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Tacrolimus-Eluting Suture Inhibits Neointimal Hyperplasia: An Experimental *In Vivo* Study in Rats

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WHAT THIS PAPER ADDS

A tacrolimus-eluting suture significantly inhibited neointimal hyperplasia at the aortotomy site without causing any local toxicity in a rat model. This finding has significant clinical implications.

Objective/Background: Neointimal hyperplasia (NIH) remains one of the leading causes of graft failure after vascular anastomoses. Cytotoxic drugs, such as rapamycin and tacrolimus, have been shown to inhibit the development of NIH. In this study, the aim was to test the impact of a sustained releasing tacrolimus—chitosan-eluting suture on the development of NIH in a rat model.

Methods: After tacrolimus—chitosan coating of a 7/0 polyvinylidene difluoride (PVDF) Trofilen® suture, the tacrolimus concentration on the coated suture and *in vitro* release trials were performed spectrophotometrically. Twelve Wistar rats were included. After midline laparotomy, a 7—8 mm longitudinal aortotomy in the infrarenal aorta was made and then closed by a bare 7/0 PVDF (group C, $n = 6$) and a 7/0 tacrolimus—chitosan coated PVDF suture (0.65 μg/cm tacrolimus [0.9 wt%] + 1.82 μg/cm chitosan [2.28 wt%]) (group T, $n = 6$). After 1 month, rats were sacrificed and aortotomy sites were examined histologically by ratio of intimal area (including neointima) and immunohistochemically by α-smooth muscle actin (ASMA) and proliferating cell nuclear antigen (PCNA) immunostaining. The PCNA positive cells were indexed to total cell number and expressed as percentage.

Results: *In vitro* tacrolimus release tests for a 7/0 tacrolimus—chitosan coated PVDF suture were confirmed for 1 month without an initial burst release. Endothelialisation over the aortotomy line occurred in both groups. The area of neointima was significantly reduced in group T compared with group C (ratio 0.22 ± 0.12 vs. 0.42 ± 0.11; $p = .017$) 1 month post-operatively. Likewise, the percentage of PCNA immunostaining significantly decreased in group C compared with group T (3.83 ± 2.85% vs. 11.17 ± 7.78%; $p = .026$). The cells constituting NIH were positive for ASMA immunostaining.

Conclusions: Tacrolimus—chitosan-eluting suture is shown to be an effective way to reduce NIH without interfering with normal endothelialisation.

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INTRODUCTION

Neointimal hyperplasia (NIH) has remained the leading cause of failure of arterial, venous and prosthetic graft anastomoses.[1,2] The etiology of NIH has been shown to be multifactorial and particularly associated with injury to the vessel wall, local anastomotic factors, such as compliance mismatch, and response of the vessel wall to suture material.[3,4] Inflammatory cells in the vessel wall secrete various pro-inflammatory cytokines. These cytokines, along with elevated expression of adhesion molecules, matrix degrading metalloproteinases, and other pro-inflammatory mediators, create an environment that promotes proliferation and migration of vascular smooth muscle cells (VSMC), eventually leading to narrowing of the anastomosis.[5]

Prevention of NIH related to anastomoses remains a challenging issue. To halt cellular proliferation in NIH,

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several techniques, such as ionising radiation, gene therapy, external stenting, and antiproliferative drugs, have been studied experimentally and clinically.[6–8] Unfortunately, there has been a conspicuous lack of a clinically available method to prevent NIH. In the last few decades, the beneficial impact of balloons and stents releasing cytotoxic agents, such as paclitaxel and sirolimus, on the inhibition of cell proliferation and related NIH in peripheral and coronary arterial disease has diverted the focus of interest towards antiproliferative agents in vascular surgery.[9]

It has been shown that peri-adventitial application of cytotoxic agents is an effective measure in the prevention of NIH around anastomoses.[10–12] However, data related to the effect of cytotoxic drug releasing sutures on the process of NIH have remained very limited in the literature.[13] Currently, chitosan coating is one of the measures used to improve the functional characteristics of silk sutures and mesh materials. As a natural polysaccharide, it is biodegradable and found to have a scar preventing property, and antiinflammatory and antimicrobial actions.[14] In this study, the aim was to test the impact of a tacrolimus–chitosan coated suture with sustained release pattern on the development of NIH in a rat model.

