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The Decellularized Vascular Allograft as an Experimental Platform for Developing a Biocompatible Small-Diameter Graft Conduit in a Rat Surgical Model

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Purpose: The present study was aimed to assess the feasibility of using decellularized aortic allograft in a rat small animal surgical model for conducting small diameter vascular tissue engineering research. **Materials and Methods:** Decellularized aortic allografts were infra-renally implanted in 12 Sprague-Dawley (SD) adult rats. The conduits were harvested at 2 (n = 6) and 8 weeks (n = 6), and assessed by hematoxylin and eosin (H&E), van Gieson, Masson Trichrome staining, and immunohistochemistry for von Willebrand factor, CD 31^{+}, and actin. **Results:** Consistent, predictable, and reproducible results were produced by means of a standardized surgical procedure. All animals survived without major complications. Inflammatory immune reaction was minimal, and there was no evidence of aneurysmal degeneration or rupture of the decellularized vascular implants. However, the aortic wall appeared thinner and the elastic fibers in the medial layer showed decreased undulation compared to the normal aorta. There was also minimal cellular repopulation of the vascular media. The remodeling appeared progressive from 2 to 8 weeks with increased intimal thickening and accumulation of both collagen and cells staining for actin. Although the endothelial like cells appeared largely confluent at 8 weeks, they were not as concentrated in appearance as in the normal aorta. **Conclusion:** The results showed the present rat animal model using decellularized vascular allograft implants to be a potentially durable and effective experimental platform for conducting further research on small diameter vascular tissue engineering.

Key Words: Tissue engineering, vessel, rat, animal model

INTRODUCTION

There is a growing need for a clinically applicable and biologically compatible small-diameter vascular conduit. Currently, the autologous reversed saphenous vein graft (SVG) is the most widely used and time proven conduit, not only for coronary

artery bypass grafting (CABG) but also for peripheral vascular surgery.[1-3] However, autologous SVGs are often limited in availability. Various prosthetic small-diameter vascular conduits have been used alternatively, but the outcomes have thus far been disappointing, including those associated with arteriovenous fistula (AVF) access creation for hemodialysis, which comprise the bulk of the demand for small-diameter vascular conduits.[4-7]

Vascular remodeling-induced premature graft failure is a major limitation of small-diameter vascular conduits. Decellularization is potentially an excellent tissue processing method for increasing the availability of non autologous biological small diameter vascular conduits. Removal of antigenic determinants may enhance the utility of vascular allograft conduits such as cadaveric radial arteries or the saphenous vein. However, the propensity for structural deterioration and adverse vascular remodeling remain limitations which must be resolved through further research. Studies using large animal models inherently pose difficulties related to higher cost and logistical problems. Therefore, the present study was conducted to investigate the feasibility of developing a small animal surgical model using the rat as a platform for conducting small diameter vascular tissue engineering research.

MATERIALS AND METHODS

Animal model and surgical procedure

A total of 24 Sprague-Dawley (SD) female rats, 12 donors and 12 recipients, each weighing 250 to 300 g, were used. Each animal was anesthetized with intra-peritoneal injection of 0.25 mL of a 2 : 1 : 2 (v/v/v) mixture of Zoletil 50 (40 mg/kg, Virbac Laboratories, Carros, France), Xylazine (10 mg/kg Bayer, Leverkusen, Germany) and 0.9% (w/v) physiologic saline. For donor preparation, the abdominal aorta was approached via a midline laparotomy incision, after which the aorta side branches were ligated using polypropylene #8-0 monofilament sutures. The aorta was thus mobilized from a point just distal to the left renal artery takeoff to the iliac bifurcation. To prevent clotting, heparin (10 IU/100 g body weight) was injected into the inferior vena cava (IVC). The aorta was then resected just distally to the renal artery and proximally to the iliac bifurcation. The harvested conduit was then flushed with normal saline and transferred for decellularization.

