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The Performance of Cross-linked Acellular Arterial Scaffolds as Vascular Grafts; Pre-clinical Testing in Direct and Isolation Loop Circulatory Models

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Abstract

There is a significant need for small diameter vascular grafts to be used in peripheral vascular surgery; however autologous grafts are not always available, synthetic grafts perform poorly and allografts and xenografts degenerate, dilate and calcify after implantation. We hypothesized that chemical stabilization of acellular xenogenic arteries would generate off-the-shelf grafts resistant to thrombosis, dilatation and calcification. To test this hypothesis, we decellularized porcine renal arteries, stabilized elastin with penta-galloyl glucose and collagen with carbodiimide / activated heparin and implanted them as transposition grafts in the abdominal aorta of rats as direct implants and separately as indirect, isolation-loop implants. All implants resulted in high patency and animal survival rates, ubiquitous encapsulation within a vascularized collagenous capsule, and exhibited lack of lumen thrombogenicity and no graft wall calcification. Peri-anastomotic neo-intimal tissue overgrowth was a normal occurrence in direct implants; however this reaction was circumvented in indirect implants. Notably, implantation of non-treated control scaffolds exhibited marked graft dilatation and elastin degeneration; however PGG significantly reduced elastin degradation and prevented aneurismal dilatation of vascular grafts. Overall these results point to the outstanding potential of crosslinked arterial scaffolds as small diameter vascular grafts.

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Disclosure statement

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1. Introduction

Almost 1.4 million vascular grafts are needed every year in the US alone to replace diseased arteries. Of these, about 200,000 are small and medium diameter grafts (4-6mm) for vascular access and to relieve lower limb ischemia and more than 600,000 are small diameter grafts (1-4mm) needed for coronary bypass procedures. The conduit of choice for small diameter vascular graft surgery is the autologous vein or artery, but these are not available in 25-30% of patients due to preexisting conditions or previous harvesting [1]. Current grafts are made of polyethylene terephthalate (Dacron) or expanded polytetrafluoroethylene (ePTFE), or biologically derived conduits such as cryopreserved saphenous vein allografts and decellularized bovine ureters [2, 3]. Synthetic grafts are being used successfully for replacements of large caliber arteries (above 8 mm internal diameter) with acceptable long term patency [4]. However when the same materials are used in small diameter applications (less than 6 mm internal diameter), they perform very poorly as peripheral arteries, with 50% of them occluding within 5 years, potentially leading to amputation. This is due to the intrinsic thrombogenicity of the materials, significant compliance mismatch leading to peri-anastomotic intimal hyperplasia and lack of remodelling and growth when implanted in young patients [5]. Short term results of biological grafts are also quite promising, but despite their “off the shelf” appeal, poor 1-year patency, extended thrombosis, aneurysmal degeneration leading to rupture and calcification have limited the use of such conduits [6]. This daunting lack of options has prompted surgeons to implant small diameter vascular grafts made of synthetic polymers with suboptimal results.

Therefore, surgeons welcome the possibility of gaining access to “off-the-shelf” small diameter grafts that would be easy to suture, exhibit adequate compliance and burst pressures, remain patent and resist thrombosis and be resistant to aneurysmal degeneration and calcification. It is believed that tissue engineering has the potential to generate such viable grafts by combining synthetic or naturally derived degradable or non-degradable scaffolds with a variety of cells followed by maturation in bioreactors. Such constructs have been tested in animal models but few of them have reached clinical trials because of their tendency to degenerate, dilate and calcify after implantation [6-9].

To overcome aneurysmal degeneration and dilatation, we hypothesized that superior vascular graft scaffolds can be produced by chemically stabilizing acellular arteries. To test this hypothesis, we pioneered the use of elastin-rich tubular vascular grafts (ETVGs) produced from porcine arteries from which all cells and most of the collagen has been selectively removed. This approach has the advantage of creating a 3-D porous structure and maintaining native tissue architecture and arterial matrix “niche” while removing xeno-antigens. We were also the first to describe treatment with pentagalloylglucose (PGG) an elastin-stabilizing polyphenolic tannin to reduce biodegradation and calcification of ETVGs [10],[11],[12]. In addition we showed that PGG-treated ETVGs exhibited adequate mechanical and biological properties *in vivo* by subdermal implantation and were non-thrombogenic in acute implantation studies in rabbits [13, 14]; recently we also showed that PGG treatment diminished the tendency of ETVGs to undergo diabetes-related alterations *in vivo* [10] which could become relevant if these grafts will be implanted in diabetic patients.

Encouraged by these results, we are now for the first time presenting data regarding pre-clinical testing of stabilized ETVG grafts in a circulatory model in the rat using direct implantation as transposition grafts with 4 and 8 week follow up and indirect implantation using the isolation-loop approach with 12 week follow-up, a recently validated approach as a high throughput model for testing mechanisms of endothelialization [15].

