

In all groups, the adaptation process of vein grafts to arterial pressure led to the formation of multiple smooth muscle cells layers, which resulted in thickening of the media (Fig. 3; see also below in Section 3.3). This was accompanied by an increased number of PCNA+ cells (Fig. 2; see also below in Section 3.2). In the venous autograft with the sirolimus-releasing mesh, the infiltration of CD4+ and CD8+ cells was remarkably decreased in the vessel wall (i.e. complex of intima, media and adventitia) compared to the untreated autologous graft and the graft wrapped with a PES mesh after 3 weeks (Table 1, Fig. 4).

The number of plasmocytes and macrophages was very small in all groups. There were only sporadic neutrophils and eosinophils in the venous grafts (data not shown). After 6 weeks, a lower number of plasmocytes was found in veins with a sirolimus-releasing mesh compared to the untreated autologous graft. However, in veins with a sirolimus-releasing mesh, the number of macrophages increased significantly between the 3rd week and the 6th week, although they were similar in number to those in both control groups after 6 weeks (Table 1). No calcium particles were found in any of the groups. No infectious wound complications were observed in any of the groups of animals. One rabbit died during the operation, and two rabbits due to systemic infection.

3.1.3. Tunica adventicia

Histological staining revealed the presence of PES meshes and sirolimus-releasing PES meshes in the adventitia of the vein grafts. In all groups, the distribution of immunocompetent cells, i.e. plasmocytes, CD4+ and CD8+ cells, was similar to the distribution of these cells in the tunica media (Fig. 4A), see Section 3.1.2 (Table 1). Small deposits of iron were sometimes observed, probably as a histological correlate of haematoma resorption (Fig. 4B). In addition, in groups with a PES mesh and a sirolimus-releasing PES mesh, foreign-body giant cells were observed around the meshes (Fig. 4C and D).

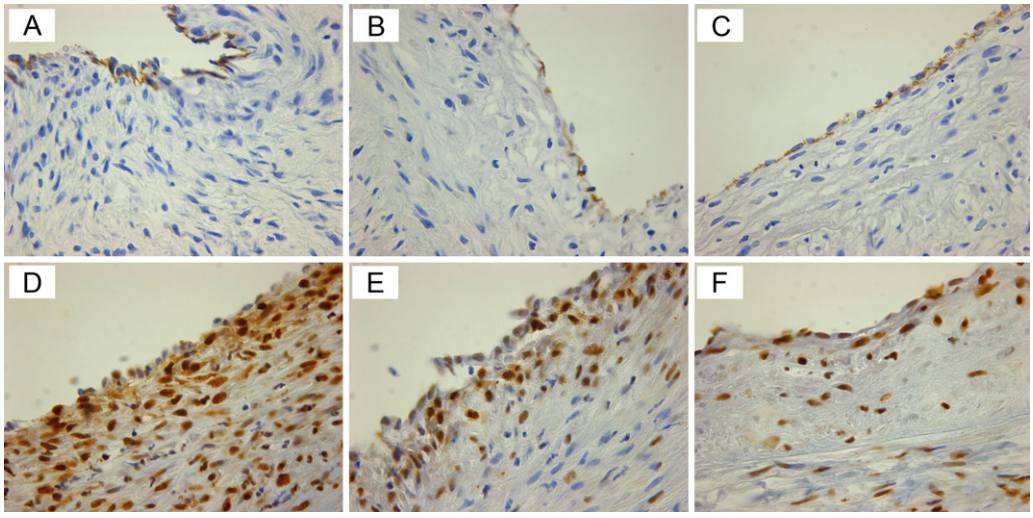

Fig. 2. Immunohistochemical staining of CD31 (A–C) and proliferating cell nuclear antigen (PCNA, D–F) in an untreated autologous graft (A, D), an autologous graft wrapped with a PES mesh (B, E), an autologous graft wrapped with a sirolimus-releasing PES mesh (C, F) 3 weeks after implantation into rabbits, Olympus BX41 microscope original magnification 400×, positively stained cells are brown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Proliferating cell nuclear antigen (PCNA)

Most of the PCNA+ cells were found in the media and intima (Figs. 2 and 5), and fewer in the adventitia. The highest number of PCNA+ cells was observed in the control untreated grafts 3 weeks after implantation (graft_3w). After 3 weeks, wrapping the vein graft with a pure PES mesh reduced the number of PCNA+ cells by 59 ± 15%, while wrapping with sirolimus-releasing PES meshes reduced the number by 84 ± 13%. After 6 weeks, the number of PCNA+ cells decreased significantly in the control untreated graft (graft_6w); however, the number did not change in grafts wrapped with a pure PES mesh or with a sirolimus-releasing mesh.