For recipient preparation, anesthesia and aortic mobilization were performed in a manner identical to donor preparation. IVC heparin (10 IU/100 g body weight) injection was performed after which the aorta was clamped just distally to the renal artery and proximally to the iliac bifurcation. The mid portion of the isolated aorta was transected. The four opposing edges of the decellularized graft (two proximal and two distal) were sutured to the corresponding edges of the native vessel with #8-0 polypropylene sutures. Mosquito clamps were applied to each suture arm for traction. The anterior margins of the proximal and distal native aorta were anastomosed with the graft using multiple interrupted Nylon #9-0 sutures. The aorta was then rotated 180 degrees by reversing the traction sutures from left to right which resulted in exposure of the unsutured posterior margins. Anastomosis was performed in an identical manner with Nylon #9-0 sutures (Fig. 1). No additional anticoagulation was performed postoperatively. Ketoprofen 5 mg/kg sc daily and Amoxicillin 150 mg/kg sc x twice daily were administered for 3 days postoperatively for pain and prophylaxis against infection. All surgeries were performed in an environment that was largely specific pathogen-free (SPF). Animals were managed according to the "Revised Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health, revised and published in 1996.[8]

Tissue decellularization

The rat infrarenal abdominal aorta was harvested for decellularization as described above. The harvested specimens, approximately 1.5 cm each in length, were thoroughly washed two or three times in Hank's balanced salt solution (HBSS)

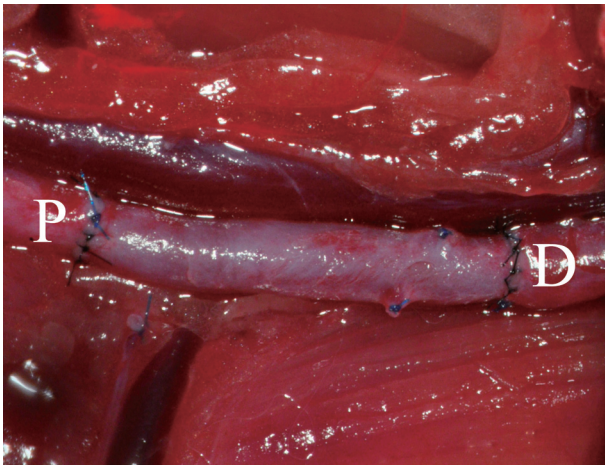

Fig. 1. Interposition of decellularized aortic conduit in the infrarenal abdominal aorta. Anastomosis was achieved with multiple interrupted Nylon #9-0 sutures. The proximal part of the graft was marked by P and distal part by D in reference to the direction of blood flow.

and incubated overnight (15-18 hours) at 37ºC in 10 mL of physiologic saline containing 0.25% (v/v) Triton X-100 (Sigma, St. Louis, MO, USA) and 0.25% (w/v) sodium deoxycholate in a 10 cm-diameter Petri dish. The aortic segments were immersed in pre-chilled HBSS and shaken vigorously, with solution replacement every 2 hours. To ensure detergent elimination, this procedure was repeated three times. Each sample was incubated for 15-18 hours at 37ºC in 10 mL of PBS containing 150 IU/mL DNase I, 100 μg/mL RNase A, and 50 mM $MgCl_2$. Samples were again shaken in pre-chilled HBSS, with solution replacement every 2 hours, as above. Each decellularized aorta was stored at 4ºC in physiologic saline until use. Decellularization efficacy was evaluated by high-power light microscopic observation of hematoxylin and eosin (H&E)-stained specimens.

Preparation for microscopic study

The implanted grafts were harvested at 2 (n = 6) and 8 (n = 6) weeks. To avoid endothelial cell sloughing by mechanical trauma, the graft specimens were fixed in 4% (v/v) buffered para-formaldehyde with gentle continuous perfusion at 60 mmHg prior to extraction. Each of the fixed infrarenal aortic specimens was removed en-bloc with a generous attachment of muscle and surrounding tissue. Stretching or otherwise traumatic instrumentation of the aorta was avoided as much as possible. Excess muscle and surrounding tissue were gently trimmed using a surgical microscope. Prior to paraffin-embedding, specimens were post-fixed in formalin for at least 3 hours. Basic light microscopic staining consisted of H&E, Elastica von Gieson (EVG), and Masson Trichrome (MT).