MATERIAL AND METHODS

Polyvinylidene fluoride (PVDF; Trofilen®, Doğsan, Turkey) was used as suture material, and tacrolimus (FK-506 monohydrate; Sigma–Aldrich, St. Louis, MO, USA) was used for coating the sutures.

Tacrolimus

Tacrolimus was dissolved in dimethyl sulfoxide to a concentration of 2 mg/mL, measured spectrophotometrically (BioSpec, Shimadzu, Japan) at 220 nm for checking the concentration and standard curve preparation, then stored at −20 °C.

Coating of suture

For *in vitro* studies, suture material was cut into 1 cm long pieces. For preparation of tacrolimus coated sutures, 30 μg tacrolimus solution was added to five pieces of 1 cm long sutures. To prepare tacrolimus–chitosan coated sutures 60 μg tacrolimus–chitosan (w/w: 1/1) solution was added to five pieces of 1 cm long sutures. The tubes were incubated at room temperature for 72 h until saturation. All studies were carried out under aseptic conditions and tacrolimus–chitosan solution was filter sterilised with a 0.22 μm membrane filter.

Determination of coated tacrolimus amount

The amount of tacrolimus included was calculated based on measurement of the tacrolimus concentration into the coating solution spectrophotometrically at the beginning and the end of coating.

In vitro *release of tacrolimus from coated sutures*

Five pieces of 1 cm long sutures were added to a tube containing 1 mL phosphate buffered saline (PBS). The tube was incubated at 37 °C with constant shaking at 120 rpm in a shaking water bath. Supernatant was removed and replaced with fresh PBS at specific time intervals and the tacrolimus concentration of the samples was measured spectrophotometrically at 220 nm.

Animals and study protocol

The animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. All procedures involving animals were approved by the Animal Experiment Advisory Committee of the Marmara University School of Medicine, Istanbul, Turkey. In this study, 12 male Wistar rats (10–12 weeks old weighing 300–350 g) were used. General anaesthesia was induced with intraperitoneal ketamine hydrochloride 100 mg/kg (Pfiser Ilacları Ltd, Sti., Istanbul, Turkey) and chlorpromazine 3 mg/kg (Eczacibasi Ilac Pazarlama, Istanbul, Turkey). After surgical prophylaxis with cefazolin sodium 20 mg/kg (Mustafa Nevzat Ilac, Istanbul, Turkey), midline laparotomy was performed and the infrarenal abdominal aorta exposed. Following systemic heparin sodium 100 IU/kg (Nevparin, Mustafa Nevzat Ilac sanayi, Istanbul, Turkey) via the tail vein, the proximal and distal infrarenal aorta was clamped with two bulldog clamps. A 7–8 mm longitudinal aortotomy was made on the superior surface of the aorta with an 11 N blade. Then, the aortotomy was closed with either a bare 7/0 PVDF (Trofilen®; Dogsan, Trabzon, Turkey) suture (group C, control, $n = 6$) or a tacrolimus–chitosan coated 7/0 PVDF suture (group T, tacrolimus–chitosan, $n = 6$) in a continuous fashion. For hemostasis, gentle compression with a surgical sponge for a short while was preferred initially. Reversal of anticoagulation by intravenous protamine chloride (Meda Pharma Ilac San. ve Tic. Ltd. Sti, Istanbul, Turkey; in a dosage equal to the dosage of heparin sodium given for anticoagulation) was given if hemostasis was not satisfactory. The external appearance of the aortotomy site immediately after closure was observed to determine residual aortic stenosis. While subtle to minimal reductions in the external diameter of the aortotomy site after repair was regarded as satisfactory, cases with a moderate to severe reduction in diameter were excluded from the study. After establishment of hemostasis, surgical layers were closed in a standard way. Oral feeding was allowed 12 h post-operatively. Post-operatively, a routine daily check-up was performed for surgical site infection and alteration in general status. Rats were sacrificed 1 month after operation and the aortotomy site, with 4–5 mm of normal aorta on each side, was excised and examined.

