The Acellular Myocardial Flap: A Novel Extracellular Matrix Scaffold Enriched with Patent Microvascular Networks and Biocompatible Cell Niches

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There is a great need for acellular, fully vascularized, and biocompatible myocardial scaffolds that provide agreeable biological, nutritional, and biomechanical niches for reseeded cells for *in vitro* and *in vivo* applications. We generated myocardial flap scaffolds comprising porcine left-anterior ventricular myocardium and its associated coronary arteries and veins and investigated the combinatorial effects of sodium dodecyl sulfate (SDS) and sodium hydroxide (NaOH) perfusion on both the myocardial extracellular matrix (ECM) and the vascular ECM. Results showed that all scaffolds displayed a fully intact and patent vasculature, with arterial burst pressures indistinguishable from native coronary arteries and perfusion to the level of capillaries. Scaffolds were free of cellular proteins and retained collagen and elastin ECM components, exhibited excellent mechanical properties, and were cytocompatible toward relevant seeded cells. SDS perfusion preserved collagen IV, laminin, and fibronectin well, but only reduced DNA content by 33%; however, this was further improved by post-SDS nuclease treatments. By comparison, NaOH was very effective in removing cells and eliminated more than 95% of tissue DNA, but also significantly reduced levels of laminin and fibronectin. Such constructs can be readily trimmed to match the size of the infarct and might be able to functionally integrate within host myocardium and be nourished by direct anastomotic connection with the host's own vasculature; they might also be useful as physiologically accurate models for *in vitro* studies of cardiac physiology and pathology.

Introduction

MYOCARDIAL INFARCTION IS the principal complication associated with cardiovascular disease and affects nearly 600,000 new individuals annually.¹ Incidences of acute myocardial infarction (AMI) are increasingly survivable. However, the associated transient ischemia causes tissue damage, scar formation, and initiates a degenerative progression toward congestive heart failure (CHF).²⁻⁵ To forestall CHF, therapies must target replacement of the infarct scar with functional cardiac muscle.^{2,6-8} Heart transplantation falls short as a solution, as demand for donor hearts outpaces supply,^{9,10} and surgical procedures such as coronary artery bypass grafting, transmyocardial revascularization, and endoventricular circular-patch plasty focus solely on revascularization and/or do not replace the non-functional scar.¹¹ Stem cell therapy for AMI in the form of intramyocardial or intracoronary delivery of pluripotent cells has great potential, but its success has been limited due to

poor cell engraftment and integration with host tissue, extensive apoptosis, and absence of cell differentiation.¹²⁻¹⁴

Tissue-engineering approaches utilizing scaffolds offer great prospects as a modality for delivery of regenerative cells to the infarcted area.⁸ Early efforts have shown promise with thinner grafts (<1 mm),^{15,16} but thicker grafts (7 to 10 mm) are necessitated because the infarct scar is nearly transmural in many cases. However, long-term survival and functionality of thicker grafts have been hindered by insufficient vascularization and lack of perfusion with the host's vasculature.⁶

Whole-heart and myocardial patch decellularization has been demonstrated by a number of groups.^{10,17-22} Typically, remnants of this decellularization process are composed of extracellular matrix (ECM) proteins native to the myocardium and the native vascular network. Few studies, however, have attempted a comprehensive, parallel analysis of both the vascular and myocardial matrices focusing on decellularization efficacy, elastic moduli and burst pressures of

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the intrinsic coronary arteries, patency of capillary perfusion, exact amounts of collagen and elastin, and identification of important basement membrane proteins, among others. In our view, complete and efficient decellularization of such complex tissues appeared problematic and required a comparative analysis of several decellularization methods.

To this end, we decellularized porcine myocardium and its constituent vasculature using four different procedures comprising combinations of sodium dodecyl sulfate (SDS) and sodium hydroxide (NaOH) in a comparative study of their ability to remove cells and cell remnants and preserve the features of the native ECM. The scaffold was uniquely shaped as a flap, so that the native vasculature could be used in a threefold manner: first, to facilitate decellularization by perfusion; second, to serve as a conduit for recellularization with appropriate cells; and third, to allow for surgical manipulation and implantation via anastomosis of the graft's vessels with the recipient vasculature (similar to coronary bypass surgery). We systematically evaluated both the myocardial and coronary arterial ECM and investigated their ability to support the attachment and survival of relevant cardiac cell types.

