Journal of Biomechanics 48 (2015) 1436–1443

Contents lists available at ScienceDirect

Journal of Biomechanics

journal homepage: www.elsevier.com/locate/jbiomech
www.JBiomech.com

Induction of fiber alignment and mechanical anisotropy in tissue engineered menisci with mechanical anchoring

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ARTICLE INFO

Article history:
Accepted 15 February 2015

Keywords:
Meniscus
Tissue engineering
Collagen
Boundary conditions
Fiber alignment

ABSTRACT

This study investigated the effect of mechanical anchoring on the development of fiber organization and anisotropy in anatomically shaped tissue engineered menisci. Bovine meniscal fibrochondrocytes were mixed with collagen and injected into molds designed to produce meniscus implants with 12 mm extensions at each horn. After a day of static culture, 10 and 20 mg/ml collagen menisci were either clamped or unclamped and cultured for up to 8 weeks. Clamped menisci were anchored in culture trays throughout culture to mimic the native meniscus horn attachment sites, restrict contraction circumferentially, and encourage circumferential alignment. Clamped menisci retained their size and shape, and by 8 weeks developed circumferential and radial fiber organization that resembled native meniscus. Clamping also increased collagen accumulation and improved mechanical properties compared to unclamped menisci. Enhanced organization in clamped menisci was further reflected in the development of anisotropic tensile properties, with 2–3 fold higher circumferential moduli compared to radial moduli, a similar ratio to native meniscus. Ten and 20 mg/ml clamped menisci had similar levels of organization, with 20 mg/ml menisci producing larger diameter fibers and significantly better mechanical properties. Collectively, these data demonstrate the benefit of using bio-inspired mechanical boundary conditions to drive the formation of a highly organized collagen fiber network.

1. Introduction

Menisci are primarily anchored by ligament-like extensions at the horns which play a major role in menisci's ability to withstand and distribute loads in the knee. These extensions prevent extrusion, while allowing movement to conform to the condyles during each gate cycle (Kale et al., 2010; Messner and Gao, 1998). Collagen organization is also fundamental to the distribution of complex loads of the knee (Kawamura et al., 2003; Messner and Gao, 1998). Native menisci consist largely of circumferentially aligned collagen bundles anchored by perpendicular radial tie-fibers (Kambic and McDevitt, 2005; Petersen and Tillmann, 1998; Rattner et al., 2011). This organization results in anisotropic properties, with the circumferential tensile modulus 3–10 times larger than the radial modulus (Makris et al., 2011; Tissakht and Ahmed, 1995).

Tears that disrupt this organization result in pain, swelling, mechanical instability and degradation of surrounding cartilage (Hasan et al., 2013). Due to the avascular nature of the meniscus, tears are slow to heal, resulting in over 1 million surgeries a year in the US (Hasan et al., 2013; Khetia and McKeon, 2007). Treatment of large tears are generally not effective, and while cadaveric meniscus transplants show promise, lack of supply, the potential for disease transmission, and geometry constraints limit allograft therapy (Hasan et al., 2013).

Although there has been great effort to generate whole tissue engineered menisci to serve as an alternative to allografts (Balint et al., 2012; Ballyns et al., 2008; Higashioka et al., 2014; Huey and Athanasiou, 2011; Kon et al., 2008; Mandal et al., 2011; Puetzer and Bonassar, 2013; Tienen et al., 2006; Zur et al., 2011), none have achieved clinical use. Such implants often lack the native collagen fiber organization and anisotropic properties essential to distribute the loads of the knee appropriately. Recently, we have developed high density type I collagen menisci that have significant improvements in biochemical and mechanical properties with time in culture, but lack native organization (Puetzer and Bonassar, 2013).

It has been well established that contraction of collagen gels under a variety of mechanical boundary conditions can guide the formation of aligned collagen fibers (Bell et al., 1979; Bowles et al., 2010; Costa et al., 2003; Grinnell and Lamke, 1984; Thomopoulos et al., 2005). It is believed cellular traction forces align collagen fibers

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http://dx.doi.org/10.1016/j.jbiomech.2015.02.033
0021-9290/

along free edges (Costa et al., 2003; Grinnell and Lamke, 1984). Further, these aligned collagen gels develop anisotropic mechanical properties (Gould et al., 2012; Lee et al., 2008; Thomopoulos et al., 2005). However, all of this work has been done with low density collagen gels (1–6 mg/ml) and very thin simple geometries. To date, mechanical boundary conditions have not been investigated in high density collagen gels (10–20 mg/ml) or used with large complex geometries such as the meniscus.