2. Materials and Methods

2.1. ETVG preparation, stabilization and characterization

Fresh porcine kidneys were obtained from the local abattoir and stored on ice while in transit back to the laboratory. The interstitial renal arteries (2.5-3.5 mm diameter, 10-15 mm length) were dissected, cleaned and decellularized by an alkaline treatment (0.1M NaOH at 37°C for 3 hours). Scaffolds were extensively rinsed with sterile ddH₂O until the pH of rinse solutions dropped to about 8 and then finally rinsed in sterile PBS. Batches of scaffolds were further treated with sterile 0.1% pentagalloylglucose (PGG, Omnicem Ajinomoto, Belgium) in 50 mM phosphate buffer pH 5.5 containing 20% isopropanol for 24 hours, rinsed and stored in sterile PBS. After sterilization for 24 hours in 0.1% peracetic acid in sterile PBS, scaffolds were rinsed in sterile PBS and stored at 4°C for up to 3 months. Scaffold decellularization efficacy was qualitatively assessed by histology using DAPI nuclear staining and Hematoxylin-eosin (H&E) for cell nuclei and general matrix morphology, Masson's trichrome for collagen and smooth muscle proteins and Voerhoff van Gieson (VVG) stain for elastin and biotinylated GS lectin histochemistry for α -Gal [16]. DNA was also extracted from fresh arterial tissue and decellularized scaffolds using a Qiagen extraction kit and samples analyzed by PicoGreen assay and by ethidium bromide agarose gel electrophoresis.

2.2. Mechanical properties

For measurement of suture retention strength, ETVG samples were cut into 5×10 mm segments (n=6 per group) and one end was clamped to an 10N MTS test frame (MTS Systems Corp., Eden Prairie, MN). A single 4-0 braided suture was placed 1 mm from the free edge and its end tied to the MTS test frame. Samples were then preloaded to 0.005 N and extended to failure at 5 mm/min; final data was expressed as grams-force. For compliance and burst pressure analysis, ETVGs (n=6 per group) were adapted on both ends with barbed Luer connectors and secured with a clamp at each connector. A peristaltic pump was used to progressively fill sections with PBS at room temperature and a pressure transducer was mounted on the distal end of the arterial scaffold to record pressures continuously via a computer interface. For diametrical compliance, segments were exposed to 80 mmHg and 120 mmHg and digital images were captured at each pressure setting. The images were then imported into SolidWorks and mean outside diameter calculated digitally using measurements at 6 positions perpendicular to the ETVG sides. Diametrical compliance was then calculated using equations published by Hamilton's group [17]. A similar setup was used to assess burst pressures (n=6 per group) using the peristaltic pump to progressively fill sections with PBS until rupture.

2.3. Scaffold heparinization

To optimize the heparinization protocol, PGG and non-PGG treated ETVG samples were rinsed in phosphate buffered saline (PBS), reacted with Jeffamine, amine-terminated polypropylene glycol, (Huntsman) 240mM in 0.25M MES buffer, pH=5, 2 hours at room temperature using a combination of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide (EDC/NHS, 300/10mM) as activator. After another PBS rinse, nitrous acid degraded heparin (Celsus; 2mg/ml; in 0.15M NaCl; pH=3.9, solution containing 1mg/ml NaCNBH₃) was coupled by reductive amination to the aminated and non-aminated control samples by a 16 hours reaction at room temperature. The treatments were performed either with or without pre-soaking in Heparin and samples were subsequently rinsed and stored in sterile PBS.

2.4. Heparin quantification

The heparin content of ETVGs (n=3) was quantitatively determined by 3-methyl-2-benzothiazoninone hydrazone (MBTH) assay adapted from Risenfeld and Roden [18] similar to that described before by Bezuidenhout et al. [19]. Heparin content (mg/g tissue) was calculated from standard curves.

2.5. Heparinization of grafts for implantation

Heparinized ETVG samples for implantation were prepared as described above via Jeffamine functionalization (without pre-soaking) and heparin attachment using 0.2µm-filtered solutions under sterile conditions in a laminar flow hood. The following four implant groups were thus generated: 1) untreated (None); 2) PGG-treated (PGG); 3) Heparin (Hep); and 4) PGG followed by Heparin (PGG, Hep).

2.6. Denaturation temperature determination

Thermograms of ETVG samples (5-10mg, n=3) in sealed aluminum sample pans were obtained at a heating rate of 10°C/min (DSC 7; Perkin Elmer) and the onset temperature of the denaturation endotherm recorded as the denaturation temperature [20].

2.7. Resistance to enzymatic degradation

ETVG samples (n=6 per group and per enzyme) of about 3×3 mm were lyophilized and their masses recorded to obtain dry tissue mass. For collagenase resistance, samples were incubated in 1 mL solution of 20 units/mL ultrapure type VII collagenase (Sigma) in 1 mM CaCl₂ 100 mM Tris (hydroxymethyl) aminomethane at pH 7.8 and 0.02% NaN₃. For elastase resistance, samples were incubated in 1 ml of 6.25 units/mL ultrapure elastase (Elastin Products Company) in the same buffer. After incubation at 37°C for 24 hours with mild agitation, samples were centrifuged at 12,000 RPM, rinsed three times with ddH₂O, lyophilized and weighed to calculate percent mass loss during enzyme digestion.