Methods and Materials

Decellularization of porcine myocardium

Whole hearts were obtained from healthy, adult pigs at a local abattoir. Immediately after harvest, 50 mM ethylenediaminetetraacetic acid (EDTA) in warm phosphate-buffered saline (PBS) was injected into the left coronary artery branch at the aortic trunk to prevent clotting in the vasculature, and the hearts were transported on ice for immediate processing. A 1-cm-thick, 6–8 cm-wide, and 12-cm-long flap of myocardium was excised from the left-anterior ventricular wall, with care not to sever the coronary artery or cardiac vein, and these vessels were cannulated with barbed Luer connectors (Cole-Parmer) secured with zip-ties to facilitate perfusion (Fig. 1). Larger, open-ended vessels severed during excision of the flap were closed with 4-0 silk suture (Ethicon). The flaps were then subjected to combined perfusion and immersion of solutions in a continuous-flow decellularization system. The system comprised a series of fluid reservoirs and a multichannel peristaltic pump (Masterflex; Cole-Parmer) that circulated 2 L of solution through and around each scaffold. The inflow reservoir was elevated 90 cm above the cannulated ends of vessels, generating a hydrostatic pressure of about 80 mmHg and a flow rate of about 200 mL/min (largely dependent on tissue resistance). A solution of 30 mM EDTA in PBS (pH=7.5) was perfused for 12 h to clear any remaining clotted blood from the vasculature, followed by 1% SDS and 0.1 M NaOH solutions in sequence and in various durations for 10–15 days, while solutions were changed every 2 days. The four separate decellularization treatment groups ($n=4$ scaffolds per group) were as follows: group 1: 2 days SDS followed by 8 days NaOH; group 2: 5 days SDS followed by 5 days NaOH; group 3: 10 days SDS followed by 2 days NaOH; group 4: 15 days SDS. Decellularization efficacy was gauged by monitoring changes in scaffold color and mass. After completion of the decellularization process, scaffolds were immersed and rinsed overnight in 70% ethanol and three changes (one overnight) of PBS on an orbital shaker. For post-SDS decel-

lularization studies, group 4 scaffolds ($n=3$) were further subjected to treatments of either 24-h perfusion with DNase/RNase (Worthington Biochemical) solution (720 mUnits/mL each) in PBS containing 5 mM magnesium chloride at pH 7.5, perfusion of 0.1 M NaOH for 2 h, or 3 days of perfusion of PBS.

Histology and Immunohistochemistry

Samples of myocardial ECM (MYO in Fig. 1A) and coronary arterial ECM (CA in Fig. 1A) were examined by histological staining with hematoxylin and eosin (H&E), Gomori's trichrome, Movat's pentachrome, and Verhoeff-Van Gieson's stains. Immunohistochemical (IHC) analysis was performed on formalin-fixed and paraffin-embedded sections using heat-mediated antigen retrieval (10 mM citric acid buffer at 95°C–100°C for 10–15 min) followed by incubation with antibodies to collagen IV (2 µg/mL; Abcam), laminin (4 µg/mL; Abcam), actin (2 µg/mL; Abcam), cardiac myosin heavy chain (MHC, 4 µg/mL; Abcam), or fibronectin (4 µg/mL; BD Biosciences), and application of Vectastain DAB kit (Vector Labs) reagents for detection. For pore size determination, images ($n=25$) of H&E-stained sections of the MYO ECM from each group were analyzed using ImageJ software (NIH) to determine the mean diameter of pores in both longitudinal ($n=210$) and transverse (cross-sectional) ($n=278$) directions.

DNA, collagen, and elastin quantification

DNA content of the MYO ECM ($n=8$) and CA ECM ($n=10$) and samples of similarly located native tissues (myocardium: $n=8$, coronary artery: $n=10$) was measured using a Quant-IT PicoGreen® kit (Invitrogen). Lyophilized samples of MYO ECM ($n=8$), CA ECM ($n=8$), native myocardium ($n=8$), and native coronary artery ($n=8$) were analyzed for desmosine content by radioimmunoassay and for hydroxyproline content by amino acid analysis as previously reported,²³ and collagen and elastin content was calculated as previously described.^{24,25}

Mechanical properties

Rectangular 2.5 × 1-cm specimens of decellularized porcine MYO ECM ($n=4$) either were left with the epicardium attached or had the epicardium removed ($n=4$). Similar samples ($n=4$) of native porcine myocardium with and without the epicardium were prepared for comparison. Specimens were secured to a frame with 10-N load cell (MTS Systems), submerged in PBS at 37°C, and preconditioned for 10 cycles between 0%–15% strain at a rate of 3.0 mm/min. After 2-min rest, they were subjected to three cycles to 40% strain at 3.0 mm/min. Tangential slopes of each stress-strain curve between 35%–40% strain were averaged and taken as the Young's modulus. Samples of native porcine coronary arteries ($n=8$) and decellularized CA ECM ($n=8$) isolated from decellularized porcine myocardial flap scaffolds were subjected to burst pressure measurements as previously described.²⁶

Evaluation of scaffold vasculature

A native porcine heart (obtained as above) was injected with 50 mM EDTA in warm PBS at the left coronary arterial branch from the aortic trunk to clear the vasculature of any

FIG. 1. A pedicled flap of (A) native myocardium excised from the left ventricular wall of the porcine heart and a representative image of the decellularized (Decelled) scaffolds resulting from perfusion decellularization. The cannulae used to connect the vasculature to the perfusion system are shown secured with orange-clip ties. Red arrows indicate the open end of the coronary artery before cannulation (Native) and the inlet to the coronary artery after cannulation (Decelled). Similarly, blue arrows indicate the same features for the cardiac vein. Arrows also indicate the direction of antegrade blood and decellularization solution flow. Areas designated by circles indicate origins of specimens of decellularized coronary arterial extracellular matrix (CA ECM) and decellularized myocardial extracellular matrix (MYO ECM) used in histological, immunohistochemical (IHC), DNA, biochemical, mechanical, and cell-seeding analyses. Time-course plot (B) of the mean recorded masses of scaffolds exposed to 4 different decellularization methods. Results of a (C) PicoGreen[®] quantitative assay for DNA are shown, which compare the DNA content of both decellularized MYO ECM and CA ECM samples generated with various decellularization treatments to that of native myocardium and native coronary arteries, respectively. DNA content of (D) MYO ECM samples from scaffolds generated with the group 4 decellularization treatment and subsequent exposure to various reagents as measured by PicoGreen assay. DNA content of group 4 and native myocardium represented in (D) are carried over from C for comparison (*: indicates statistical significance from all other groups, $p < 0.0001$). Images of 4',6-diamidino-2-phenylindole (DAPI)-stained sections (E) from MYO ECM and CA ECM of decellularized porcine myocardial scaffolds from group 4 and group 3, showing residual nucleic acids (blue, white arrows; 10 $\times$, scale bar=100 μ m). Images of hematoxylin and eosin (H&E)-stained sections (F) from MYO ECM and CA ECM of scaffolds from group 4 and group 3, showing residual pools (black arrows) of nucleic acids (blue; 10 $\times$, scale bar=100 μ m).

