The fabrication and characterization of a multi-laminate, angle-ply collagen patch for annulus fibrosus repair

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Abstract

One major limitation of intervertebral disc (IVD) repair is that no ideal biomaterial has been developed that effectively mimics the angle-ply collagen architecture and mechanical properties of the native annulus fibrosus (AF). Furthermore, it would be beneficial to devise a simple, scalable process by which to manufacture a biomimetic biomaterial that could function as a mechanical repair patch to be secured over a large defect in the outer AF that will support AF tissue regeneration. Such a biomaterial would: (1) enable the employment of early-stage interventional strategies to treat IVD degeneration (i.e. nucleus pulposus arthroplasty); (2) prevent IVD re-herniation in patients with large AF defects; and (3) serve as a platform to develop full-thickness AF and whole IVD tissue engineering strategies. Due to the innate collagen fibre alignment and mechanical strength of pericardium, a procedure was developed to assemble multi-laminate angle-ply AF patches derived from decellularized pericardial tissue. Patches were subsequently assessed histologically to confirm angle-ply microarchitecture, and mechanically assessed for biaxial burst strength and tensile properties. Additionally, patch cytocompatibility was evaluated following seeding with bovine AF cells. This study demonstrated the effective removal of porcine cell remnants from the pericardium, and the ability to reliably produce multi-laminate patches with angle-ply architecture using a simple assembly technique. Resultant patches demonstrated their inherent ability to resist biaxial burst pressures reminiscent of intradiscal pressures commonly borne by the AF, and exhibited tensile strength and modulus values reported for native human AF. Furthermore, the biomaterial supported AF cell viability, infiltration and proliferation. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: annulus fibrosus; scaffold; decellularization; angle-ply laminate; tissue engineering; intervertebral disc repair; collagen fibres

1. Introduction

The intervertebral discs (IVDs) of the spine support complex loads and motions during activities of daily living. This function is imparted by the structure of the IVD, which is comprised of two distinct anatomical regions: (1) the centrally located aggrecan- and collagen type II-rich core [known as the nucleus pulposus (NP)]; which is sequestered by (2) 12–25 concentric multi-laminate lamellae composed of type I collagen [known as the annulus fibrosus (AF)]; (Urban and Roberts, 2003). Evaluation of the microarchitecture of the AF reveals an alternating angle-ply microarchitecture in which the collagen fibre-preferred direction within each subsequent lamellae is oriented at ± 28–43° relative to the transverse (horizontal) axis of the spine, thus providing an optimal structure for resisting tensile loading and intradiscal pressures (IDPs) generated by the hydrophilic NP (Urban and Roberts, 2003). During IVD degeneration and herniation, the competency of the AF is often compromised resulting in fissures and extrusion of the NP, which can impinge on or chemically irritate adjacent nerve roots contributing to discogenic low back pain and muscle weakness (Hughes et al., 2012). The AF has limited capacity for self-repair and thus, in the aforementioned clinical scenarios, restoring AF integrity is critical (Bron et al., 2009). It has been demonstrated that patients with AF tears measuring greater than 6 mm are at increased risk for re-herniation (Carragee et al., 2003). Additionally, the use of NP implants as early-stage interventions to mitigate the progression of IVD degeneration require an intact AF to prevent implant migration (Bron et al., 2009). To further illustrate the criticality of AF repair, the AO Foundation (a preeminent musculoskeletal research institution) launched a collaborative research programme in 2011 aimed at developing engineered solutions to combat AF rupture.