2.8. Implant groups

For direct implants, a total of 52 rats were implanted with vascular grafts to complete a targeted n=12 per ETVG group, with n=6 per time point. Three rats were replaced due to premature graft rupture at 6 weeks. For isolation-loop implantation, we first prepared two

groups of 2.5-3 mm diameter, 10-15 mm long acellular grafts, namely Hep and PGG, Hep (n=6 per group) as described above. Then we heat set 90 mm long low-porosity ePTFE segments for 3 minutes at 100°C over a spiralled 1.5 mm nylon cord to generate a stable alpha-loop structure without kinking. A 10 mm midway segment was then removed from the ePTFE loop and replaced with a ETVG segment by end-to-end anastomosis with interrupted 9-0 nylon (Ethilon; Johnson & Johnson, New Brunswick, NJ) using an operating microscope (Zeiss Universal S3 OPMI 6-SFC, Oberkochen, Germany).

2.9. Graft implantation

All animal experiments were approved by the Animal Research and Ethics Committee of the University of Cape Town and were in compliance with the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, South Africa. Male Wistar rats weighing 350-510 grams were induced with isoflurane 5% anaesthesia and were maintained with 2% isoflurane spontaneously breathing via a conical mask. Sterility was maintained throughout the procedure and a warming pad was used to regulate temperature. Following a mid-line laparotomy, the aorta was dissected free of the inferior vena cava and surrounding tissues and all perforating arteries between the left renal artery and the iliac bifurcation were ligated. In all cases the inferior mesenteric artery was preserved. A single dose of intravenous heparin (1mg/kg) was administered and the direct grafts were implanted into the infrarenal aorta by end-to-end anastomoses using 9-0 Nylon interrupted sutures. The isolation-looped grafts were similarly implanted by anastomoses to the infrarenal aorta and further secured with a suture to the lumbar muscles to prevent twisting. The abdomen was closed in layers and Buprenorphine (0.1mg/kg) was administered subcutaneously twice daily for three days. No anticoagulation medication was administered after surgery.

2.10. Graft removal

Following 4 and 8 weeks for direct implants and 12 weeks for isolation-loop implants, the abdomen was opened and the grafts inspected for patency by observation of pulsation in the distal aorta, under general anaesthesia as for the implant procedure. Animals were euthanized by exsanguination following 1mg/kg heparin administration via the inferior vena cava. The aorta was flushed with phosphate-buffered solution via the apex of the left ventricle until clear of blood (cca.150ml), following which the aorta was perfusion fixed with formalin. The graft was then excised, cross-sectioned for macro-photography and processed for histology.

2.11. Histology and quantification

Tissue samples were post fixed in zinc solution [21], dehydrated, embedded in paraffin, sectioned and processed for histological examination. Hematoxylin-eosin and Miller and Masson's trichrome stain (ELMAS) were used for basic light histological analysis. Immunofluorescent identification of endothelial cells was performed with antibodies to Factor 8 (Dako), CD3 for lymphocytes (Dako), ED1 for pan-macrophages (Serotec), CCR7 for M1 macrophages (Abcam), ED2 for M2 macrophages (Serotec), alpha-smooth muscle cell actin (Dako), using Cy3 conjugated streptavidin or Alexa Fluor 488 GxR (Invitrogen). Immunohistochemistry for endothelium was also performed with anti-CD31 antibodies

(Fitzgerald). Elastin and calcification was identified with Orcein and Alizarin red stain respectively, and glycosaminoglycans were stained with Alcian blue. Quantitative measurements of wall thickness, lumen area, internal elastic lamina (IEL) diameter and neo-intimal pannus overgrowth were performed on trichrome stained sections with Visiopharm Integrated Systems software (VIS, Visiopharm A/S, Hoersholm, Denmark) and Adobe Photoshop CS6 on mid-graft cross-sections.

2.12. Statistical analysis

Statistical tests were performed with STATA (StataCorp. 2011. *Stata Statistical Software: Release 11* College Station, TX: StataCorp LP) Results were expressed as mean $\pm$ SD for continuous variables. All continuous data was confirmed as nonparametric (Shapiro-Wilkinson) interrogated with Mann Whitney test for statistical significance. Kruskal-Wallis equality-of-populations rank test was used for multiple group analysis. Categorical data was tested with the Fishers exact, and the level of significance was set at $P < 0.05$. Burst pressures, diametrical compliance and enzyme resistance were compared using ANOVA, and effect of heparinization and denaturation temperatures with student's t-test.

3. Results

3.1. Scaffold preparation and characterization

To generate 2.5-3.5 mm diameter, 10-15 mm long vascular grafts, we dissected segmental and inter-lobar arteries from adult porcine kidneys and decellularized them with NaOH as reported previously with minor modifications [22]. Completeness of decellularization was confirmed lack of extractable DNA, absence of cell nuclei in histological DAPI staining (Fig. 1) and histological analysis including H&E, Trichrome, and α -Gal antigen lectin histochemistry, all of which depicted cells in native tissues and their absence in decellularized ETVGs. Acellular renal arteries showed good preservation of overall arterial matrix morphology including native collagen and elastin (Fig. 1). Notable histological features of the acellular renal arteries included a distinct internal elastic lamina (IEL), a relatively thin media containing thin elastin and collagen fibers and a very thick elastin and collagen-rich adventitia. Suture retention strengths and burst pressures of acellular arteries were not different from those of native arteries (Fig. 2). However, decellularization reduced diametrical compliance by about 50% (Fig. 2).