clots. The left coronary artery and superior portion of the cardiac vein were cannulated with barbed Luer connectors (Cole Parmer). A cannulated, acellular flap scaffold was embedded in concentrated (126 mg/mL) Knox gelatin to seal any open-ended vascular channels created during the excision portion of the decellularization process. Red- and blue-pigmented polymethyl methacrylate (PMMA) from Batson's #17 Anatomical Corrosion Kit (Polysciences, Inc.) was sequentially injected into the vasculature of both the native heart and scaffold. Red PMMA was infused antegradely through the coronary arterial inlet, and blue PMMA retrogradely through the cardiac vein inlet. After polymerization, a cast of the vasculature was obtained by tissue maceration. Vascular casts were imaged on a dissection microscope, and ImageJ software was used to measure the diameters of blood vessels.

For injection and imaging of fluorescent dyes, fluorescein isothiocyanate (FITC)-dextran and rhodamine B isothiocyanate (RITC)-dextran (Sigma) were simultaneously injected into the scaffold vasculature (FITC-dextran antegradely through the arterial inlet and RITC-dextran retrogradely through the venous inlet). The scaffolds were immediately imaged macroscopically using an *in vivo* imaging system (IVIS[®] Lumina XR; Caliper Life Sciences). The system-generated images of photon density on a gray scale, which were artificially colored using the accompanying Living Image[®] software (Caliper Life Sciences).

Carbon black spherical particles of 2–12 μm in diameter (Sigma) were passivated via overnight incubation in 100% fetal bovine serum (Atlanta Biologicals), washed $2\times$ in PBS, suspended in PBS at a concentration of 2×10^6 particles/mL, and injected into the vascular inlets of a group 4 scaffold. Movat's pentachrome stain was used to examine 5- μm -thick sections of MYO ECM distal (at the apical end of the scaffold) to the inlet cannulae.

Cell seeding and analysis

Cylindrical, 5-mm-diameter punch biopsies ($n=4$) of MYO ECM were taken from decellularized porcine myocardial scaffolds from group 4, sterilized in 0.1% peracetic acid (Sigma) in PBS for 1 h, washed $3\times$ in sterile PBS, and lyophilized. For initial cytotoxicity studies, $\sim 5\times 10^6$ rat dermal fibroblasts (Cell Applications) at passage 11 were seeded onto the dry scaffold samples and cultured statically for either 7 or 14 days. At each time point, seeded scaffold samples were stained for viability using a Live/Dead kit (Invitrogen).

For seeding of neonatal rat cardiomyocytes (Lonza), 1-mm³ samples of MYO ECM from group 4 scaffolds ($n=3$) were sterilized with peracetic acid and washed as described above, seeded with 2×10^6 cells at passage 2, and cultured statically for 7 days. Samples were analyzed by immunofluorescence with primary antibodies to connexin43 (2 $\mu\text{g}/\text{mL}$; Abcam), actin (2 $\mu\text{g}/\text{mL}$; Abcam), cardiac MHC (4 $\mu\text{g}/\text{mL}$; Abcam), and α -sarcomeric actinin (2 $\mu\text{g}/\text{mL}$; Abcam), secondary antibodies Alexa-Fluor 488 and 594 (4 $\mu\text{g}/\text{mL}$; Invitrogen), and 4',6-diamidino-2-phenylindole (DAPI) nuclear counterstain.

Statistical analysis

Statistical analysis was performed using JMP software (SAS Institute, Inc.). One-way analysis of variance and

Fisher's least significant difference method for *post hoc* comparison of mean values (with $\alpha=0.05$) were used. All quantitative data are represented as mean $\pm$ standard deviation. Error bars represent $\pm$ standard error.

Results

Removal of cellular components

Scaffold color and mass loss were indicative of effective muscle cell removal, as scaffolds initially of red-brown color became progressively paler throughout the decellularization process before becoming white and translucent (Fig. 1), and wet masses decreased with time. Notably, NaOH-treated scaffolds displayed a higher rate of mass removal than SDS-treated scaffolds (Fig. 1B). Upon switching from SDS to NaOH as called for by each treatment, the mass removal rate increased by more than a factor of three. The treatment for group 4 (SDS alone) removed $49.6\pm 1.4\%$ of the original tissue mass, while the other three treatments, which all incorporated varying lengths of NaOH exposure, resulted in an average $58.8\pm 1.3\%$ removal of the initial mass ($p=0.0006$). These results indicate that NaOH is a more rapid and more efficient decellularization agent as compared to SDS.