To date, few synthetic AF repair devices have been marketed and, while development of suturing techniques, adhesives, and various natural and synthetic biomaterials continues, none has yet illustrated comparable structural and mechanical characteristics of the native AF concomitant with the ability to support tissue regeneration (Guterl et al., 2013; Schoenfeld and Weiner, 2010). Herein, we hypothesized that the pericardium, the connective tissue that surrounds the heart, would serve as an optimal material with which to construct a multi-laminate angle-ply patch to repair the outer AF. This tissue is a durable, thin sheet of collagen fibre-reinforced matrix that has been extensively used in the manufacture of medical devices, including bioprosthetic heart valves and tendon grafts.
The pericardium itself is comprised of both dense regular and irregular connective tissue (predominantly type I collagen) that is organized into fibrils, fibres, fibre bundles and laminates (Rémi et al., 2011). The fibrous pericardium is the outermost layer of the pericardium containing aligned type I collagen fibres, which is fused to an adjacent layer of parietal pericardium containing a multi-directional network of fine collagen fibres and elastin. Despite the presence of this multi-directional network, a discrete fibre-preferred or predominant fibre directionality is evident within the fibrous pericardium (Braga-Vilela et al., 2008). Thus, we hypothesized that multiple sheets of pericardium could be oriented relative to each other and overlaid such that the preferred fibre alignment could be tailored within each layer of the AF patch in order to achieve varying angle-ply orientations and mechanical properties similar to the human AF. Thus, the objectives of the studies described herein were to: (1) confirm the complete decellularization of porcine pericardium; (2) demonstrate a simple and scalable method by which to create a multi-laminate angle-ply AF repair patch; and (3) assess its basic mechanical characteristics and cytocompatibility relative to the native AF and its cells, respectively. Quantitative results were expressed as a mean ± standard error, and were statistically compared via one-way analysis of variance with significance denoted as $p < 0.05$.

### 2. Materials and methods

Porcine pericardium was obtained from a local abattoir and transported to the lab within 3 h of harvest. Tissue was cleaned of extraneous fat and subjected to a decellularization process previously described by Tedder et al. (2009) with modifications. Briefly, the pericardium was submerged in distilled water for 24 h at 4 °C to lyse porcine cells via hypotonic shock. Tissue specimens (three pieces ~2 × 5 cm each) were then transferred to 100 ml decellurization (decell) solution (pH 7.8) containing 50 mM Tris, 0.15% (v/v) Triton X-100, 0.25% (w/v) deoxycholic acid, 0.1% (w/v) ethylenediaminetetraacetic acid and 0.02% (w/v) sodium azide, while maintained at room temperature under constant agitation (150 rpm) for 3 days. Decell solution was changed on day 3 and the process was continued for a total of 6 days prior to sequential washes in 70% ethanol and distilled water (two washes each for 10 min while agitating at room temperature). Tissues were placed in a solution (pH 7.5) of DNase/RNase (720 U/ml each) containing 5 mM magnesium chloride at 37 °C for 24 h at 150 rpm.

### 3. Results

Efficacy of decellularization was assessed via routine, paraffin-embedded histology (5 μm sections) followed by haematoxylin and eosin (H&E) staining for the evaluation of cell nuclei, as well as agarose gel electrophoresis and Nanodrop spectrophotometry for residual porcine DNA that was isolated from tissue via a Qiagen DNeasy Blood and Tissue kit according to manufacturer’s instructions. Additionally, immunohistochemistry (IHC) for the porcine antigenic epitope alpha-gal (biotinylated GSL1-isolectin B4; 2.5 μg/ml; VectorLabs) was performed. Histology results indicated the complete absence of intact porcine cell nuclei in decellularized samples with some evidence of tissue swelling, and minor matrix disruption indicated by an increase in overall tissue thickness to ~250 μm as compared with fresh pericardium (~150 μm; Figure 1A and B). Furthermore, staining with 4',6-diamidino-2-phenylindole (DAPI) for cell nuclei was absent (data not shown). Additionally, IHC for alpha-gal, the xenogenic epitope responsible for acute rejection of porcine-derived materials in humans was below the detection limit of the antibody in the decellularized pericardium (Figure 1C and D). One percent agarose gels stained with ethidium bromide demonstrated the absence of residual DNA greater than 300 base pairs (bp), concomitant with a significant ($p < 0.05$) 95% reduction in double-stranded DNA content in decellularized pericardium compared with fresh tissue as determined by spectrophotometry (96.2 ± 13.4 and 2051 ± 112.7 ng/mg dry weight, respectively; Figure 1E and F). Removal of xenogenic DNA is required when developing biomaterials for implantation into humans so as not to elicit an immune reaction. Our results are in alignment with Crapo et al. (2011) and Gilbert et al. (2009), who provide initial benchmarks defining minimal criteria for effective tissue decellularization. Furthermore, herein we corroborate the effectiveness of the decellularization technique developed by Tedder et al. (2009) illustrating the repeatability of the procedure.