3.2. Stabilization and heparinization

To increase biocompatibility, untreated and PGG-treated ETVGs were heparinized by covalent immobilization of nitrous acid activated heparin (which contains aldehyde end-groups for end-point attachment). To monitor efficacy, we measured heparin content in each group of tissues. Simple soaking in activated heparin did not result in heparin binding because acellular arteries did not contain sufficient exposed amine groups. Thus, we first aminated the ETVGs using diamines (Jeffamine) and carbodiimide chemistry. Control samples showed low baseline levels of heparin, irrespective of PGG treatment. No differences were seen between the tissue samples which were pre-soaked in heparin or not (Fig. 2). Significantly and much higher heparin content values were observed for samples that were first aminated with Jeffamine / carbodiimide prior to the reaction with heparin.

PGG did not interfere with the reaction, and pre-soaking in heparin did not significantly affect the heparin content of ETVGs (Fig. 2). Jeffamine / carbodiimide treatment alone resulted in significant increases in denaturation temperature ($p < 0.001$) of the grafts (Fig. 2) indicative of crosslinking. Further reaction of aminated scaffolds with activated heparin did induce further chemical crosslinking in both -PGG or +PGG scaffolds. These results indicate that covalent immobilization of heparin cross-links the ETVGs and that PGG does not impede heparinization. Analysis of resistance to collagenase and elastase showed that decellularization did not significantly change tissue susceptibility to enzymes ($p > 0.05$). PGG treatment of ETVGs reduced their susceptibility to elastase by about 50% while heparinization stabilized collagen by more than 80% (Fig. 2), indicating that PGG stabilizes elastin while heparinization treatment cross-links collagen.

3.3. Direct implants; surgical handling and implants statistics

Although the surgeon was not blinded at the time of graft implant, surgical handling was adequate for all vascular grafts and subjectively superior in the heparinized set of grafts (Hep and PGG, Hep groups). The goal of this study was to ensure completion of $n=6$ implants per time point per group. The implant statistics (Table 1) show that only 3 grafts ruptured prior to their scheduled 8 week explant, all of which occurred after 6 weeks. Two of them were from the non-stabilized graft group ("Non") and one from the PGG-treated group. Rupture was confirmed at autopsy with abdominal blood and macroscopic evidence of wall defect. These grafts were subsequently replaced. After replacement, all of the animals survived to their designated explant time points and had 100% patent grafts (Table 1). An overview of implantation and explantation macroscopic aspects is shown in Fig. 3.

3.4. Direct implants; histological evaluation

All grafts, irrespective of the treatments they were subjected to, demonstrated concentric peri-graft tissue infiltration and formation of a well vascularized collagenous granulation tissue of about 600-800 μm in thickness (Fig. 4). Cells were seen infiltrating and completely re-populating the (initially acellular) graft adventitia without penetrating the media. This cellular infiltration was composed of CCR7-positive (M1) and ED2-positive (M2) macrophages, α -smooth muscle cell actin-positive myofibroblasts, foreign body giant cells and very few CD3-positive lymphocytes (Fig. 4). The thickness of this capsule did not increase from 4 to 8 weeks and did not appear to reduce the lumen diameter, suggesting little if any constrictive remodeling. A thin layer of neo-intimal tissue with the appearance of "intimal hyperplasia" but probably derived from peri-anastomotic pannus over growth, was also present in all direct implants. The neo-intimal tissue was rich in myofibroblasts and covered by a continuous layer of F8-positive endothelial cells (Fig. 4). In most explants analyzed, the neo-intimal tissue was stable and prevented formation of clots. However, in about 1/3 of all implants, irrespective of graft pre-treatment, the neo-intimal tissue underwent detachment or remodelling at 4 weeks, allowing for thrombus formation. At 8 weeks, the heparinized grafts (with or without PGG) did not exhibit any thrombus formation. Three neo-intima samples (out of total 45 analyzed) also exhibited chondroid metaplasia with Alcian Blue positive (not shown) and Alizarin Red positive structures (Fig. 4). Two additional samples stained positive for calcium in the neointima, in the absence of

chondroid metaplasia. None of the acellular grafts showed any evidence of calcium deposition within the implanted graft wall.

To gather more quantitative data, we analyzed representative ELMAS-stained midway graft sections by digital morphometry and measured the perimeter of the IEL, wall area, lumen area, wall thickness and neo-intimal pannus area for each sample (Fig. 5). We also used Orcein stain for elastin and quantified the integrity of implanted elastin as a function of graft treatment.