Histological analysis

After excision of aortotomy sites, aortas were fixed with 10% neutral buffered formaldehyde at room temperature for 24 h. Then, tissues were dehydrated in alcohol series,

cleared in toluene, and embedded in paraffin. Short axial, consecutive 5 μm serial sections were obtained along the aortotomy line by microtome (Leica RM 2125RT). Five random sections from the aortotomy line were examined. The sections were stained with hematoxylin and eosin for evaluation of general vascular morphology and endothelialisation. The area of the intima (including neointima) and media at the aortotomy site was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA) on the sections stained with hematoxylin and eosin, as described.[10] Briefly, to characterise NIH the ratio of intimal area was calculated using the following formula: ratio of intimal area = intima area/intima + media area. The non-sutured reference sites on the proximal and distal side of the aortotomy were also evaluated and used as a control in all rats. Stained sections were examined and photographed with a digital camera (DP72; Olympus, Tokyo, Japan) attached to a photomicroscope (BX51; Olympus).

Immunohistochemical analysis

Immunohistochemical analysis was performed to identify the smooth muscle cells affected by NIH, and to evaluate cellular proliferation at the aortotomy site. To represent mesenchymal cells (e.g., smooth muscle cell or myofibroblast), α-smooth muscle actin (ASMA) antibody was used as the primary antibody. To represent proliferation related to NIH in VSMC, proliferating cell nuclear antigen (PCNA) was used. Three randomly selected sections per graft were assessed. The number of PCNA positive cells was expressed as a percentage of the total cell number (PCNA index). The number of PCNA positive cells and total nuclei in the area of intima (including neointima) and media at the aortotomy site in each section were counted and the PCNA index (positive nuclei/total nuclei × 100) was calculated. Immunoexpression of ASMA and PCNA antigens was performed using the standard streptavidin—biotin—peroxidase immunohistochemical staining method. Briefly, serial sections of 3 μm thickness were cut from the paraffin blocks and placed on positively charged slides. The sections were dried overnight at 37 °C and deparaffinised with xylene and rehydrated in ethanol. Endogenous peroxidase activity was blocked with hydrogen peroxide. Antigen retrieval was performed by microwaving sections in 10 mM citrate buffer (pH 6.0). Tissue sections were washed with PBS and were blocked with protein blocking solution (Histostain Bulk Kit [catalog number: 959943B]; Invitrogen LAB-SA Detection System, Paisley, UK). After diluting in antibody diluent solution (catalog number: 15-M35T; Bio-Optica, Milan, Italy), primary antibodies to ASMA (dilution 1:50 [NCL-L-SMA, liquid mouse monoclonal antibody]; Leica Biosystems, Newcastle, UK) and PCNA (dilution 1:100 [NCL-L-PCNA, liquid mouse monoclonal antibody]; Leica Biosystems) were applied to the slides and incubated at room temperature for 60 min. Sections were washed with PBS, followed by incubation with the biotinylated secondary antibody and streptavidin peroxidase (Histostain Bulk Kit [catalog number: 959943B]; Invitrogen LAB-SA Detection System) and subsequently washed in PBS. Staining was visualised with 3.3′-diaminobenzidine tetrahydrochloride (DAB Plus Substrate Staining System, TA-060-HDX; Thermo Fisher Scientific, Waltham, MA, USA). The slides were counterstained with Mayer hematoxylin, dehydrated in ethanol, cleared in xylene, and mounted. To test the specificity of the staining, the primary antibody was replaced with non-immune serum in negative control sections. Staining sections were examined and photographed with a digital camera (DP72; Olympus) attached to a photomicroscope (BX51; Olympus).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). Continuous experimental results are expressed as mean ± SD, while categorical values are given as percentages (%). Data were analysed by the Mann—Whitney *U* test and *p*-values were regarded significant if $< .05$.