3.3. Thickness of the intima, media, media–intima complex, and the media/intima ratio

The intima measurements (Fig. 6A) after 3 weeks showed the highest intima thickness value in the control graft. In grafts wrapped with a PES mesh or with a sirolimus-releasing mesh, the thickness of the intima was reduced by 73 ± 8% and 73 ± 11%, respectively, compared to the untreated graft.

Between the 3rd and 6th weeks, the intimal thickness of the untreated autologous grafts, the grafts with a pure PES mesh and the grafts with a sirolimus-releasing PES mesh remained constant. After 6 weeks, the relative reduction of the thickness of the intima in the grafts wrapped with a PES mesh or with a sirolimus-releasing PES mesh was 59 ± 12% and 73 ± 9%, respectively, in comparison to the untreated graft. The sirolimus-releasing meshes had a significantly stronger effect reducing intimal hyperplasia than the pure PES meshes after 6 weeks.

The increase in media thickness (Fig. 6B) was significantly reduced by 65 ± 9% by the use of sirolimus-releasing meshes after 3 weeks of implantation. Concurrently, a pure PES mesh did not influence the hyperplasia of the tunica media compared to the control untreated autologous graft, at both time intervals. After 6 weeks, the media was significantly thinner by 20 ± 12% in grafts wrapped with a sirolimus releasing PES mesh than in the untreated autologous grafts. The thickness of the intima–media complex

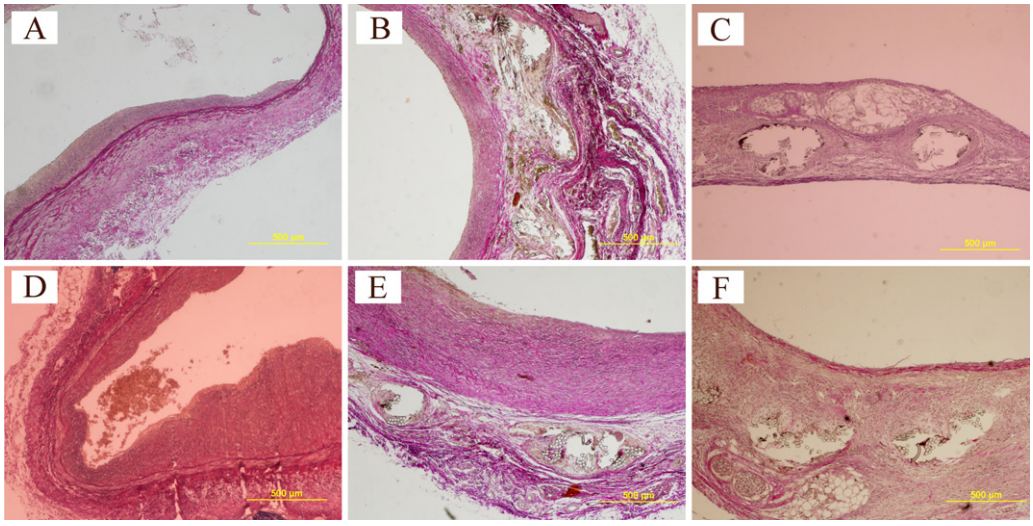

Fig. 3. An autologous vein graft A, D), an autologous vein graft wrapped with a polyester mesh (B, E) and an autologous vein graft wrapped with a polyester mesh releasing sirolimus (C, F) after 3 (A–C) and 6 weeks (D–F) in rabbits, van Gieson and elastica staining, objective 4×, scale bar = 500 μm, Olympus IX51 microscope, DP70 digital camera.