Immunohistochemistry

Three um thick paraffin-embedded slices were deparaffinized and rehydrated in ethanol. The specimens were then placed in a solution, containing 0.1% (v/v) Triton X-100 and 0.05% (v/v) Tween-20 in 0.01 M sodium citrate buffer, for antigen retrieval. Immunohistochemistry to detect von Willebrand factor and alpha-smooth muscle actin were performed using rabbit anti-human von Willebrand factor antibody (1 : 200, catalog no. F3520, Sigma, St. Louis, MO, USA) and mouse anti-human smooth muscle actin antibody (1 : 100, catalog no. M0851; Dako, Glostrup, Denmark). Antibody reaction was assessed using an Envision DAB+ kit (catalog no. K4006, K4010, Dako). Samples were subsequently counterstained with Mayer's hematoxylin (catalog no. S3309, Dako). Immunohistochemistry for CD31 and von Willebrand factor was performed using goat anti-PECAM-1 antibody (1 : 100, catalog no. sc-1506; Santa Cruz Biotechnology Inc.) and rabbit anti-von Willebrand factor. Antibody reaction was assessed using a rabbit anti-Goat IgG conjugated Cy3 (catalog no. 305-165-006, Jackson immune research laboratories inc., West Grove, Pennsylvania, USA) and goat anti-rabbit IgG conjugated alexa 488(catalog no. A-11034, Invitrogen inc., Eugene, OR, USA). Samples were subsequently counterstained with DAPI (catalog no. D21490, Invitrogen Inc., Eugene, OR, USA).

RESULTS

Development and efficacy of animal model

A pre-study pilot trial was conducted to develop the current rat surgical model. High operative and late mortalities were encountered during this period due to various forms of technical failures resulting from inexperience. Surgical bleeding accounted for 15 early deaths and suture occlusion of the ureter causing a large intra-abdominal renal cyst resulted in 1 late death. Accidental suturing of the posterior and anterior walls accounted for graft failure in another 6 animals. However, after completion of this developmental phase, a standardized surgical protocol which resulted in the predictable and reliable outcomes of the present study was established. Accordingly, the data for the present study were collected from animals operated on after this period, and therefore, did not include the animals from the pre-study trial. In the present series, there were no operative and late deaths, and surgery was conducted consecutively in which all animals survived the experiment.

Assessment of decellularization efficacy

The present decellularization protocol was considered to be effective based on the absence of cellular or nucleic components in both the luminal surface and the underlying matrix scaffolding under high powered light microscopic examination (400× magnification) of the H&E-stained specimens (Fig. 2). The absence of DNA content was also confirmed through DAPI staining (Fig. 2C and D)

Vascular remodeling

Intimal hyperplasia, which appeared as early as 2 weeks post-implantation, was characterized by the accumulation of collagen and alpha actin-staining smooth muscle cells which appeared to be progressive up to 8 weeks. As a result, the in-