When compared to pre-implant samples, the IEL diameter in all implanted grafts (irrespective of treatment) did not change after 4 weeks but increased significantly to almost 100% at 8 weeks in non-treated grafts (Fig. 6). The IEL diameters in grafts treated with PGG, Hep or PGG followed by Hep remained unchanged at 8 weeks ($p>0.05$). Similarly, the luminal area was statistically larger only in the non-treated grafts at 8 weeks; grafts treated with PGG alone, Hep alone or PGG followed by Hep exhibited similar luminal areas at 4 weeks without signs of progression at 8 weeks ($p>0.05$). The graft wall thickness (collagenous granulation tissue) was similar in most groups (600-800 μm) except for PGG, where it was thinner ($p<0.01$). Neo-intimal pannus thickness increased across all four groups from 4 weeks to 8 weeks as a result of peri-anastomotic tissue ingrowth (Fig. 6); however the differences among the four groups at 8 weeks were not statistically significant. Notably, statistically significant preservation of elastin with sequential stabilization treatments was measured in ETVGs (Fig. 6E). Both heparinization and PGG treatment independently resulted in a statistically significant improvement in elastin preservation (when compared to untreated controls) with an additive effect in their combination demonstrating that elastin, unless stabilized, is susceptible to in vivo degeneration. Elastin stabilization only correlated statistically to dilatation at 8 weeks (Bivariate Fit, $P=0.0427$).

3.5. Evaluation of isolation-loop (indirect) implants

To test the hypothesis that the neo-intimal pannus observed in direct implants is related to trans-anastomotic tissue ingrowth and not to trans-mural cell infiltration, we sutured 10 mm-long graft segments midway in 9 cm long ePTFE loops such that the implanted grafts were 4 cm away from the anastomosis to the native abdominal artery (Table 2). The grafts were easy to suture onto the ePTFE material and they did not kink when forced into an alpha-loop configuration (Fig. 7). At explantation, the grafts were well integrated into host tissues, namely the retroperitoneum. In the Hep group 5/6 grafts were patent while in the PGG, Hep group 4/6 grafts were patent.

Similar to the direct implants, all isolation-loop grafts demonstrated formation of the external, well vascularized collagenous granulation tissue (Fig. 7). Quantitative morphometric comparisons between the two pre-treatments applied to the indirect grafts (Hep vs PGG, Hep) showed that there were no significant differences in wall (capsule) thickness, IEL diameter (dilatation), elastin preservation and lumen area ($p>0.05$). Similarly, no statistical differences were noted in these four parameters when indirect implant data were compared to the direct implant results. The major differences between direct and indirect grafts were 1) the complete lack of neo-intimal pannus tissue formation in the isolation-loop samples, irrespective of the graft pre-treatment and 2) significantly reduced

thrombus formation. Since the arterial sub-endothelial basement membrane in the indirect grafts was not covered by pannus, this experiment also gave us the unique opportunity to evaluate the intrinsic thrombogenicity of the luminal surface of acellular arteries. As seen in the histology, immunofluorescence and SEM images, the surface of the 12 week indirect implants appeared wavy but relatively smooth and free of adhered cells, fibrin strands or microthrombi (Fig. 7).

4. Discussions

We have shown earlier that alkaline decellularization of carotid segments removes all cells and some of the collagen; however the NaOH technique was never applied to muscular arteries. Current results on renal arteries demonstrate that the alkaline decellularization method was effective, with complete cell removal as seen on DAPI stained sections and lack of extractable DNA, as well as lack of cells on histology stains (H&E, trichrome, VVG). Furthermore, acellular arteries lacked α -Gal staining which confirmed complete decellularization and also revealed the absence of the powerful xeno-antigen throughout the tissues.

We chose renal arteries as the tissue source in order to generate size-matched grafts which would be implanted into the abdominal artery of rats. In current studies we focused on acellular arteries as biological scaffolds for several reasons. First, because we believed that the biggest asset of acellular scaffolds prepared from target tissues is the presence of an architecturally accurate extracellular matrix network capable of presenting cell adhesion motifs for repopulation with differentiated cells as well as to serve as “niches” and cues for stem cell differentiation into desired cell types [23, 24]. While cell removal reduced immunogenicity and generated pores allowing cell infiltration, it also raised concerns regarding the exposure of the vascular sub-endothelial layer to flowing blood. In current studies we showed that the sub-endothelial basement membrane of ETVGs was essentially non thrombogenic. Second, preservation of major tissue components (elastin and collagen) in their original configuration ensured adequate mechanical properties. Our results showed that suture retention strength, handling properties and burst pressures were unaltered by decellularization, while compliance values were reduced by about 50% as compared to fresh arteries. These results suggest that load-bearing structural components were well preserved, while other elements responsible for recoil (elastin associated microfibrils, proteoglycans) were probably removed by decellularization. We and others have shown that among matrix components, the proteoglycans are rapidly lost during decellularization [14]. It remains to be determined whether these compliance levels (5-6%) will be important in the long run; however these values are similar to those reported by Hamilton’s group for human femoral and popliteal arteries (4.7-6.1%) [17]. Third, maintenance of intact, mechanically functional elastin within the structure of scaffolds is a very important aspect of vascular tissue engineering. Elastin is almost impossible to incorporate into sheets or lamellae using a bottom-up approach and new mature elastin fibers are notoriously difficult to synthesize in vitro or in vivo [25, 26]. Elastin is important for recoil properties of arterial tissues and thus its presence within our scaffolds may ensure extended mechanical durability. Moreover, elastin has a tendency to calcify and degenerate upon implantation and once lost in vivo, it will not likely be re-synthesized by resident or infiltrating cells. For this reason we used

