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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.

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

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Conflict of interest

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

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 $\pm 28\text{--}43^\circ$ 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 $\pm$ 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 $\sim 2 \times 5$ cm each) were then transferred to 100 ml decellularization (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 μ m 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 μ g/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 μ m as compared with fresh pericardium (~ 150 μ m; 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 $\pm 30^\circ$ (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 plies having an aligned collagen fibre-preferred direction oriented at $\pm 30^\circ$ 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 obliquely (Figure 1H) across the sample in order to observe multiple layers. Histological inspection of patch sections illustrated the presence of 'chevrons' (Figure 1H*), indicating the location at which the collagen-preferred directions in immediately adjacent layers of fibrous pericardium intersect at opposing $\pm 30^\circ$ angles (Figure 1I and J). While one of the major hurdles of AF regeneration is to promote the development of a highly oriented multi-laminate structure, one must also consider that any biomaterial developed to assist the repair/regenerative process must also have the appropriate dimensions in order to adequately cover large (~6–6.5 mm) defects resulting from NP herniation while allowing for attachment to lumbar IVDs. Considering the dimensional constraints of lumbar IVD heights, AF patches as small as 4×4 mm and upward of 15×15 mm were made using this technique, and thus were able to meet the aforementioned critical design requirements for use in either the anterior or posterior AF due to their inherent micro-architecture and customizable dimensions. It should be noted that mechanical evaluations of AF patches described below were carried out on 10×10 mm multilaminate patches (Figure 1K) consisting of one–six sheets (or plies). The approximate thickness of each single sheet of decellularized pericardium was 0.25 mm, whereas six-ply patches were approximately 1.60 mm in total thickness.

The native AF is subject to complex stretching and loading, including biaxial strains and circumferentially directed tensile hoop stresses developed due to spinal motions and IDP generated by the NP. Thus, it is critical to assess the inherent strength of the AF patches to resist biaxial burst. Briefly, single layers of pericardium and AF patches of two-, three- and six-ply ($n = 6$ each) were assembled and subjected to biaxial ball burst directed radially (perpendicular to axis of the patch fibres) generating/simulating a potential large AF herniation or expulsion of an NP replacement out through the patch thickness. Briefly, patches were placed in a custom-designed stationary test fixture, which centred and secured the AF patch between two restraining blocks lined with course grit sandpaper and contained a 6.25-mm diameter thru hole. The fixture and patches were secured to the base of an Instron mechanical test frame fitted with a 1000 N load cell. A 6-mm-diameter stainless steel ball was welded to a push rod, which was secured to the test frame crosshead. The crosshead was

lowered such that the rod and ball were directed into the thru hole of the 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).

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

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

$$f = \sqrt{(b^2 + a^2 - d^2)} \quad (3)$$

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, ϕ 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 $\pm 30^\circ$ 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 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 1×10^5 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 1×10^5 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 AxioVert 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 3×10^5 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 $10.31 \pm 0.48\%$, $28.72 \pm 1.22\%$ and $29.94 \pm 0.90\%$, 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.

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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 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 $\pm 30^\circ$ 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 $\pm 30^\circ$ ‘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 ($p < 0.05$)

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Figure 2.

(A) Graph of the average maximum biaxial burst strength of one-, two-, three- and six-ply annulus fibrosus (AF) patches. (B) Histological image of a three-ply patch (with layers separated for clarity) illustrating the presence of AF cells on the surface and penetrating (insert, total magnification: 200 ×) the AF patch after 6 days of *in vitro* culture (purple = nuclei; pink = extracellular matrix; total magnification: 50 ×). (C) Graph illustrating bovine AF cell DNA content within three-ply AF patches following 6 and 15 days of culture, respectively, as compared with a DNA standard curve generated from known numbers of viable AF cells. (D) Graph illustrating percent lactate dehydrogenase (LDH) produced by bovine AF cell-seeded three-ply patches immediately following seeding (day 0), and after 6 and 15 days of culture relative to a positive cell death control (i.e. AF cell-seeded three-ply patches subjected to snap-freezing with liquid nitrogen to induce 100% cell death). Solid lines connecting different study groups on graphs indicate a significant difference ($p < 0.05$)