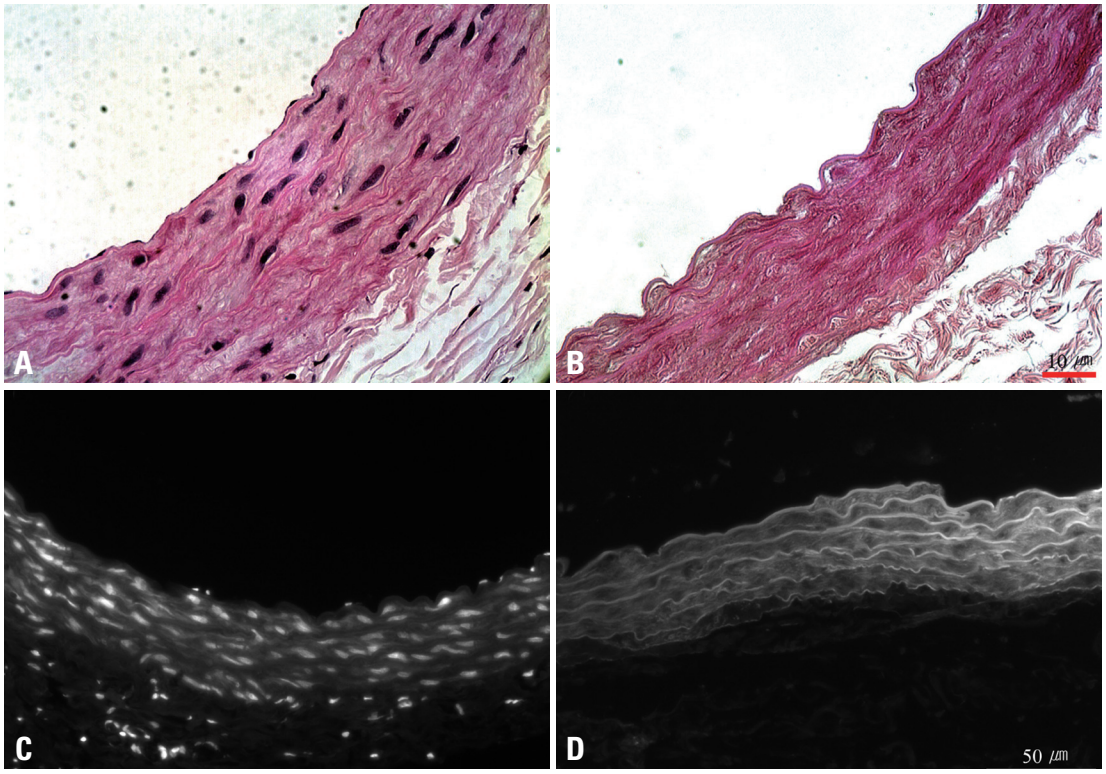

Fig. 2. Histologic assessment of the decellularized scaffold. (A) Normal aortic wall with a confluent endothelial cell lining and abundant cellularity in the native vascular wall matrix. (B) Decellularized vascular scaffold with absence of endothelial cell lining and cellularity or nuclear staining within vascular matrix. (C) DAPI staining against DNA content in the aorta with dense cellularity. (D) Post decellularized aorta showing only the supporting matrix scaffold with complete absence of DNA content.

tima at 8 weeks was noticeably thicker with greater interstitial deposition of collagen in the vascular media. Elastic fibers in the normal vascular media appeared to have an undulating pattern while those in the decellularized vascular implants appeared more straightened with an overall appearance of being more compressed than normal. With regards to cellularity, the normal aortic wall media appeared highly cellular, whereas the decellularized media appeared sparsely cellular with little improvement over time (Fig. 3).

Immunohistochemical staining

In the normal aorta, a confluent layer of flattened endothelial like cells staining for von Willebrand factor were observed on the luminal surface. At 2 weeks after implantation of the decellularized vascular implant, however, the surface was almost devoid of endothelial like cells. At 8 weeks, the endothelial like cells were more prominent, but showed fewer cells than in the normal aortic surface. Similarly, the von Willebrand factor staining was more prominent at 8 weeks than at 2 weeks, but with noticeably weaker activity than in the normal aorta (Fig. 4). To further assess the characteristics of the endothelial like cells, CD 31^+ was stained in addition to that for von Willebrand factor. The results showed the von Willebrand factor staining endothelial like cells to stain for CD 31^+ as well (Fig. 5).

DISCUSSION

The development of a biologically compatible small-diameter vascular conduit may have far-reaching clinical implications, especially in situations where autologous conduits are either unusable or unavailable. In contrast to the central hemodynamic flow conditions of "large-diameter" arterial graft conduits, the decreased flow velocity, shear stress, and higher vascular resistance of the peripheral vascular system contribute to the poorer long term patency of small-diameter vascular conduits.[9] Hemodynamic flow conditions have been shown to influence vascular remodeling and graft patency, but properties specifically related to the graft material have also been reported to affect graft thrombosis, intimal hyperplasia, aneurysm formation, infection, and progression of atherosclerosis.[10] Therefore, improvement of the bio-compatibility of vascular scaffolding remains an important research objective.