arteries as a starting material. After decellularization, elastin is evident in the form of a distinct layer in the IEL, as fine fibrils within the media and as an extensive 3D network of fibers within the adventitia. We then chose to treat ETVGs with PGG, an elastin binding polyphenol which reduces elastin's susceptibility to enzymatic degradation and also diminishes its calcification potential [16, 22, 27-30]. Recently we also showed that PGG treatment of elastin-rich scaffolds protected implants from diabetes-related glycation, crosslinking and alterations in mechanical properties, which might prove very beneficial for diabetic patients requiring peripheral vascular surgery [10].

Acellular arterial scaffolds derived from renal arteries also contained a significant amount of collagen specifically in the adventitia layer. In order to stabilize the collagen component and to increase implant hemocompatibility, we heparinized the scaffolds via carbodiimide chemistry. The optimal protocol employed Jeffamine / carbodiimide tissue amination, followed by reaction with activated heparin, which yielded incorporation of significant amounts of heparin within the ETVGs (about 10% heparin by tissue weight). Results also showed that PGG did not interfere with the heparinization reaction and that collagen was effectively crosslinked as evidenced by increased denaturation temperatures. Resistance to enzymatic degradation further confirmed that PGG stabilized elastin by reducing its susceptibility by almost 50%. This effect was demonstrated earlier by us for grafts derived from carotid arteries [22]. Heparinization stabilized and crosslinked collagen and increased its resistance to collagenase by 3-4 fold. Using these two selective stabilization techniques, we generated 4 main scaffold groups which were selected for implantation as direct transposition grafts in the abdominal aorta of rats and analyzed after 4 and 8 weeks: untreated, PGG-treated (elastin stabilized), heparinized (collagen stabilized) and PGG, heparin treated (elastin and collagen stabilized). Initial studies showed that the scaffolds exhibited good handling properties and were easy to implant. Survival was above 85% reaching 100% in some groups and overall patency at explantation was close to 100% in all groups.

Samples were analyzed by histology and the findings of this study could be separated into two categories; those which were unrelated to the implant treatment and those specific to the implant.

In the first category, one major finding was encapsulation of the grafts in a concentric, peri-graft vascularized granulation tissue which integrated well with the scaffold adventitia but not the media. The newly formed collagenous tissue stabilized at about 800 μ m thickness after a few weeks and did not grow in thickness with time. It is known from previous work by Campbell et al. that implantation of solid objects (rods, tubes) into the peritoneal cavity or pleural cavity induces activation of macrophages and initiation of a foreign body response [31, 32]. Infiltrating cells differentiate into myofibroblasts which generate a new connective tissue capsule rich in collagen surrounding the implant. These collagen tubes were later used as vascular grafts with promising results [31, 32]. A similar approach was used to manufacture heart valves by implanting tricuspid valve-shaped molds subdermally in rabbits and detaching the collagenous capsule tissue [33]. In pilot studies, we also observed that essentially the same type of capsule is formed when ETVGs are implanted as direct vascular grafts in the femoral artery in minipigs (data not shown); thus it appears that this

encapsulation reaction is non-specific and could be beneficial after implantation of tubular grafts. Notably, porcine ETVGs did not elicit an immune reaction after implantation in rats, strengthening the hypothesis that xenogeneic matrices, if well decellularized, can serve as excellent tissue scaffolds [34, 35].

Graft encapsulation occurred irrespective of the graft pre-treatment, indicating that stabilization of collagen and elastin does not prevent cell infiltration in the adventitia. While the adventitia was extensively repopulated with host cells and capillaries, few cells were found within the media of the implanted grafts. The mechanisms underlying this phenomenon are not known at this point, but it is possible that the compact IEL and adventitial elastin fibers averted cell infiltration from the lumen and adventitial sides, respectively.

Another major finding was the omnipresence of neo-intimal tissue which grew in thickness with time, remodelled and in some instances calcified. The neo-intima, potentially derived from trans-anastomotic tissue overgrowth [15, 36] was covered by endothelium and thus was non-thrombogenic if intact; however, in few cases the neo-intimal tissue appeared disturbed, activated and associated with local thrombi. In all samples analyzed calcification was absent from the scaffold graft wall, denoting the fact that decellularized arteries are less prone to calcification in this model.

Several aspects were noted to depend on the scaffold pre-treatment. Only non-stabilized grafts exhibited dilatation, associated with visible elastin degeneration *in vivo*, specifically the IEL and the adventitial elastin fibers. PGG treatment as well as the heparinization crosslinking procedure both reduced elastin degeneration and the combination of the two was very effective in maintaining matrix integrity, pointing to stabilized arterial scaffolds as viable small diameter vascular grafts.