RESULTS

Coating of suture

The amount of coating was calculated spectrophotometrically at 220 nm by measuring tacrolimus concentration values at the beginning and the end of 72 h. The amount of coating was calculated 0.71 μg/cm in tacrolimus-coated sutures and 2.58 μg/cm in tacrolimus—chitosan-coated sutures. It was observed that the amount of coating increased approximately fourfold in tacrolimus—chitosan coated sutures by addition of the chitosan polymer (Table 1).

In vitro *release of tacrolimus from the coated sutures*

Release profiles of tacrolimus and tacrolimus—chitosan from coated sutures are given in Fig. 1. Release of tacrolimus from coated sutures was followed for 30 days and it was observed that release of tacrolimus from coated sutures was faster than from tacrolimus—chitosan coated sutures. The addition of the chitosan polymer in the coating prolonged release of tacrolimus.

In vivo *study*

Only one animal in group T was excluded from the study owing to the development of moderate or severe diameter reduction immediately after aortotomy repair. All of the animals survived to the end of the protocol. After sacrifice

Table 1. *In vitro* evaluation of the amount of tacrolimus used for coating. After 24 h, the decrease in drug concentration *in vitro* reflects the amount used for coating.

	Tacrolimus	Tacrolimus + chitosan
In vitro drug concentration (μg)		
Baseline	30	30 + 30
After 24 h	22.63	16.4
Amount of coated drug (μg/cm)	0.65	2.47
Coated area (%)	0.89	3.38

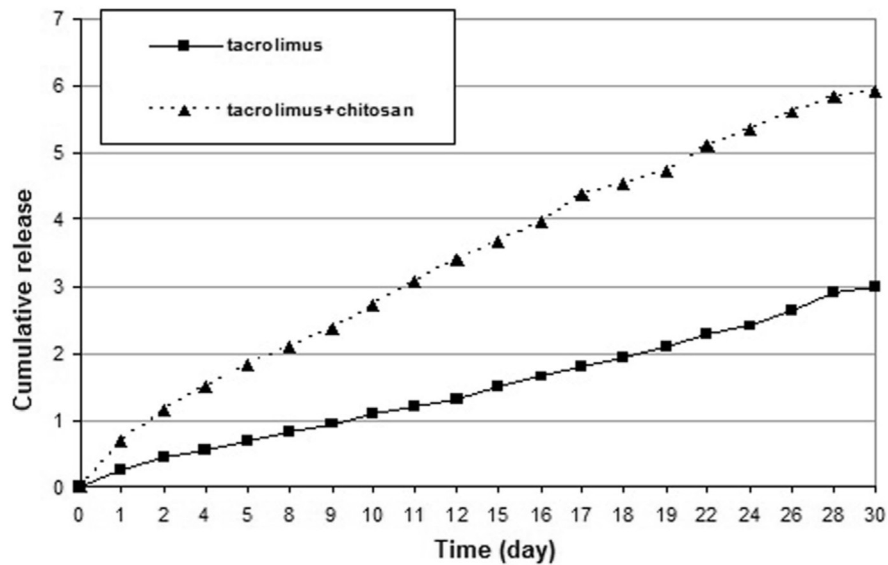

Figure 1. Drug releasing profiles of tacrolimus and tacrolimus—chitosan from the coated suture.

of the animals, none revealed intraperitoneal infection, abscess formation, or fluid collection. There were no occlusions of the graft or necrosis in the limbs, as visualised at postmortem examination.