Histological analysis of MYO and CA ECM (Fig. 2A) illustrated removal of cell nuclei and cytoplasmic proteins from native tissues, leaving voids in the resulting scaffold. IHC analysis confirmed complete removal of actin and cardiac MHC muscle proteins from both tissues (Fig. 2B). Data in Figure 6 also reflect effective decellularization, as the disparity between the weight percentages of matrix proteins in decellularized and native tissues was caused by removal of cell proteins.

Quantification of DNA showed that all decellularization treatments that employed NaOH (groups 1–3) cleared 95%–98% of DNA from myocardial and arterial tissues. However, DNA content was not significantly different in groups 1–3. The treatment in group 4 (SDS alone) reduced DNA content by 33.2% in the myocardium and only 27.5% in the coronary arteries (Fig. 1C). H&E- and DAPI-stained sections of group 4 scaffolds showed positive staining for nucleic acids that had pooled in subepicardial and subintimal areas of the MYO and CA ECM, respectively, while group 3 scaffolds were completely devoid of nucleic acids (Fig. 1E, F). Quantification of DNA in MYO ECM from group 4 scaffolds perfused with additional solutions, described as post-SDS procedures, including DNase/RNase or NaOH, resulted in further reduction of DNA content (Fig. 1D).

Preservation of matrix architecture and mechanical integrity

H&E-stained histological sections cut in the longitudinal and transverse planes of decellularized MYO ECM reflected retention of the native myocardial structure and organization (Fig. 2A). Taken together, images from these two planes illustrated the cylindrical voids left within scaffolds after removal of cardiomyocytes. Quantitative analysis of the pores in these images showed that they had mean dimensions of about $20\times 40\mu\text{m}$ among all treatment groups (Table 1). Histological sections of decellularized CA ECM displayed elliptical and oblong pores vacated by smooth muscle cells

FIG. 2. Histological images of **(A)** native porcine myocardium and decellularized (Decel) porcine myocardial ECM (MYO) stained with H&E and sectioned in the longitudinal and transverse (cross-sectional) directions with respect to cardiomyocytes, depicting the cylindrical voids left in their absence ($40\times$, scale bar = $20\mu\text{m}$); H&E-stained native porcine coronary artery and decellularized porcine coronary arterial ECM (CA; $10\times$, scale bar = $100\mu\text{m}$). Decellularized sections are representative of all four treatment groups. Cell nuclei (blue), cytoplasmic proteins (dark pink), and collagen (light pink) are shown. **(B)** IHC staining of native and decellularized (Decel) porcine myocardium (MYO) for actin and cardiac myosin heavy chain (cardiac MHC; $40\times$, scale bar = $20\mu\text{m}$). IHC of native and decellularized porcine CA for actin ($10\times$, scale bar = $100\mu\text{m}$). Decellularized sections are representative of all four treatment groups. IHC positive staining = brown. Inserts = IHC negative controls. L = lumen and black arrows indicate the internal elastic lamina. Color images available online at www.liebertpub.com/tec

and fibroblasts, intact internal elastic lamellae, and little delamination of the vessel tunics (intima, media, and adventitia; Figs. 3–5). Uniaxial tensile testing of decellularized MYO ECM samples showed that scaffolds from all groups had a higher elastic modulus than that of native tissue ($p < 0.0001$). Group 1 scaffolds (with epicardium attached) had a significantly greater modulus than scaffolds from other groups ($p = 0.0011$). Separation of the epicardium from MYO ECM samples was not found to have a significant effect upon the modulus (Fig. 6C).

Tubular segments of decellularized CA ECM from all groups exhibited burst pressures which were not signifi-

cantly different from those of native coronary arteries and which exceeded 2000 mmHg (Fig. 6D).

Preservation of ECM proteins and basal lamina

Gomori's trichrome-, Movat's pentachrome-, and Verhoeff-Van Gieson's-stained sections illustrated the preservation of collagen and elastin after decellularization in both the MYO ECM and CA ECM (Fig. 3). Notably, elastin was diffuse within the CA ECM (Fig. 3), but was also observed in the epicardium and microvascular channels within the MYO ECM (Fig. 7). These results were consistent among scaffolds from all treatment groups and indicate excellent preservation of collagen and elastin. To evaluate preservation of basement membrane components, we performed IHC for collagen IV, laminin, and fibronectin. These were all present within native tissues and localized to the pericellular interstitium (Figs. 4 and 5). IHC of sections from group 4 confirmed excellent retention of collagen IV, laminin, and fibronectin in both the MYO and CA ECM after decellularization (Figs. 4 and 5, Table 2). Scaffolds from groups exposed to NaOH (groups 1, 2, and 3), however, displayed a trend of increasingly diminished staining of these proteins as length of NaOH exposure increased (Figs. 4 and 5, Table 2). IHC of sections from group 1 scaffolds displayed sparse labeling of collagen IV in comparison to other groups and little-to-no labeling of either laminin or fibronectin. Images of these

TABLE 1. DIMENSIONS OF PORES WITHIN MYOCARDIAL SCAFFOLDS

	Transverse diameter (μm)	Longitudinal diameter (μm)
Group 1	21.27 ± 0.26	44.38 ± 0.71^a
Group 2	19.94 ± 0.25^b	42.19 ± 0.74
Group 3	20.67 ± 0.30	41.16 ± 0.79
Group 4	22.01 ± 0.28^c	40.73 ± 0.73

^aSignificant from all other groups ($p = 0.0022$).