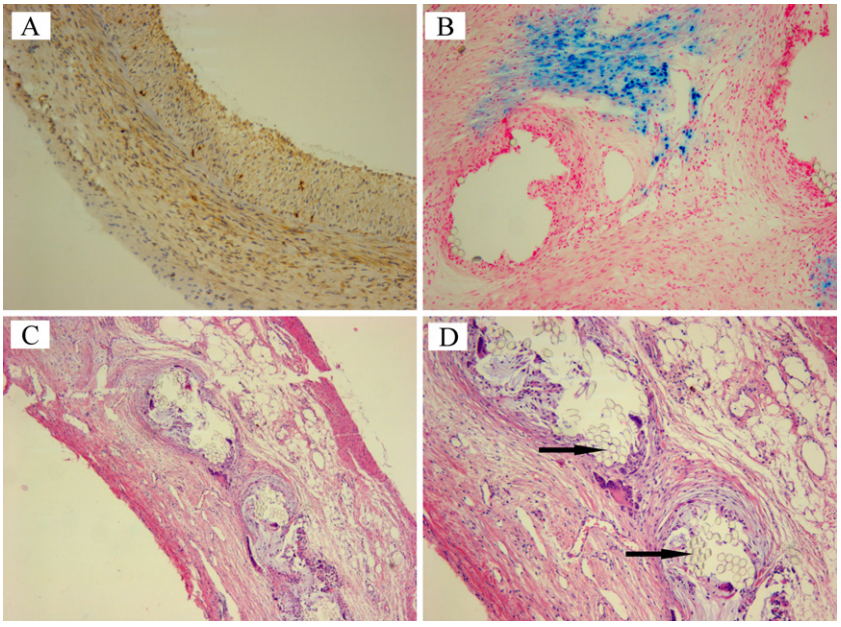

Fig. 4. Immunohistochemical staining of CD4 in an untreated autologous graft after 3-week implantation in a rabbit (A), Pearl's staining of iron (B) in an autologous graft wrapped with a sirolimus-releasing PES mesh after 3-week implantation in a rabbit, and haematoxylin-eosin staining of an autologous graft wrapped with a sirolimus-releasing PES mesh after 3-week implantation in a rabbit (C and D), magnification 50× (A, C), 200× (B) and 100× (D), Olympus BX41 microscope; CD4+ cells are stained brown (A), Fe is stained blue (B), arrows (D) indicate PES mesh fibres grown in the vein graft; the foreign-body giant cells are localized around the PES mesh fibres. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 6C) was remarkably reduced by the sirolimus-releasing mesh by 76 ± 5% and 37 ± 9% in comparison with the control grafts after 3 and 6 weeks, respectively. At 3 weeks, a pure PES mesh reduced thickening of the intima–media complex by 41 ± 6% compared to the untreated autologous graft.

The control autologous grafts and the sirolimus treated graft after 3 weeks had an intima to media ratio that was similar to the ratio in a healthy vein, i.e. graft_0. After 3 weeks, the sirolimus releasing mesh was able to keep a media/intima ratio similar to that of graft_0. After 6 weeks, selective thickening of the media caused an increase in the media/intimal ratio in a graft wrapped with sirolimus-releasing meshes, probably due to the minimal concentration of sirolimus in the meshes at this time interval [14]. The highest media/intima ratio was observed in grafts wrapped with a pure PES mesh after 3 and 6 weeks. In these groups the ratio was higher than the ratio of the untreated autologous graft.

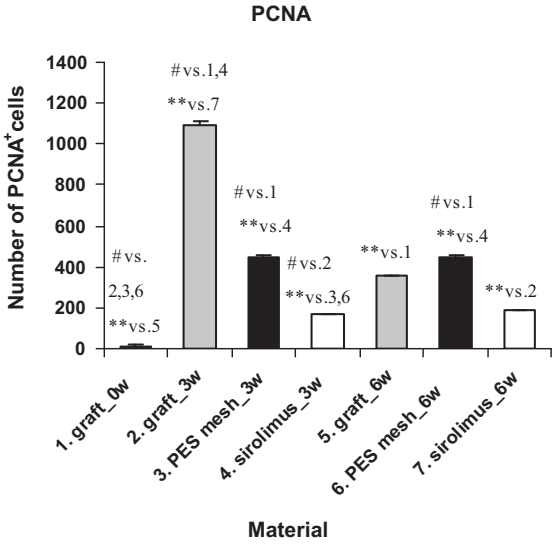

Fig. 5. The number of proliferating cell nuclear antigen-positive (PCNA+) cells in an untreated autologous vein graft (*graft*), an autologous vein graft wrapped with a polyester mesh (*PES mesh*), and an autologous vein graft wrapped with a polyester mesh releasing sirolimus (*sirolimus*), after 3 and 6 weeks (3w, 6w) in rabbits. The values are presented as mean ± SEM (standard error of mean). Two-way ANOVA and a Tukey pairwise comparison were used for statistical analyses. Statistical significance: $^{*}p \leq 0.01$ and $^{\#}p < 0.05$ in comparison with the sample labelled with the same number.