Hematoxylin and eosin staining

Proximal and distal non-sutured arterial sites next to the aortotomy showed no NIH. NIH was prominent on both sides of the aortotomy line in control rats. In areas with NIH, increased VSMC and accumulation of fibroblasts were observed. In group T, NIH around the suture line was found to be significantly decreased compared with the control group. While the ratio of intimal area (including neointima) in the control group was 0.42 $\pm$ 0.11, it was found to be 0.22 $\pm$ 0.12 in group T ($p = .017$; Fig. 2).

PCNA and ASMA immunohistochemistry

The percentage of PCNA positive cells (PCNA index) significantly reduced in group T compared with the controls (3.83 $\pm$ 2.85% vs. 11.17 $\pm$ 7.78%; $p = .026$) (Fig. 3).

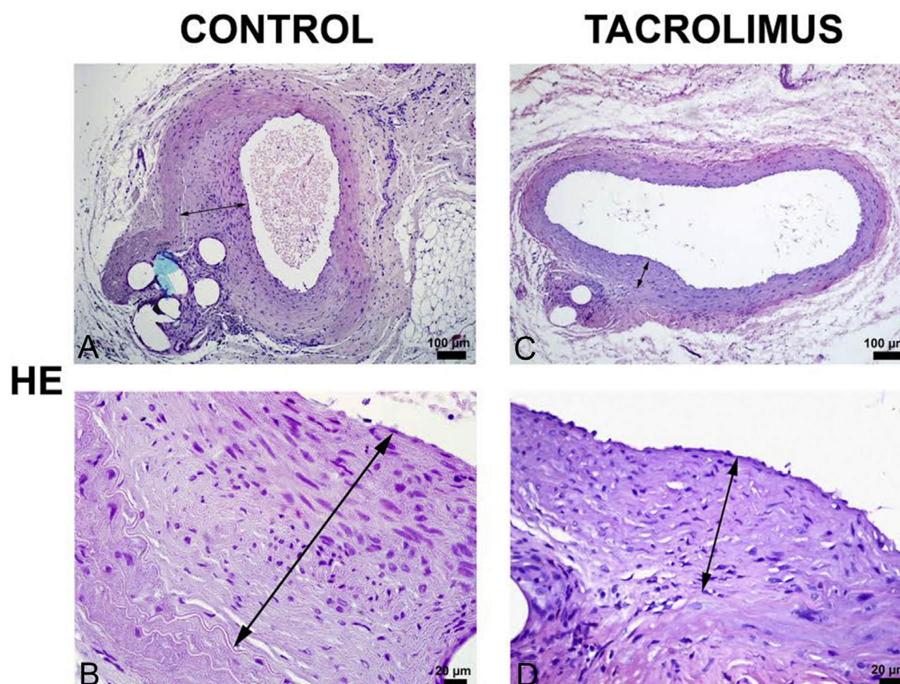

Figure 2. Hematoxylin and eosin (HE) staining. (A, B) In the control group, neointimal hyperplasia (double headed arrows) was observed around nearly all endothelium, whereas significantly decreased neointimal hyperplasia (double headed arrows) was observed at suture sites in the tacrolimus group (C, D). Original magnification (A, C): ×100; (B, D): ×400.