^bSignificant from group 1 ($p = 0.0001$).

^cSignificant from all other groups ($p = 0.0001$).

FIG. 3. Histology of native porcine myocardium and decellularized (Decelled) porcine myocardial ECM (MYO) from group 4 scaffolds (40 $\times$, scale bar=20 μ m) and native porcine coronary artery and decellularized porcine coronary arterial ECM (CA) from group 4 scaffolds (10 $\times$, scale bar=100 μ m) stained with Movat's pentachrome, which shows nuclei (dark red), collagen (yellow-orange), elastin fibers (maroon), and cytoplasmic proteins (red). Native porcine myocardium and decellularized porcine myocardial ECM from group 4 scaffolds (40 $\times$, scale bar=20 μ m) and native porcine coronary artery and decellularized porcine coronary arterial ECM from group 4 scaffolds (20 $\times$, scale bar=50 μ m) stained with Gomori's trichrome, which shows nuclei (dark purple), collagen (blue-green), elastic fibers (purple), and cytoplasmic proteins (red). Native porcine myocardium and decellularized porcine myocardial ECM with epicardium from group 4 scaffolds (20 $\times$, scale bar=50 μ m) and native porcine coronary artery and decellularized porcine coronary arterial ECM from group 4 scaffolds (10 $\times$, scale bar=100 μ m) stained with Verhoeff-Van Gieson's (VVG) stain for visualization of nuclei (gray/black), elastin fibers (black), and collagen (pink). Images of decellularized sections for all stains are representative of all four treatment groups. L=lumen and black arrows indicate the internal elastic lamina.

sections are not shown due to their similarity to images of sections from group 2 scaffolds.

Quantification of collagen and elastin content using total hydroxyproline and desmosine analysis showed that MYO and CA ECM were composed mainly of collagen and elastin, with slight differences among the various groups (Fig. 6). Elastin content was significantly different only between group 2 and group 4 in the MYO ECM ($p=0.0005$; Fig. 6A) and between group 2 and group 3 in the CA ECM ($p=0.0432$; Fig. 6B). Both types of matrices from all decellularized treatment groups had mean collagen and elastin weight percentages which were significantly different from those of native tissue ($p<0.0001$; Fig. 6A, B). The ratio of mean collagen weight percentage to mean elastin weight percentage for native myocardium was 19.5. This ratio increased significantly in samples of decellularized MYO ECM from all treatment groups, and, among treatment groups, was only significantly different between groups 1 and 4 ($p=0.0457$, Table 3). This same ratio was 4.3 in native coronary arteries, and it was not significantly different from that of the CA ECM in any of the groups (Table 3).

Preservation of vascular integrity and patency

Simultaneous injection of FITC- and RITC-conjugated dextran into arterial and venous vascular inlets showed extensive perfusion of scaffolds with fluorophores (Fig. 7A, B). An overlaid image of both emission spectra demonstrated that the fluorophores had actually mixed within the same vessels (Fig. 7C), and an overlaid image of the fluorescent channel with a white-light photograph of the scaffold (Fig. 7D) illustrated localization of fluorophores to the scaffold vasculature. Corrosion casts of a scaffold from group 4 demonstrated retention of structure and patency of both macro- and microvasculature after decellularization. Intact capillaries 5–8 μ m in diameter were observed in casts of native myocardium as well as decellularized scaffolds (Fig. 7). Carbon microspheres injected through the vascular inlets of a scaffold from group 4 were observed to accumulate within capillaries in histological sections of distal portions of the scaffold (Fig. 7M, N). Microvasculature was also routinely observed in histological sections, and was illustrated by staining of internal elastic lamina or laminin via VVG and IHC, respectively (Fig. 7K, L).

FIG. 4. Immunohistochemistry (IHC) of native porcine myocardium and decellularized porcine myocardial ECM, showing the degree of preservation of collagen IV (Coll IV), laminin, and fibronectin in group 2, group 3, and group 4 (40 $\times$, scale bar = 20 μ m). IHC positive staining = brown. Inserts = IHC negative controls. Color images available online at www.liebertpub.com/tec

FIG. 5. Immunohistochemistry (IHC) of native porcine coronary artery and decellularized porcine coronary arterial ECM, showing the degree of preservation of collagen IV (Coll IV), laminin, and fibronectin in group 2, group 3, and group 4 (20 $\times$, scale bar = 50 μ m). IHC positive staining = brown. Inserts = IHC negative controls. L = lumen and arrows indicate the internal elastic lamina. Color images available online at www.liebertpub.com/tec