4. Discussion

In the present study, we created a unique periadventitial system with controlled release of the antiproliferative drug sirolimus to prevent neointimal hyperplasia in the vein graft interposed to the arterial system in rabbits. In addition, our study confirmed the hypothesis of the synergistic effects of a PES mesh and sirolimus on vein wall remodelling.

Several experimental and clinical studies have investigated the effects of external wall support on reducing the vessel wall stress and inhibiting neointimal proliferation (Mehta et al., 1998). The external torlen/dacron mesh tubing around an autologous vein graft in sheep retarded overgrowth of both the intima and the media within 12 weeks; the effect was especially pronounced in the intima (Krejca et al., 2002). The external wrap acts as a barrier against distension of the vein graft. The polyester periadventitial mesh works in the same way as the external elastic membrane of native arterial vessels, and allows them to withstand higher pressures in arterial circulation. It has been suggested that haemodynamic forces, especially excessively high wall shear stress, promote intimal hyperplasia. The wall thickness in grafts tends to adapt to the same value as the wall thickness in the grafted artery, which indicates that wall thickening occurs to normalize the tangential wall stress (Dobrin et al., 1989). While shear stress is a dominant regulator of lumen caliber, wall tension is a more critical determinant of wall thickness. Animal models indicate that there is a structurally optimal lumen radius/wall thickness ratio to support arterial pressure with minimal wall stress (Owens, 2010).

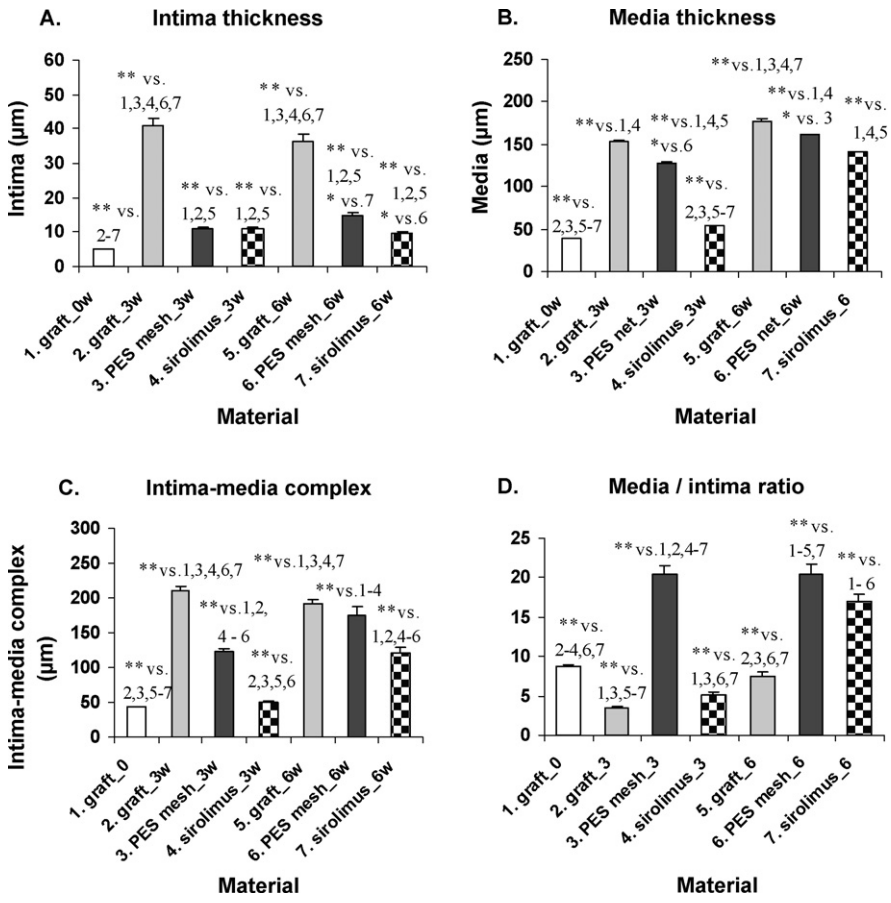

Fig. 6. The thickness of the intima (A), the media (B), and the intima–media complex (C) of an untreated autologous vein graft (*graft*), an autologous vein graft wrapped with a polyester mesh (*PES mesh*), and an autologous vein graft wrapped with a polyester mesh releasing sirolimus (*sirolimus*), after 3 (A–D) and 6 weeks (3w, 6w) in rabbits (B–D); media/intima ratio (D). The values are presented as mean ± SEM (standard error of mean). The Tukey studentized range method was used for the statistical analyses; $^{**}p \leq 0.01$ and $^{*}p \leq 0.05$ in comparison with the sample labelled with the same number.