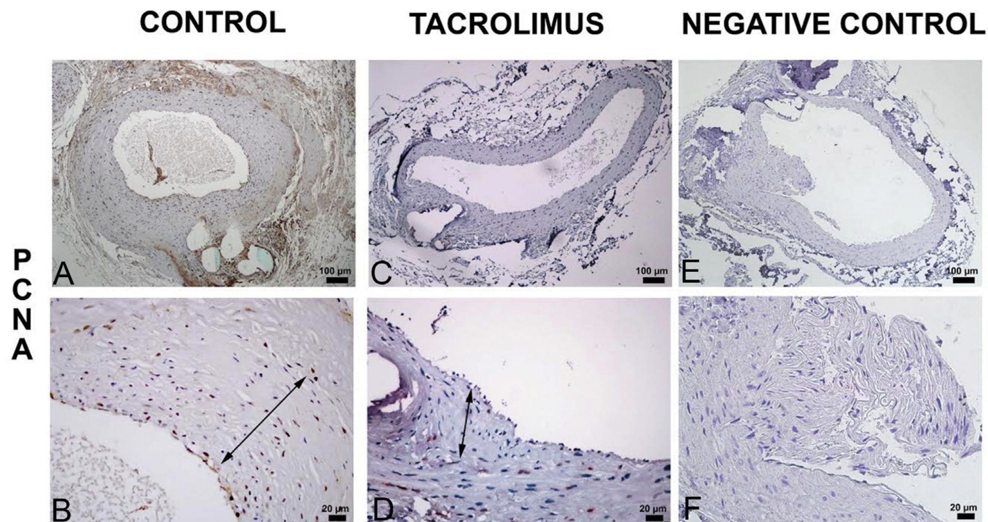

Figure 3. Proliferating cell nuclear antigen (PCNA) immunohistochemical staining. PCNA positive cells were seen in all layers. (A, B) In the control group, PCNA positive cells were dense. (C, D) PCNA positive cells were significantly reduced in the tacrolimus group. Double headed arrows show neointimal area. (E, F) Negative controls. Original magnification (A, C, E): ×100; (B, D, F): ×400.

Similarly, the cells that constituted NIH were positive for ASMA immunostaining (Fig. 4).

DISCUSSION

The results showed that the use of a tacrolimus-chitosan suture (TES) caused a sustained release pattern of tacrolimus for 1 month *in vitro*. It inhibited NIH to a great extent 1 month post-operatively *in vivo*. Moreover, endothelialisation occurred normally in both groups. No local toxicity, like loss of endothelial cells and necrosis or rupture of the vessel wall, was seen in group T.

Tacrolimus is a broad-spectrum immunosuppressant and antiproliferative agent.[13] A growing body of evidence suggests that tacrolimus inhibits several steps of the cascade leading to neointimal proliferation. Tacrolimus inhibits the expression by VMSC of calcineurin, nuclear factor of activated T cell, and interleukin-2, which have been shown to be the most important factors in the development of NIH.[15]

For cytotoxic drug delivery, different forms of periadventitial application methods have been described, such as direct application, hydrogels, wraps, and nanoparticles.[4] In a study by Mutsuga et al.,[10] the efficacy of a periadventitial tacrolimus-eluting biodegradable nanofibre was tested in an aortic anastomosis model. Three different dosages of the nanofibre were tested (tacrolimus content varied from 0.04 weight percent (wt%) to 1.0 wt% of the 5 mg nanofibre). It was found that the 1% wt tacrolimus-eluting nanofibre group revealed a significant reduction in