Next, in order to create multi-laminate AF patches (Figure 1G), decellularized pericardium sheets were gently dried with tissue paper, and sections of the tissue with a clearly defined collagen fibre-preferred/aligned direction were identified in the fibrous pericardium and cut out into squares. The fibre aligned direction of each square was then oriented ± 30 ° (verified via a protractor) relative to a stationary grid containing a common horizontal axis. Once aligned, sections were stacked and the multi-laminate sheets were placed upon a dissolvable embroidery backing material (Sulky Fabri-Solvy; 100% polyvinyl alcohol), which allowed for easy positioning within a sewing machine (Brother JX2517) and enabled sewing needle penetration through all pericardium layers. A square pattern was sewn around the periphery of the sheets using a thread diameter equivalent to a 2-0 suture followed by removal of excess tissue and backing material. The patches, along with the backing, were soaked in saline for 30 min to ensure that the backing had completely dissolved. To illustrate that patches could be made with adjacent plys having an aligned collagen fibre-preferred direction oriented at ± 30 ° relative to each other, polarized light microscopy was performed in conjunction with employing a red fluorescence wavelength filter to visualize multi-laminate patches that had been sectioned.
Figure 1. Representative histological images of (A) fresh and (B) decellularized porcine pericardium stained with haematoxylin and eosin (H&E), respectively (pink = extracellular matrix; arrowheads = location of cell nuclei; total magnification: 200 ×). Representative histological images of (C) fresh porcine pericardium demonstrating positive (brown) immunohistochemistry (IHC) staining for alphagal epitope and (D) decellularized pericardium illustrating a lack of positive staining (blue = matrix; black = cell nuclei; total magnification: 200 ×; inserts = negative IHC controls). (E) Ethidium bromide-stained agarose gels for DNA isolated from fresh (lanes 1–5) and decellularized (lanes 6–10) pericardium (white bands = presence of DNA). A 300–24 000-bp DNA standard ladder (lanes 11–12) is shown for comparison. (F) DNA quantification of fresh and decellularized pericardium performed with Nanodrop spectrophotometry. (G) Diagrammatic representation of multi-laminate annulus cellular matrix; arrowheads = location of cell nuclei; total magnification: 200 ×; inserts = negative IHC controls). (E) Ethidium bromide-stained agarose gels for DNA isolated from fresh (lanes 1–5) and decellularized (lanes 6–10) pericardium (white bands = presence of DNA). A 300–24 000-bp DNA standard ladder (lanes 11–12) is shown for comparison. (F) DNA quantification of fresh and decellularized pericardium performed with Nanodrop spectrophotometry. (G) Diagrammatic representation of multi-laminate annulus fibrosus (AF) patch formation in which at least three-plys of decellularized pericardium were stacked (red tubes = aligned collagen type I fibres in the fibrous pericardium layer of each ply) oriented at ± 30° to each other. (H) Schematic representation depicting histology sectioning of AF patches using an oblique cut (dotted red line) across multiple layers performed to microscopically visualize collagen fibre alignment in fibrous pericardium surfaces stacked directly adjacent to one another demonstrating a ± 30° ‘chevron’ (*) pattern. Alternatively, when fibrous and parietal pericardium surfaces were directly adjacent to each other, a ‘half chevron’ (#) was achieved. (I) Chevron and (J) half chevron patterns (dashed white outlines), respectively, were observed within each patch via polarized light microscopy confirming the presence of oriented collagen fibre alignment (total magnification: 100 ×). (K) Macroscopic image of a six-ply AF patch sewn with suture (black outline). Solid lines connecting different study groups on the graph indicate a significant difference (p < 0.05).