The adventitia plays an important role in the neointimal formation mechanism (Shi et al., 1996; Scott et al., 1996). Mechanical injury of the vessel induces an adventitial angiogenic response. It has been suggested that adventitial myofibroblasts play a role in proliferation, in synthesizing growth factors and finally in migration into the neointima. Applying the antiproliferative drug from the outer space of the vessel lumen has the advantage of hitting the target area for administering the drug in a controlled manner.

In our study, both a PES mesh and a sirolimus-releasing PES mesh significantly reduced intimal thickness in comparison with the control untreated grafts 3 and 6 weeks after implantation. Between the 3rd and 6th weeks, there was no difference in intima thickness in all groups.

There is no convincing evidence of the durability and the maximal effective period of external sheaths or wraps, due to the unfavourable inflammatory response to the synthetic material used as a permanent mechanical support (Bunt, 1983). In addition, biodegradable materials may cease to fit, and may allow the vein graft to expand under arterial pressure. Then the increased level of circumferential wall stress would not be controlled, due to the loss of mechanical support. The mechanical support in our study was provided by a mesh made from polyester yarns. A yarn 90 µm in width was formed by fibres 17.5 µm in diameter; the area of an individual hole in the mesh was approximately 0.44 mm^2. This material was used on the basis on the results of previously published studies of the porosity, flexibility, biocompatibility and non-inflammatory response (Mehta et al., 1998; Hinrichs et al., 1994). Macroporosity is crucial for the efficacy of external stents in reducing neointima formation in porcine vein grafts. In addition, macroporous stents allow adventitial microvessels to connect with the vasculature outside the stent, thereby potentially improving oxygenation. Although external stenting is highly effective in reducing neointima formation after vein grafting, the properties of the stent material necessary for this effect have not been defined (Georgie et al., 2001). Non-degradable wraps could elicit long-term mechanical damage, especially in coronary artery bypass grafting, where the moving heart will be directly juxtaposed to an external wrap. New types of biodegradable wraps could provide a future option for solving this problem.

Another important role of the external periadventitial wrap developed in our study is in delivering sirolimus to the vein wall. Sirolimus is used as immunosuppressant for patients with organ transplants. This drug has been observed to inhibit IL-2 induced T cell proliferation (Ballou and Lin, 2008). This can explain the reduced numbers of CD4+ and CD8+ cells that we observed in the graft wall wrapped with a sirolimus-releasing mesh after 3 weeks in rabbits. After 6 weeks, the difference among the groups of rabbits disappeared, probably due to the kinetics of sirolimus release from the purasorb coating. In our earlier *in vitro* study, 67% of the sirolimus was released after 11 days of incubation in a phosphate-saline buffer (Filova et al., 2011).

The counts of other cells in our grafts, such as macrophages, plasmocytes, neutrophils and eosinophils, were very low, and their distribution was similar to that in normal tissues. A slightly increased number of macrophages is associated with the presence of foreign material, i.e. a PES mesh. The foreign-body giant

cells correlate with tissue reaction to the presence of a PES mesh.

The presence of thrombi in all tested groups of grafts probably resulted from the activation of blood coagulation due to damage to the endothelium during surgery and expansion of the graft lumen by relatively high blood pressure in the venous graft interposed into the arterial bed. This expansion then exposed tissues underlying the endothelium to the blood stream. A second reason might be blood coagulation due to reduced blood flow in the vein graft. Sirolimus did not increase the incidence of thrombi in either of the time intervals.

Sirolimus inhibits vascular smooth muscle cell migration and proliferation, and decreases the reocclusion of coronary arteries following angioplasty (Moses et al., 2003). There is a significant inhibitory effect of sirolimus on the proliferation of endothelial cells (Liuzzo et al., 2005). Regeneration of the injured endothelium after surgery depends on growth factor, such as FGF, VEGF, PDGF, and other molecules derived from endothelial cells or circulating cells. Sirolimus inhibits FGF-2 stimulated proliferation of human endothelial cells. Endothelium re-establishment is completed in the range of weeks after extensive injury. We observed a confluent layer of endothelial cells in the lumen of all vein grafts after 3 and 6 weeks. Due to the final position of the periadventitial wrap in the tunica adventitia, smooth muscle cells in the vicinity of the sirolimus-releasing mesh were exposed to high sirolimus concentrations, while distant endothelial cells were affected by a lower sirolimus concentration. Earlier findings that there was an increased rate of apoptosis in the graft adventitia and a lower rate of apoptosis in the neointima after perivascular application of pluronic gel with rapamycin may also be explained by the sirolimus concentration gradient (Schachner et al., 2005).