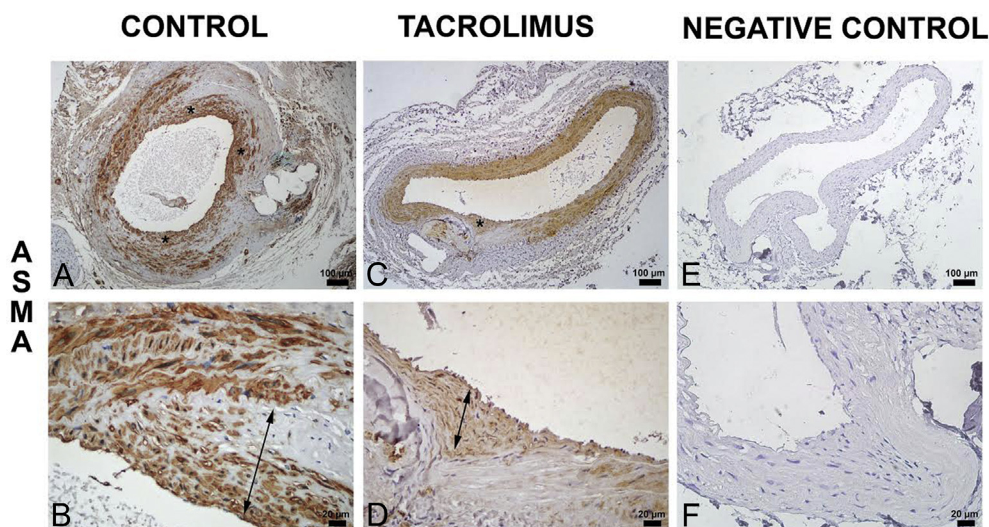

Figure 4. α-Smooth muscle actin (ASMA) immunohistochemical staining. (A, B) In the control group, ASMA positive cells were present throughout the lumen in the smooth muscle layer and neointimal hyperplasia areas (asterisk and double headed arrows). (C, D) In the tacrolimus group, ASMA positive cells were present in the smooth muscle layer and in the limited area near the surgical site where there was neointimal hyperplasia (asterisk and double headed arrows). (E, F) Negative controls. Original magnification (A, C, E): ×100; (B, D, F): ×400.

the ratio of intimal area at 4 weeks when compared with the groups with lower dosages of tacrolimus (0.04 and 0.4 wt%). In another study, the efficacy of a peri-adventitial tacrolimus-eluting nanofibre (1 wt%) on NIH in a rat venous anastomotic model was tested. Similarly, peri-adventitial application of the nanofibre reduced NIH at 1, 2, and 4 weeks after operation and preserved endothelialisation.[2] However, Rajathurai et al. revealed that while peri-adventitial application of sustained release rapamycin microspheres (low dose [60 μg/cm] and high dose [120 μg/cm]) decreased NIH in 4 week old grafts, the inhibition was not found to be sustained in 12 week old grafts.[11] Similar findings were reported by Kawatsu et al.[12] They showed that peri-adventitial rapamycin-eluting biodegradable poly-L-lactic acid and epsilon—caprolactone co-polymer films inhibit NIH of both arterial and vein grafts, although the inhibitory effect was more pronounced in the arterial graft than the vein graft. Clinically, Mátyás et al. studied the efficacy of a bioresorbable paclitaxel-eluting wrap around anastomosis in patients undergoing femoropopliteal bypass with a polytetrafluoroethylene graft.[16] After 2 years, they found that the wrap group had fewer limb amputations than controls (15.5% vs. 18.4%). The time to amputation for patients who required amputation was twice as long (153 days vs. 76 days), and the benefits were found to be more pronounced in diabetic patients. This effect was pronounced, with 13.8% of treated patients requiring limb amputations versus 23.5% of controls. Moreover, the diameter at the distal graft anastomosis was greater in the wrap group than in controls (difference of 2.1 mm after 2 years; $p = .03$).[16]

The adventitia has been shown to contribute substantially to the formation of NIH and vascular remodelling in the process of restenosis after angioplasty.[17,18] The main advantages of peri-adventitial drug delivery have been claimed to be that the effect is mostly local, with no systemic toxicity related to cytotoxic drugs.[13] Moreover, the amounts of drug that reach the intimal layer are markedly diminished, minimising the risk that drug application will inhibit re-endothelialisation of the vessel lumen after anastomosis.[15] However, the amount of the drug delivered to the inner layers including intima and media *in vivo* remains unclear in the peri-adventitial mode of drug delivery. Alternatively, the anastomosis could be constructed using cytotoxic drug-eluting sutures. Theoretically, the transmural nature of cytotoxic drug release from coated sutures seems to have more potential in inhibition of NIH as it has the capacity to do battle with all layers of the vessel wall involved in the pathogenesis of NIH.