[Colour figure can be viewed at wileyonlinelibrary.com]
stationary test fixture until contact was made with the secured AF patches (indicated by the generation of a 0.1 N preload). Testing was performed at a rate of 300 mm/min until patch rupture. The resultant ball-burst pressure at failure was calculated given the maximum force at rupture, and its relationship with ball-burst pressure according to established equations [equations (1)–(3)] given the geometric constraints of our test set-up (Freytes et al., 2005).

\[ P = \frac{F}{A} = \frac{F}{(2\pi d^2(1 - \cos(\phi)))} \quad (1) \]

\[ \phi = \pi - \left( \frac{\pi}{2} - \tan^{-1}\left(\frac{d}{f}\right) \right) - \tan^{-1}\frac{a}{b} \quad (2) \]

\[ f = \sqrt{\left(b^2 + a^2 - d^2\right)} \quad (3) \]

where \( P \) is the ball-burst pressure, \( F \) is the maximum recorded burst force, \( A \) is the contact area between the patch and the surface of the steel ball, \( \phi \) is the contact angle between the AF patch material and ball, \( d \) is the radius of the steel ball, \( f \) is the magnitude of the vector representing the stretching material, which is geometrically determined from: \( a \) – the distance between the central axis of the ball and tissue clamp set-up (3.25 mm); \( b \) – the position of the steel ball and push rod relative to its starting point (3 mm), which maintains the tangential relationship between the patch material and surface of the ball as described by Freytes et al. (2005).

Burst strength results illustrated a positive correlation between the number of layers used in patch construction and biaxial ball-burst strength (Figure 2A). AF patches of one-, two-, three- and six-ply exhibited average biaxial burst strengths of 1.28 ± 0.12 MPa, 2.25 ± 0.16 MPa, 2.92 ± 0.46 MPa and 5.53 ± 0.23 MPa, respectively. All values were significantly different from each other (\( p < 0.05 \)), except between two- and three-ply patches. The three-ply patch material inherently exhibited biaxial burst strengths exceeding the highest reported in vivo IDP value of 2.3 MPa measured in human lumbar IVDs when lifting a 20 kg load with flexed back (Wilke et al., 1999).

Uniaxial tensile testing was performed on three-ply AF patches (\( n = 6 \)) according to methods described by Green et al. (1993) who assessed the circumferential tensile properties of human AF. Briefly, patches were affixed between two tensile grips such that the fibre alignment of the patches was oriented ± 30° to the axis of applied tension. Additionally, herein single layer sheets of pericardium (\( n = 6 \)) were tested in the fibre-preferred (tensile loading applied in the direction of collagen fibre alignment) and cross-fibre (tensile load applied perpendicular to collagen fibre alignment) directions. The testing protocol consisted of applying five preconditioning cycles to 10% strain followed by testing to failure at a rate of 240 mm/min. Modulus values were determined from the linear region of the resultant stress–strain curves. Stress–strain curves demonstrated a non-linear profile as is reminiscent of a viscoelastic material (data not shown). The average ultimate tensile strengths (UTSs) of the

![Figure 2.](image-url)
three-ply AF patch, single-ply decellularized pericardium in the fibre-preferred and cross-fibre directions were 5.9 ± 0.3 MPa, 5.6 ± 1.1 MPa and 2.9 ± 0.2 MPa, respectively. The UTS of the single-ply pericardium tested in the cross-fibre direction was significantly different (p < 0.05) from the single-ply sample tested in the fibre-preferred direction as well as the multi-laminate AF patch. Average linear circumferential moduli of the three-ply AF patch, single-ply decellularized pericardium tested in the fibre-preferred and cross-fibre directions were 16.4 ± 3.5 MPa, 62.0 ± 13.6 MPa and 23.6 ± 6.0 MPa, respectively. Overall, the UTS and modulus values of the three-ply AF patches mirror the values reported for posterolateral human AF tissue (3.8 ± 1.9 MPa and 12–24 MPa, respectively; Green et al., 1993; Long et al., 2015; O’Connell et al., 2012). Furthermore, the average linear modulus of a single sheet of decellularized pericardium in the fibre-preferred direction matched values reported for single AF lamellae from the outer AF (64.8 MPa; Long et al., 2015).