Expanded polytetrafluoroethylene (ePTFE) was widely

used as an artificial substitute for autologous saphenous vein grafts, however, the results have generally been disappointing due to the tendency for accelerated neointimal hyperplasia and premature graft failure.[3-5,11] Efforts to overcome these limitations included coating with heparin or recombinant human thrombomodulin. Nevertheless, the outcomes with these methods have not yet been substantiated through long term studies.[12,13]

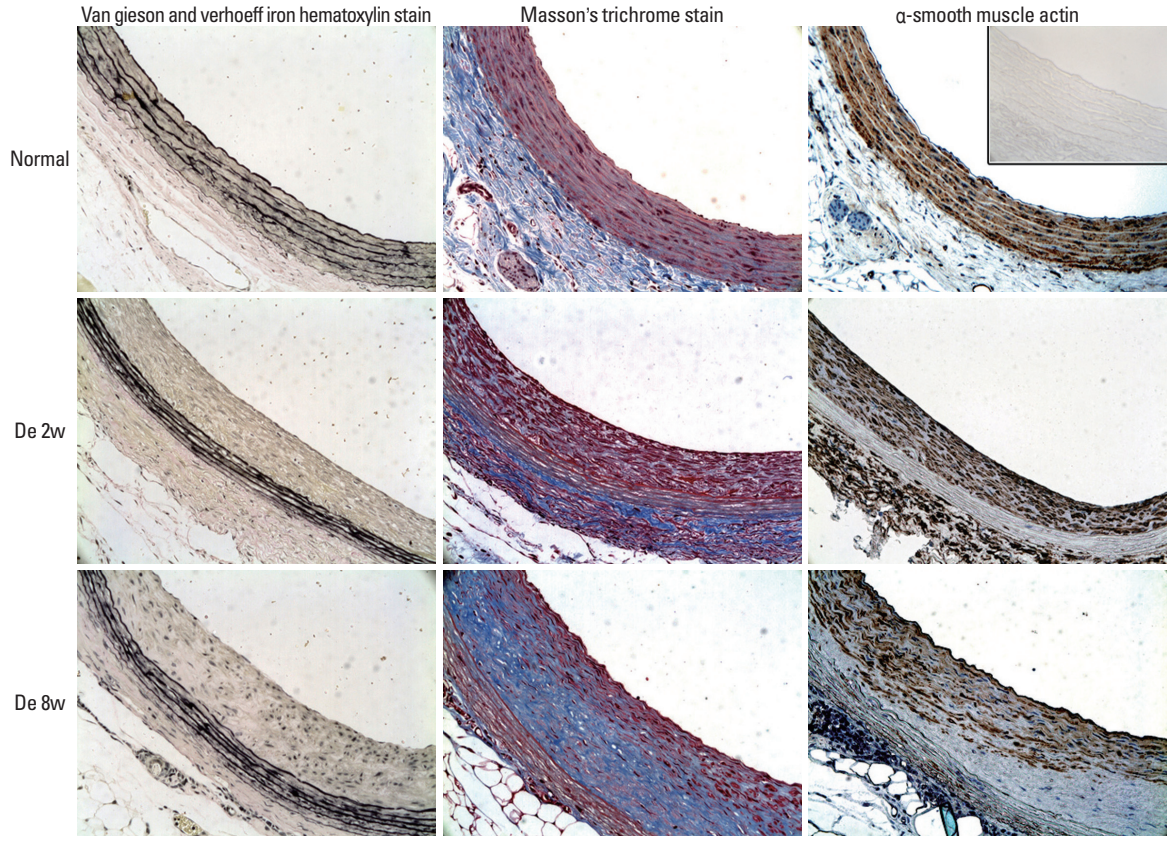

Fig. 3. Von Gieson's stain (black, far left column) shows absence of elastin fibers within the intimal proliferative layer. Collagen accumulation (middle column) and actin-positive smooth muscle cells (far right column) are present at 2 weeks. Both the thickness of collagenous matrix layer and cells staining positively for actin are increased at 8 weeks. Insert image shows staining without primary antibody.