FIG. 6. Biochemical quantification of collagen and elastin content of (A) decellularized myocardial ECM samples generated with four different decellularization treatments as compared to that of native myocardium (*: indicates statistical significance from all other groups, $p < 0.0001$; ** $p = 0.0275$; *** $p = 0.0005$). Collagen and elastin content of (B) decellularized coronary arterial ECM samples generated with four different decellularization treatments as compared to that of native coronary arteries (*: indicates statistical significance from all other groups, $p < 0.0001$; **: indicates significance from all groups except group 3, $p < 0.0001$; *** $p = 0.002$; # $p = 0.0419$; ## $p = 0.0432$; ### $p = 0.0009$). (C) Young's elastic moduli of decellularized myocardial ECM samples generated with four different decellularization treatments as compared to that of native myocardium (*: indicates statistical significance from all other groups, $p < 0.0001$; **: indicates statistical significance from all other groups in without epicardium group, $p = 0.0011$). Groups of samples with and without the epicardium attached were tested. (D) Burst pressures of decellularized coronary arterial ECM segments generated with four different decellularization treatments as compared to that of native coronary arteries. Color images available online at www.liebertpub.com/tec

Cytocompatibility

Samples of MYO ECM from scaffolds exhibited excellent cytocompatibility toward several types of cells normally present in the myocardium, fibroblasts and cardiomyocytes, after 7 and 14 days in culture (Fig. 8A). Rat neonatal cardiomyocyte-seeded samples labeled for connexin43, actin, cardiac MHC, α -sarcomeric actinin, and DAPI appeared viable and positive for these markers. Although no tests of functionality were performed, cells appeared to interconnect and self-organize on the scaffolds (Fig. 8B).

Discussion

Evaluation of decellularization efficiency

In this study, we have taken a systematic and thorough approach to develop a naturally vascularized acellular

myocardial flap, addressing the key features and characteristics of the myocardial matrix, the major arteries and veins, and the intrinsic microvasculature. Several groups have successfully decellularized the myocardium in either whole or excised portions of both rat and pig hearts using a wide range of chemical agents and physical methods.^{10,17–22,27} Many reported on characteristics (e.g., acellularity, biochemical composition, vascularity, and thickness) of the resulting scaffolds and their applicability to tissue engineering efforts. However, with regard to optimization of a decellularization method, these studies vary in the comprehensiveness of their analysis.

Our choice of chemical agents for decellularization was predicated upon previously published results from studies that used SDS^{10,18,20,28} and upon our previous experience with NaOH as a decellularization and DNA removal agent for carotid arteries.²⁶ Thus, it was of interest to investigate

FIG. 7. Top left panel: Macroscopic images of a decellularized porcine myocardial scaffold from group 4 after injection with (A) fluorescein isothiocyanate (FITC, red) through the arterial inlet and (B) rhodamine B isothiocyanate (RITC, blue) through the venous inlet. (C) Merged image of images A and B showing colocalization of fluorescent dyes within arteries and veins. (D) Merged image of image (C) and a white-light image of the scaffold displaying fluorescent dyes confined to the vasculature. Bottom left panel: Corrosion casts of the vasculature of (E, F) native left-ventricular porcine myocardium and a (G, H) decellularized porcine myocardial scaffold from group 4, showing preservation of both (G) macro- and (H) microvasculature after the decellularization process. A 500- μ m-diameter needle was included in the frame as a scale reference (black scale bar in E–H = 500 μ m). Right Panel: Representative images across all decellularization treatments of (I) H&E- and (J) VVG-stained sections of decellularized vasculature from scaffolds showing the CA and adjacent cardiac vein (CV; 2.5 $\times$, scale bar = 500 μ m). Micrographs of (K) VVG staining and (L) IHC for laminin in sections of microvasculature from group 4 scaffolds showing preservation of elastin (black) and laminin (brown) around vessels, respectively (40 $\times$, scale bar = 20 μ m). Micrographs (M, N) of decellularized myocardial scaffold vasculature from group 4 after injection of 2–12- μ m diameter, carbon particles via the vascular inlets. Sections were from sites distal (apical portions of scaffold) to inlets, and black carbon particles are shown localized to small vessels (M: 10 $\times$, scale bar = 100 μ m; N: 20 $\times$, scale bar = 50 μ m).

FIG. 8. (A) Images of decellularized porcine myocardial ECM from group 4 scaffolds seeded with rat dermal fibroblasts (FB) and cultured for either 7 (top) or 14 (bottom) days. Cells were labeled with Live/Dead reagents at each time point. Live cells were labeled with calcein AM (green) and dead cell nuclei were stained with ethidium homodimer (EthD-1, red; 20 $\times$, scale bar = 50 μ m). (B) Immunofluorescence (IF) images of decellularized porcine myocardial ECM from group 4 scaffolds seeded with neonatal rat cardiomyocytes (CM) and cultured for 7 days. Scaffolds were stained for α -sarcomeric actinin, cardiac MHC, actin, and connexin43, and nuclei were stained with DAPI (40 $\times$, scale bar = 20 μ m).