Morizumi et al. coated 7/0 PVDF sutures with two different doses of tacrolimus (0.13 wt% as the low dose and 0.40 wt % as the high dose) in a transverse femoral arteriotomy model.[13] The drug coated sutures (both low and high dose) caused a significant reduction of neointimal thickness without interfering with normal endothelialisation. It was claimed that because of the sustained release of tacrolimus from the suture using a bioabsorbable polymer directly into all layers of the blood vessel wall, including the intima, media, and adventitia in the model, the drug coated sutures were more advantageous than the peri-adventitial method in NIH. In the present study, the natural biopolymer chitosan was used for drug coating, to establish a sustained release pattern. Coating sutures with chitosan offers several advantages in drug delivery, including control of drug release, slowing the degradation rate, and improving biocompatibility.[19] In group T, in accordance with the study by Morizumi et al.,[13] the indices of NIH including the ratio of intimal area and the degree of anti-PCNA immunostaining reduced significantly compared with group C. Additionally, in the current study, the dosage of the coated drug was twice as high as that used in the study by Morizumi et al. (0.9 vs. 0.4 wt%).[13] No local or systemic toxicity related to the drug was seen after 1 month. However, a high rate of local toxicity (25%) like graft rupture, inhibition of adventitial neoangiogenesis, and paradoxical acceleration of vein graft disease was reported by Rajathurai et al.,[11] with the use of a higher dose of peri-adventitial rapamycin (120 μg/cm). However, the amount of tacrolimus used for coating in the present study (0.65 μg/cm) was much less than the dose used by Rajathurai et al.,[11] which could explain the difference. Passing the needle and then suture from the wall of artery may wash out the coated surface of the suture. The amount of drug that was washed out from the suture during anastomosis was not tested. However, it has been shown that chitosan is a bioadhesive polymer at physiological pH values and once it is bound to the surface of the suture it is never released.[20]

In the present experimental model, a longitudinally limited infrarenal aortotomy was preferred. This model was previously described by Ogata et al. and Yamamoto et al.[21,22] Intimal hyperplasia models have been done by (i) partial transverse or limited segment longitudinal aortotomy; (ii) end to end anastomosis; (iii) graft interposition; (iv) aorto-caval (arteriovenous) anastomosis; and (v) balloon endothelial injury.[5,8,10,11,13,21,22] Models (ii), (iii), and (iv) are regarded as anastomotic stricture models in which, in addition to intimal hyperplasia related to vessel wall injury, the degree of luminal narrowing is evaluated. Therefore, anastomosis models (e.g., end to end or end to side) have the ability to simulate the real hemodynamic alterations seen in clinical practice, and are therefore more clinically relevant than type (i). The advantage of selecting a longitudinal aortotomy model for the induction of NIH is reported to be that residual stenosis is inevitable with this type of aortotomy repair and it stimulates VSMC proliferation and migration to a great extent.[22] In the authors' opinion and as stated by Mutsuga et al.,[10] the main disadvantage of transverse partial aortotomy and anastomosis models (both end to end or end to side types) is that owing to the transverse configuration of aortotomy, the chance of cutting axial histological sections obliquely is high and consequently causes over-estimation of the extent of NIH. To prevent this issue, serial histological sectioning at the line of aortotomy is mandatory. However, serial cutting is not always technically feasible in such a limited area of aortotomy. Moreover, in histological evaluation of axial

aortotomy sections, it is difficult to evaluate both sides of the suture line in a single tissue section in transverse aortotomy. In the authors' opinion, this issue could be overcome with longitudinal aortotomy models.

The present study has some limitations. First, the sustained release pattern of the TES *in vivo* was not tested. Second, although the inhibitory affect of the TES on NIH was confirmed at 1 month, its long-term effect is unknown. Third, the experimental longitudinal aortotomy model was done only to establish NIH and was not a real anastomosis model. Finally, compared with transverse aortotomy models, longitudinal aortotomy creates a higher risk of turbulence and residual stenosis after repair. Control angiography immediately after closure of aortotomy was not performed.

In conclusion, TES inhibited NIH at the aortotomy site to a great extent without causing any local toxicity in the rat model. It is believed that this finding has significant clinical implications. The transmural nature of the sustained release pattern might offer a promising solution for early anastomosis failure related to NIH in cardiovascular surgery.

CONFLICTS OF INTEREST

None declared.

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