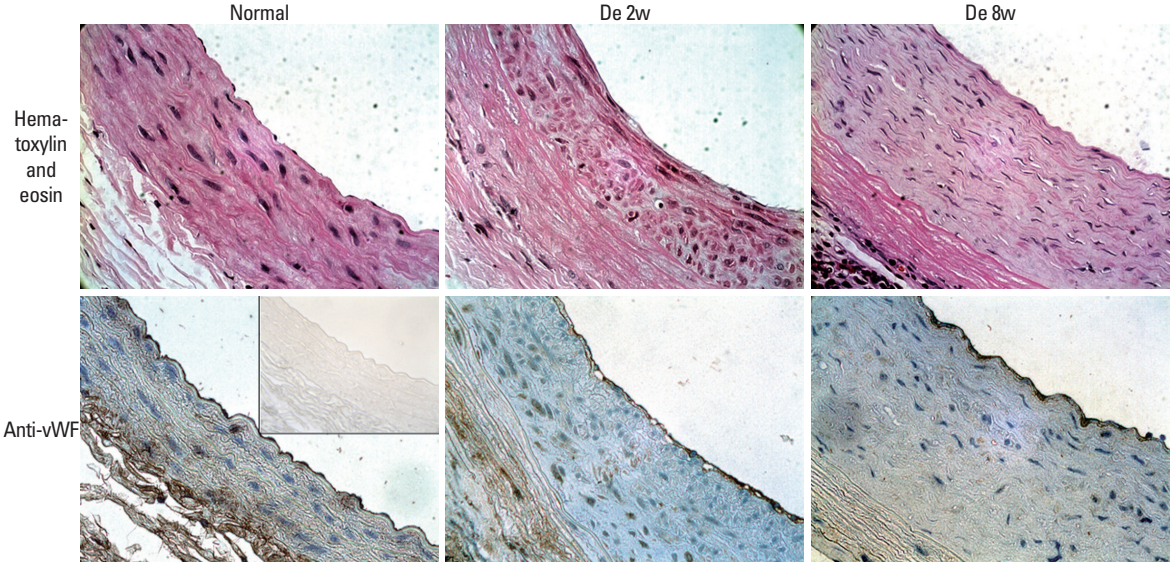

Fig. 4. Luminal surfaces of normal and decellularized aortic implant at 2 and 8 weeks. Greater numbers of endothelial cells are seen at 8 weeks, compared to 2 weeks, but in fewer concentrations than the normal aorta (upper row). Factor VIII staining shows a similar pattern correlating with the degree of endothelialization (lower row). Insert shows image of staining without primary antibody.