TABLE 2. IMMUNOHISTOCHEMICAL STAINING OF BASEMENT MEMBRANE PROTEINS AND FIBRONECTIN

	Group 1	Group 2	Group 3	Group 4	Native
Myocardial ECM					
Collagen IV	+	+	++	+++	+++
Laminin	–	–	–	+++	+++
Fibronectin	–	+	++	+++	+++
Coronary arterial ECM					
Collagen IV	+	++	+++	+++	+++
Laminin	–	–	–	+++	+++
Fibronectin	–	+	++	+++	+++

Grade: + + +, intense and highly localized; + +, diffuse, but less localized; +, visible, but faint, sporadic, and/or nonlocalized; –, not visible; ECM, extracellular matrix.

the combinatorial effect of SDS's ability to solubilize plasma membranes and denature proteins with NaOH's affinity for solubilizing protein and degrading nucleic acids.²⁸

Removal of cellular material

Despite its reputation as an effective decellularization reagent,^{9,10,20,28,29} it has been suggested by some that SDS may leave cellular remnants when used in decellularization of the MYO ECM. However, there is scant evidence furnished by the authors in support of this contention.²¹ Our own histology results demonstrated that every decellularization treatment we investigated, including that which used solely SDS after EDTA (group 4), was effective in disrupting the cell and nuclear membranes and in completely removing intracellular proteins from both the MYO ECM and CA ECM. Measurements of the dimensions of pores within our decellularized scaffolds are consistent with those previously reported.¹⁹ DNA was quantified to provide an additional measure of acellularity, and with the exception of the group 4 treatment, all decellularization methods left only small amounts of residual DNA. Although quantitative assays, as well as H&E- and DAPI-stained sections, confirmed that group 4 scaffolds contained visible amounts of residual DNA (Fig. 1) in areas adjacent to denser portions of the matrix, none of the corresponding histological stains showed muscle proteins or other apparent cellular remnants (Fig. 2). Post-SDS exposure to DNase/RNase and/or NaOH was effective in removing the residual DNA within group 4 scaffolds.

TABLE 3. RATIO OF COLLAGEN WEIGHT PERCENTAGE TO ELASTIN WEIGHT PERCENTAGE

	Myocardial ECM	Coronary arterial ECM
Group 1	24.8 ± 2.4	4.37 ± 0.55
Group 2	27.5 ± 1.8	3.67 ± 0.39
Group 3	26.0 ± 1.8	3.51 ± 0.80
Group 4	30.5 ± 1.8 ^a	4.8 ± 0.61
Fresh	19.48 ± 1.4 ^b	4.27 ± 0.78

^aSignificant from group 1 ($p=0.0457$).

^bSignificant from all other groups ($p=0.0007$).

In replicating a similar SDS-based protocol developed by Ott *et al.*, Akhyari and colleagues showed only a 43% reduction of native DNA content.^{10,27} Weymann *et al.* decellularized an intact porcine heart using perfusion of 4% SDS over 12 h, concluded with perfusion of PBS for 24 h, and reported an 82% reduction in DNA content.²⁰ While SDS was effective in lysing the nuclear membranes, it likely did not interact with this newly extranucleated or free DNA. This lack of interaction is explainable, given that SDS is a strongly anionic molecule and DNA is negatively charged along its phosphate backbones. NaOH, by contrast, is exceedingly effective in breaking down DNA, as it causes both denaturation³⁰ and hydrolysis of the molecule.³¹

Preservation of basal lamina components

Basement membrane proteins contain important cell-binding moieties, and any alteration of their conformation by chemical or physical means during decellularization could render them nonfunctional.^{27,32} Laminin is a vital link in the chain which binds the cardiomyocyte cytoskeleton to the collagenous ECM components, thereby facilitating transfer of mechanical forces and stimuli between the two.^{33,34} It has multiple domains that allow it to simultaneously bind integrins, proteoglycans, and collagen IV, making laminin the all-purpose glue of the basement membrane.^{35,36} Attachment to laminin has also been shown to be necessary for cardiomyocyte survival *in vitro*, indicating its ability to mediate important cell-signaling pathways.^{37,38} Collagen IV assembles into an open network overlaying the predominant structural ECM proteins.³⁰ It serves as the linkage between fibrillar collagens and both laminin and fibronectin. Fibronectin acts as an important intermediary between cardiomyocytes and the basal lamina, binding integrins, proteoglycans, and collagens.³⁶ Although these are minor components within the ECM, they will likely play a major role in the attachment, mechanotransduction, differentiation, maintenance of phenotype, and ultimately, functionality of reseeded cells.

In our studies, decellularization treatments that employed NaOH altered and/or removed basement membrane proteins. While collagen IV and fibronectin appeared less affected by this exposure, laminin was aggressively degraded by NaOH (full degradation at 48 h exposure to 0.1 M NaOH). Scaffolds from group 4 (SDS alone) retained all basement membrane proteins we attempted to identify by IHC.

Preservation of collagen and elastin

Quantitative analysis showed that collagen and elastin were the predominant components of both myocardial and coronary matrices within scaffolds from all groups. Aside from studies that reported Western blotting results²⁷ and qualitative or semiquantitative results,^{17–22} to our knowledge, no other attempts were made to quantify these important matrix proteins. Elastin plays an important mechanical role in normal cardiac tissue, as it facilitates passive recoil of the matrix after muscle contraction and distension of the collagenous components within both the MYO and CA ECM.^{36,39} Akhyari *et al.* reported that decellularization with a similar method reduced elastin content relative to native tissues,²⁷ but our data show that elastin content is largely preserved, particularly within the CA ECM.