Overall, this is an accepted screening model for vascular grafts with the caveat that the grafts were relatively short and thus were most likely endothelialized intra-luminally by trans-anastomotic tissue overgrowth. Because of this phenomenon, it was difficult to assess the intrinsic thrombogenicity of the graft material; thus we pursued a second set of implants where the trans-anastomotic tissue over growth was avoided by separating the grafts from the anastomoses by two low porosity ePTFE segments (isolation-loop model or indirect grafts) with follow-up at 12 weeks as described recently [15, 36]. When compared to the direct grafts these were covered by same collagenous vascularized tissue. However, by contradistinction to direct implants, the indirect grafts lacked neo-intimal tissue overgrowth with absence of thrombi. These results highlight the fact that the exposed sub-endothelial basement membrane of acellular arteries (derived from removal of the endothelium during decellularization) was essentially non thrombogenic in this animal model.

All together, these results provide data which allow us to speculate on the outcome of clinically applicable lengths of stabilized arterial grafts as viable small diameter vascular grafts. Since in most clinical applications, segments of at least 8-10 cm long would be implanted, we expect the proximal and distal peri-anastomotic regions of the ETVGs to behave similar to the direct implants described in this paper and the midway section of the

graft, at least 3-4 cm away from the anastomosis, to behave similar to the indirect implants. Currently we are testing >10 cm-long, chemically stabilized small diameter ETVGs in sheep as carotid interposition grafts.

Finally, we recommend that the optimal approach to pre-clinical testing of potential materials to be used as small diameter vascular grafts should include the following steps. If the material is biologically derived, one should first perform adequate quality controls to ensure complete decellularization as well as preservation of the major extracellular matrix components, including DNA analysis, extensive histology and α -Gal analysis. Biologic or synthetic materials of about 2.5 mm in diameter and 10 mm length could be then tested by: 1) evaluating mechanical properties, including suture retention strength, burst pressure and compliance, 2) implanting samples subdermally in rats for initial biocompatibility, as described before [22], 3) testing tubular grafts as direct vascular graft implants to evaluate peri-anastomotic reactions, 4) testing as indirect isolation-loop implants to assess host reactions away from the anastomosis and 5) implant as >10 cm long grafts in large animals for pre-clinical validation.

5. Conclusions

In the current study, we show that gentle decellularization of porcine muscular arteries generated implantable non-immunogenic small diameter ETVGs endowed with adequate mechanical properties and lack of susceptibility towards calcification. To increase stability *in vivo*, we chemically treated the elastin component with PGG and the collagen component with carbodiimide/heparin. Implantation of ETVGs as vascular grafts in rats resulted in high patency and animal survival, possibly due to the ubiquitous encapsulation of the grafts within a stable vascularized collagenous capsule and to the lack of thrombogenicity of the exposed sub-endothelial basement membrane. Peri-anastomotic neo-intimal tissue overgrowth was a normal occurrence in direct implants; however this reaction was circumvented in indirect, isolation-loop implants. Implantation of non-stabilized ETVGs exhibited marked graft dilatation and elastin degeneration; however chemical stabilization of grafts significantly reduced elastin degradation and prevented aneurismal dilatation of vascular grafts *in vivo* without altering formation of the external capsule. Due to their resistance to thrombosis, dilatation and calcification, stabilized acellular arteries are promising candidates as small diameter vascular grafts.

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Fig. 1. Vascular scaffold data

(A) a whole porcine kidney arterial tree is shown after it was dissected and cleaned manually. Segmental and interlobar arteries (red squares) of 2-3 mm diameter and 10-15 mm length were dissected and decellularized as described in the manuscript. Bar is 10 mm. (B) DNA was extracted from fresh and decellularized (Decell) arteries and quantified using Picogreen ($\mu\text{g}/\text{mg}$, $n=6$ samples per group) and verified with ethidium bromide agarose gel electrophoresis (insert, $n=3$, S, DNA standard; F, fresh; D, decellularized). (C) DAPI nuclear stain (blue) superimposed over green elastin autofluorescence was used to highlight cells in fresh arteries and (D) lack of cells in decellularized (Decell) grafts. Images are representative for $n=6$, bars are $50\ \mu\text{m}$. Lower panels show histology of fresh (E-H) and decellularized (J-M) arteries using H&E, (E, J), Masson's trichrome (F, K), Verhoeff van Gieson (G, L), and α -Gal histochemical detection using GS lectin (H, M). Images are representative for $n=6$ per group, bars are $50\ \mu\text{m}$. (I, N) are α -Gal histochemistry negative controls for fresh and Decell, respectively.