Cytocompatibility of multi-laminate AF patches was assessed after seeding patches with bovine caudal IVD AF cells. Briefly, cow tails were collected within 2 h of slaughter and caudal IVDs were isolated via blunt dissection. AF tissue was minced into 2–4-mm² pieces and digested in Dulbecco’s modified Eagle’s medium (DMEM) containing 0.2% collagenase (Type I: 125 U/mg) and 1% antibiotic/antimycotic (Ab/Am; 10 000 IU penicillin, 10 000 µg/ml streptomycin, 25 µg/ml amphotericin) for 18 h at 37 °C. Prior to seeding, multi-laminate AF patches were sterilized using 0.1% peracetic acid in phosphate-buffered saline solution (pH 7.5) for 2 h at room temperature prior to thorough rinsing in sterile saline and overnight neutralization in a solution of 48% fetal bovine serum, 50% DMEM and 2% Ab/Am. AF cells were seeded drop-wise in 75 µl of media containing 1 × 10⁵ cells (passage 4) on to the surface of 10 × 10 mm patches. Cells were allowed to attach for 3 h prior to being flipped and seeded on the opposite surface. Additionally, following surface seeding, patches were injected with 1 × 10⁵ cells between the layers using a 20G syringe. Cells were allowed to culture under standard conditions for up to 15 days. Histological analysis of cell-seeded patches (n = 3/time-point) was completed on paraffin-embedded, 5-µm sections stained with H&E, which were imaged on a Zeiss Axiolab A1 microscope with Axiovision software. DNA content and cell death on the patches was assessed via Picogreen (n = 3 patches/time-point) and lactate dehydrogenase (LDH; n = 3 patches/time-point) assays, respectively, according to manufacturer’s instructions. To determine the number of cells attached to the multi-laminate AF patches, a standard curve was developed from known numbers of bovine AF cells seeded in well plates subjected to Picogreen analysis. Additionally, LDH values were expressed as a percentage of a positive cell death control developed by snap-freezing AF cell-seeded patches 3 days prior to LDH analysis on the culture media. Histological results confirmed AF cell attachment to the surfaces of the multi-laminate patches forming a monolayer. Furthermore, there was evidence of cellular infiltration into the lamellae of the patches as well (Figure 2B). DNA content of multi-laminate patches significantly (p < 0.05) increased between day 6 and day 15 (0.264 ± 0.081 and 0.625 ± 0.090 µg DNA/ml, respectively), suggesting AF cell proliferation over time in culture (Figure 2C). Interpolation from a standard curve developed from known numbers of bovine AF cells demonstrated greater than 3 × 10⁵ cells on each patch by day 15. LDH content of culture media surrounding patches immediately following seeding (day 0) and after 6 and 15 days of culture was 10.31 ± 0.48%, 28.72 ± 1.22% and 29.94 ± 0.90%, respectively, as compared with positive controls at each respective time-point (Figure 2D). While LDH values at days 6 and 15 were both significantly different (p < 0.05) compared with day 0, there was no difference between days 6 and 15 indicating that there was no increase in cell death with increasing time in culture therefore illustrating cytocompatibility of multi-laminate AF patches.

4. Discussion

In conclusion, this is the first study to report the development of an angle-ply multi-laminate AF repair patch using a simple and scalable process resulting in a biomaterial that demonstrates structural and mechanical properties comparable to that of native human AF tissue. Furthermore, this multi-laminate biomaterial supports the viability and proliferation of AF cells, thus illustrating its regenerative potential. Taken together, the potential clinical value of this multi-laminate AF patch for patients undergoing surgical procedures for IVD degeneration and herniation is immense. Ongoing investigations of this biomaterial include: (1) assessing the fatigue properties (tensile and torsional); (2) developing a surgical fixation technique to secure the patch in place; (3) evaluating matrix deposition and cell phenotype following seeding with an alternative cell source (i.e. human amniotic mesenchymal stem cells); and (4) further developing a multi-laminate implant to fill defects throughout the depth of the AF, which could be accomplished by combining the patch with a mechanically competent and cytocompatible material.

Acknowledgments

R. McGuire and R. Borem contributed equally to this work. Funding was provided in part by the National Institute of General Medical Science of the NIH (SP20GM103444-07) and Clemson University Department of Bioengineering start-up funds provided to J. Mercuri.

Conflict of interest

The authors have declared that there is no conflict of interest.
References


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