Evaluation of mechanical properties

Overall, the elastic moduli of MYO ECM and CA ECM did not differ significantly from native tissues, which suggest that decellularization procedures assessed in this study did not alter their mechanical properties. The elastic moduli we report here (0.4–0.9 MPa) for decellularized MYO ECM are somewhat lower than those reported by other groups with a similar SDS-based protocol (moduli of around 5 MPa); it is possible that the disparity can be attributed to differences in testing parameters such as strain rates (6.0 mm/min vs. the rate used in our study).¹⁸ Our mechanical testing procedure was most comparable to that described by Eitan *et al.* in terms of preconditioning and strain rate, but it was not clear as to which specific portion of the stress–strain curve was used for calculation of their reported elastic modulus.²² Importantly, both the above decellularization protocols employed trypsin extensively (Sarig *et al.*: 0.05% [w/v] for 96 h and Wang *et al.*: 0.01% for 2.5 weeks).^{19,21} Trypsin is a serine protease known to partially degrade elastin, an observation confirmed by our own work with this enzyme (data not shown). Acellular myocardial matrix generated by the first trypsin protocol²¹ was reported to be completely devoid of elastin, and while recently published results from the second group confirm the presence of elastin histologically, no quantitative data were provided.¹⁹

Vascular integrity and patency

Corrosion casts of scaffolds demonstrated that our decellularization procedure leaves the inherent vasculature intact to the extent of preserving the patency of microvasculature and capillaries. Simultaneously antero- and retrogradely injected fluorescent dyes were able to move freely through the scaffolds' vascular tree and mix with one another. In addition, carbon microspheres appeared within distal microvascular channels after their injection into the scaffold's main vascular inlets. These observations confirm that the microvasculature and capillary beds linking the arterial and venous circulation are left intact by our decellularization process. Using a similar perfusion SDS-based protocol, Ott *et al.* presented evidence of vascular preservation with corrosion casting results, but also by heterotopically connecting the vasculature of their whole, decellularized rat heart to the aorta of a recipient rat and observing perfusion of the scaffold with host blood.¹⁰ Microvasculature is absent in trypsin-exposed scaffolds, possibly complicating subsequent efforts

to maintain viability of reseeded cells and limiting feasibility of reperfusion or graft integration upon implantation.^{21,27}

Cytocompatibility and cardiomyocyte cell seeding

Several groups have already established decellularized MYO ECM as an acceptable substrate for supporting reseeded cells.^{10,17,18,21,22,27} Relevant cardiac cell types seeded onto samples of decellularized MYO ECM from scaffolds in group 4 attached, maintained a cardiac phenotype, and remained viable for up to 14 days in static culture. These data support the cytocompatibility of our scaffolds and their suitability for maintaining cardiomyocyte cell phenotype, although more definitive studies must be conducted to substantiate the latter claim.

Analysis of optimal decellularization

The acellular myocardium is an outstanding matrix scaffold with promising tissue engineering applications for *in vitro* testing, as well as for *in vivo* regeneration. Our results suggest that a rational design and selection of decellularization methods for myocardium need to take into account several criteria that serve the intended final application: (1) remove all potentially immunogenic proteins and molecules, rendering the implant biocompatible; (2) preserve the biochemical structure, composition, architecture, and relative quantities of all matrix proteins, including basement membrane, thus generating an agreeable biological and biomechanical niche for reseeded cells; (3) provide a means of nourishing reseeded regenerative cells by preserving the natural vasculature; and (4) allow for convenient surgical manipulation, implantation, and reconnection to the host vasculature.

These criteria were used to comment on some of the positive and negative effects of each of the decellularization reagents we investigated, including EDTA (Table 4). Ongoing studies focus on cell seeding and preconditioning of constructs made from our scaffolds and on implantation studies to assess biocompatibility and feasibility of implantation techniques.

Conclusions

To the best of our knowledge, this is the first study that has carried out a comprehensive, parallel analysis of both the vascular and myocardial matrices in a myocardial flap

TABLE 4. SUMMARY OF OBSERVATIONS ON THE EFFECTS OF VARIOUS CHEMICAL REAGENTS IN DECELLULARIZATION OF PORCINE MYOCARDIUM

	Positive effects	Negative effects
EDTA	Chelates Ca ⁺⁺ , Mg ⁺⁺ , and Zn ⁺⁺ ; inhibits integrin function, disrupting cell adhesion to ECM ²⁸ ; prevents unwanted degradation of ECM by residual matrix metalloproteases; prevents and solubilizes blood clots	Unknown
SDS	Lyses and solubilizes plasma membranes; solubilizes and denatures cellular proteins	Does not denature or remove nucleic acids; removes GAG
NaOH	Solubilizes and denatures cellular proteins; denatures and removes nucleic acids; removes residual SDS	Removes laminin, degrades collagen IV and fibronectin

EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; NaOH, sodium hydroxide.

containing intact coronary arteries, veins, and associated microvasculature. Our scaffolds are porous, retain the original collagen and elastin content and structure, and exhibit excellent mechanical properties. Acellular scaffolds retain laminin, fibronectin, and collagen IV as well as the intrinsic vasculature's integrity and patency down to the capillary scale. Together with our demonstration of cytocompatibility and support of cardiomyocyte survival, these results indicate that our scaffold is ideally suited for repopulation with relevant cell types as a tissue-engineered construct for myocardial repair. Given its intact vasculature tree, mechanical properties, and geometry, constructs produced from this scaffold have the potential to functionally integrate with healthy host myocardium and to be nourished by direct anastomotic connection with the host's own vasculature. We also believe that such constructs may be useful as physiologically accurate models for *in vitro* studies of cardiac physiology and pathology.

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Disclosure Statement

No competing financial interests exist.

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