In our experiments, the vein grafts were wrapped after dilatation in the arterial system. In another study (Hinrichs et al., 1994), they were wrapped before dilatation of the vein graft, which prevents intimal tears; however, similar results were observed in the thickness of the intima–media complex after 3 and 6 weeks.

In rabbits, the reduction of intimal hyperplasia depended on the sirolimus dosage in stents containing 60–200 μg of sirolimus (Suzuki et al., 2001). The sirolimus concentration used in our study was 140 μg/cm^2, and a maximum of 3 cm^2 of PES mesh was used per graft. Reduced thickness of the tunica intima and also the tunica media was observed in veins wrapped with a sirolimus-releasing mesh after 3 and 6 weeks. The effective concentration of sirolimus necessary for inhibiting smooth muscle cell growth is relatively low (IC 50 = 5 ng/ml) (Owen et al., 2010), and allows a sufficient amount of the drug to be dispersed for long-term release (in the range of 4–6 weeks). The content of sirolimus in Cypher^{TM} stents is in the range from 70 to 300 μg (Venkatraman and Boey, 2007). 100 or 200 μg of sirolimus was applied in a pluronic gel into the perivascular space on a model of interposition of the inferior vena cava into the common carotid artery in mice. After 1 and 2 weeks, but not after 4 and 6 weeks, a significant decrease in intima thickness was observed only in the group with a higher sirolimus concentration (Schachner et al., 2004a). Our sirolimus-releasing mesh decreased intimal hyperplasia to the non-wrapped graft by 73 ± 11% and 73 ± 9% after 3 and 6 weeks, respectively. A perivascular wrap prepared from poly(ε-caprolactone) with rapamycin (sirolimus) reduced the thickness of the intima by 76% after 3 weeks (Pires et al., 2005). Rapamycin-eluting homogeneous films made of copolymer of L-lactide and ε-caprolactone with 8 μg, 80 μg, and 800 μg (i.e. 1.4, 14 and 140 μg/cm^2; Kawatsu et al., 2007) of the drug were applied externally to a femoral vein graft anastomosed to the proximal femoral artery in end-to-end fashion in dogs. After 4 weeks, only the highest rapamycin concentration significantly attenuated intimal hyperplasia compared to the control. This was accompanied by a decreased number of PCNA positive cells in the neointima and adventitia.

The intima thickness values were almost the same in the grafts wrapped with a sirolimus-releasing mesh after 3 and 6 weeks. This is in agreement with the observation that maximum initial proliferation accrual of both smooth muscle and endothelial cells appears during the first weeks after implantation of a vein graft into the arterial bed. The cell proliferation returns to standard values after 12 weeks (Kalra and Miller, 2000; Zwolak et al., 1987). According to the described time course of intimal hyperplasia, it is probable that the intitial burst release of sirolimus during the first week can have a stronger protective effect against the development of intimal hyperplasia than a stable but lower sirolimus concentration released during the following weeks.

5. Conclusions

We have developed a novel device with a sustained release of sirolimus, made of a polyester mesh coated with a degradable copolymer of L-lactide and ε-caprolactone loaded with sirolimus, and we have evaluated its effect on inhibiting intimal hyperplasia *in vivo* in a rabbit model. After wrapping the mesh around the autologous vein graft, sirolimus was released into the vascular wall through the tunica adventitia. This perivascular sirolimus-releasing wrap significantly decreased the thickness of the intima and prevented neointimal hyperplasia of the autologous vein graft in a rabbit model within 6 weeks.

The mechanical support of the wrapped periadventitial polyester mesh and the antiproliferative effect of the released sirolimus have a joint synergetic effect on preventing thickening of the intima. Thus this system is a promising method for prolonging the "lifetime" of the vein grafts in arterial circulation.

Both the sirolimus content in the polymer coated mesh and the kinetics of its release seem to be favourable, since there is no intimal hyperplasia in the rabbit model in the course of the study. In clinical practice, this device could be effective in preventing restenosis of autologous vein grafts.

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