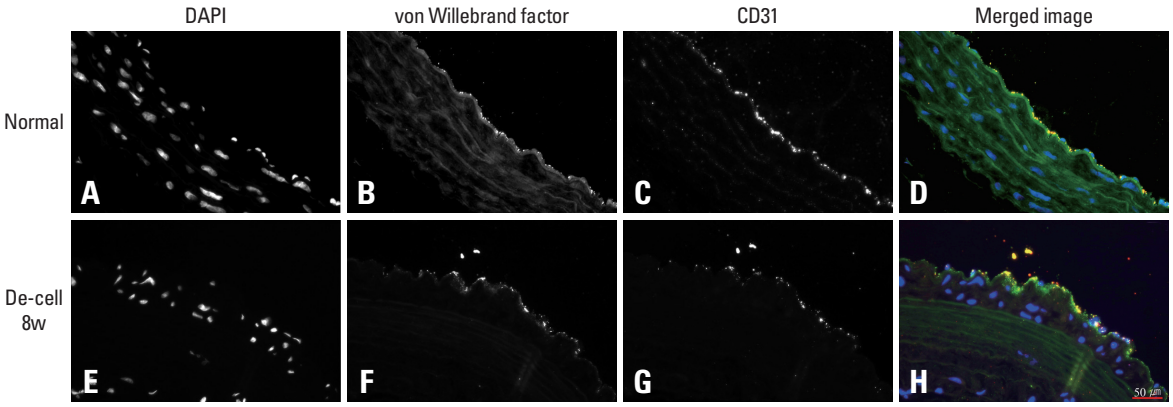

Fig. 5. DAPI study at 8 weeks showed evenly distributed cellular nuclei in the media of the normal aorta (A and E). In the De-cell specimen it was evident mostly in the neo intima layer with sparse cellularity in the media layer. Both the normal and De-cell specimen shows positive staining for von WIllebrandt factor (B and F) and CD 31^{+} (C and G). These signals were clearly co-localized on the innermost confluent cell layer in both groups albeit more strongly present in the normal aorta compared with the decellularized graft. Merging of the von Willebrand factor and CD 31 stains enhanced visualization of the endothelial cell markers (D and H).

Conceptually, decellularization may minimize the incidence of graft failures and complications related to immune rejection.[14] This contention is supported by the decreased mononuclear cell infiltrates observed in decellularized vein allograft implants at 2 weeks,[15] contrasting with the maximal inflammatory reaction occurring in fresh venous allografts harvested at similar time points.[16] The suitability of decellularized vein allograft implants as scaffolding for vascular tissue engineering was suggested earlier by Martin et al., showing repopulation of the medial matrix by recipient cells at 8 weeks post implantation with excellent retention of strength and structural integrity.[15] Accordingly, decellularization was considered to be a potentially good method for increasing the availability and utility of allograft vein grafts, however, it has thus far shown dismal results secondary to graft rejection and propensity for degenerative destruction.[17-19] Considering the importance of a functional endothelial-cell layer in preventing thrombosis and adverse vascular remodeling, further efforts to enhance re-endothelialization and repopulation of the decellularized matrix appear warranted.

Foremost is the need to develop a surgical model conducive to efficient and effective data generation and competence assessment. An animal model using a severely immune-compromised mouse model was previously described for such a purpose.[20] However, the possibility of graft thrombosis and other unforeseen factors that may have been otherwise independently affected by the influence of innate platelet dysfunction present in the immune-compromised mouse could not be ruled out. Accordingly, the present model using rats with no such deficiencies may be viewed as more accurately representing the normal *in vivo* biological conditions. The relatively larger size and the anticipated greater ease of handling for surgery further support the usefulness of the present model. The ability to accommodate a longer conduit than would be possible in the mouse, the amenability to procedural standardization, the ability to generate consistent, predictable, and reproducible operative outcomes, and the simplification of the anesthesia protocol which obviates ventilator requirements, all endorse the rat as being the more favorable small animal model. The demonstration of the ability of human mesenchymal stem cells (MSCs) to differentiate, survive, and function in a xenogeneic non-immune compromised rat environment[21] suggest further possibilities for using human-origin mesenchymal stem cells in a rat vascular implant environment.

For immunohistochemical assessment, several antibodies including anti-human antibodies were (anti-vWF and anti-SMA) used. These anti-human antibodies, which have generally been adopted in many other animal studies,[22,23] led us to follow their experimental protocols. In addition, according to the manufacturer's protocols, the antibodies have been verified to cross-react against several mammalian species such as mouse, rat and chicken, and therefore, have been recommended for use with animal tissues.

From a technical standpoint, the rat aorta, despite its small size, does not represent the hemodynamic environment of the peripheral vascular system. However, as a platform for conducting further research to enhance the utility of decellularized small diameter vascular conduits, the present model was suitable for this purpose. Thinning and dilatation with eventual development of aneurysmal changes are prominent degenerative findings of small diameter vascular conduits. Therefore, we speculated the more densely packed

appearance of the elastic fibers in the aortic implants to indicate early degenerative changes which may suggest changes representing aneurysmal progression. Although findings which are commonly present in established aneurysmal transformation such as disruption and fragmentation of the elastin fibers were not observed, further long term studies are nevertheless warranted to resolve the arguments related to this issue.[24,25]

In conclusion, the present rat small animal model was found to be an effective and efficient animal model for conducting vascular tissue engineering research, aimed at enhancing the availability and utility of decellularized allograft small diameter vascular conduits.

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