Fig. 2. Characterization and stabilization of acellular arterial scaffolds

(A) Suture retention strength, (B) diametrical compliance and (C) burst pressure values for fresh arteries, decellularized (Decell) arteries and PGG-treated Decell arteries (PGG). Data was obtained from $n=6$. *-statistically significant as compared to fresh, $p<0.05$. (D) Heparin content of untreated ETVGs (-PGG) and PGG treated scaffolds (+PGG). Groups were: Controls (C) not subjected to treatments, (H) treated with activated heparin alone and treated with Jeffamine (J) with and without pre-soaking in heparin (P). (E) Graft crosslinking as evaluated by DSC test for untreated (-PGG) and PGG treated samples (+PGG) before (Control) and after treatment with Jeffamine (Jeff) or Heparin (Hep), or Jeff followed by Hep (Jeff+Hep). (F) Five ETVG groups were prepared and tested for resistance to enzymes: freshrenal arteries, decellularized arteries (Decell), and Decell treated with PGG alone (PGG), Heparin alone (Hep), and PGG followed by Hep (PGG, Hep). $n=6$ per group; *-statistically significant as compared to Decell, $p<0.05$.

Fig. 3. Intra-circulatory implantation of ETVGs as direct grafts

(A-B) macroscopic images showing grafts during implantation in the rat infrarenal abdominal aorta; white arrows depict the anastomoses; bar is 1 mm. (C-F) representative macroscopic aspects of grafts before explantation (top) and macro images of midsections after perfusion fixation of corresponding grafts in each group (bottom). The groups were ETVGs without pre-treatment (Non) or treated with PGG alone (PGG), Heparin alone (Hep), and PGG followed by Hep (PGG, Hep). White arrows depict the anastomoses and bar in (C-F) is 1 mm.

Fig. 4. Histology of explanted direct grafts

Representative sections shown after staining with (A) H&E, (B) Masson's trichrome, (C) Alizarin red, (D) CCR7 immunofluorescence (red) for M1 macrophages, (E) ED2 stain (red) for M2 macrophages, (F) α -smooth muscle cell actin (red) for activated myofibroblasts in the adventitia and (G) in the neo-intima; (H) F8 endothelial stain and (I) CD3 lymphocyte stain. (D-I) nuclei were counterstained with DAPI (blue); Images are representative for n=6, bars are 50 μ m. AC, adventitial capsule; G, initial graft; P, neo-intimal pannus. Lumen is at lower right in all images.

Fig. 5. Evaluation of explanted direct graft histology

Panel of representative Masson's stained mid-graft histology cross-sections from each individual implant retrieved at 4 weeks (4w) and 8 weeks (8w). ETVG groups were: untreated (Non), PGG-treated (PGG), Heparin treated (Hep) and PGG followed by Heparin (PGG, Hep). Histology images were digitized and color coded to measure wall thickness (green), pannus area (yellow), lumen area (red) and clot (blue). Stained sections are shown at left and corresponding digitized images at right.

Fig. 6. Quantitative morphometric data obtained from direct implants

Digitized images (as shown in Fig. 5.) were used to measure: (A) IEL diameter, (B) lumen area, (C) graft wall thickness and (D) pannus area in untreated ETVGs (Non), PGG-treated (PGG), Heparin treated (Hep) and PGG followed by Heparin (PGG, Hep). *-statistically significant as compared to 4 weeks, $p < 0.05$. (E) Orcein stain for elastin (top row) and quantification of elastin content from the histological images (bottom). *-statistically significant as compared to non-treated scaffolds (Non), $p < 0.05$. G, graft. Lumen is at top or top/right corner, bar is 50 μm .

Fig. 7. Indirect isolation-loop graft evaluation

(A) Macroscopic image of indirect graft during implantation in between two ePTFE looped segments (white) and (B) at explantation. Bar is 3 mm. (C, D) SEM analysis of the scaffold lumen surface after 12 weeks implantation. G, cross section of the graft. Bar is 100 μm in (C) and 10 μm in (D). (E) Panel of representative Masson's stained mid-graft histology cross-sections from each individual implant and ETVG group: Heparin treated (Hep) and PGG followed by Heparin (PGG, Hep). Histology images were digitized and color coded to measure wall thickness (green), pannus area (yellow), lumen area (red) and clot (blue). Stained sections are shown on top and corresponding digitized images below them. (F) Quantitative morphometric data obtained from the indirect implants. Groups were: Heparin treated (Hep) and PGG followed by Heparin (PGG, Hep) treated ETVGs. IEL diameter, wall thickness, elastin and lumen area are shown. Representative images of (G) Masson trichrome stain and (H) CD31 stain for blood vessels; bar is 50 μm. AC, adventitial capsule; G, initial graft. Lumen is at left in both images.

Table 1
Summary of direct grafts

	4w	8w	Ruptured	Replaced	Survival at 4w (%)	Survival at 8w (%)	Excluded*	Analyzed
Non	6	8	2	2	85	92	2 ^{a)}	12
PGG	6	8	1	1	100	100	3 ^{b)} +1 ^{a)}	10
Hep	6	6	0	0	100	100	1 ^{b)}	11
PGG, Hep	6	6	0	0	100	100	0	12

* excluded from quantitative analysis:

^{a)} ruptured grafts (n=3),

^{b)} grafts explanted at designated time points which exhibited outside diameter >3.5 mm and an aortic to graft diameter ratio <0.4 at implant.

Table 2
Summary for isolation-loop (indirect) grafts

	Implanted	Ruptured	Patent	Analyzed
Hep	6	0	5	5
PGG, Hep	6	0	4	4