The objective of this study was to investigate the effect of long-term anchored culture on the organization and development of tissue engineered meniscal constructs at multiple high density collagen concentrations. We hypothesize that high density type I collagen menisci, cultured with native horn-anchored boundary conditions, will result in alignment of collagen fibers and development of anisotropic tensile properties

2. Methods

2.1. Construct fabrication

Sixty-eight meniscal constructs were fabricated as previously described (Puetzer and Bonassar, 2013). Briefly type I collagen was extracted from Sprague-Dawley rat tails (Pel-Freez Biologicals, Rogers, AZ) and reconstituted at 20 or 30 mg/ml using previously established techniques (Bowles et al., 2010; Cross et al., 2010). Meniscal fibrochondrocytes were isolated within 24 h of slaughter of 1–3 day old bovine with 0.3% collagenase digestion and suspended in media. Sets of menisci were created from 15 different batches of cells and divided equally between clamped and unclamped, with each batch consisting of cells pooled from 2–3 bovine. The stock collagen solution was mixed with appropriate volumes of 1 N NaOH, 10× phosphate-buffered saline (PBS), and 1× PBS to begin the gelling process at pH 7.0 and 300 mOsm osmolarity (Bowles et al., 2010; Cross et al., 2010). This collagen solution was immediately mixed with the cell/medium solution and injected into ovine meniscus micro-computed tomography-based molds (Ballyns et al., 2008) to obtain 10 and 20 mg/ml meniscal constructs at 25×10^6 cells/ml. The molds were allowed to gel for 1 h at 37 °C (Fig. 1). After 1 h, the meniscal constructs were removed from the molds and cultured in media composed of DMEM, 10% FBS, 100 μg/mL penicillin, 100 μg/mL streptomycin, 0.1 mM non-essential amino acids, 50 μg/mL ascorbate, and 0.4 mM L-proline, changed every 2–3 days.

2.2. Application of boundary Conditions

In order to achieve anchorage of the implants at the horns, meniscal molds (Ballyns et al., 2008) were redesigned with a $12 \times 2.5 \times 2$ mm^3 (L × W × H) extension at each horn. Further, previously described ABS loading trays (Ballyns and Bonassar, 2011) were redesigned to have a trough at the base of the meniscus that extensions lay in. Stainless steel clamps infused with bronze (Shapeways, NY) were screwed down over the extensions to secure them throughout culture (Fig. 1). This boundary condition of anchoring the meniscus at the horns was chosen to mimic the native tibial attachment sites in vivo and to restricted contraction of collagen gels circumferentially. By restricting contraction circumferentially we hope to induce a residual hoop stress to encourage alignment. After a day of static culture, 10 and 20 mg/ml collagen menisci were either clamped or unclamped and cultured for up to 8 weeks.

2.3. Construct analysis

At 0, 2, 4, and 8 weeks 4–6 samples from each group (clamped or unclamped, 10 mg/ml or 20 mg/ml) were removed from culture. Upon removal from culture all meniscal constructs were analyzed for organization, biochemical composition, and mechanical properties, with attention to radial vs. circumferential directions. For gross analysis, serial photographs taken throughout culture were analyzed using ImageJ to calculate change in area of samples, and the final weight of the constructs were compared to determine change in mass with time.

2.3.1. Confocal image analysis

Cross-sections from throughout the scaffolds were fixed in 10% buffered formalin, stored in 70% ethanol, and imaged with confocal microscopy in the circumferential and radial direction to visualize collagen fiber and cell organization as previously described (Bowles et al., 2010; Carey et al., 2012; Puetzer and Bonassar, 2013). Briefly, confocal reflectance imaging was performed in conjunction with fluorescence imaging by splitting a 488 nm laser on a Zeiss 710 confocal microscope using a 40×/1.2 C-Apochromat objective. Confocal reflectance microscopy was performed at 475–510 nm by collecting backscattered light reflected by collagen fibers through a 30 μm pinhole and using a pixel dwell time of 1.58 μs. Auto-fluorescent cells were captured at 500–580 nm. In the images, green represents collagen content, while cells are red.

Four to six circumferential confocal images from 4–6 cross-sections throughout the meniscus (total 16–36 images/treatment group) were analyzed with a custom MATLAB code based on a series of 2D fast Fourier transforms (FFT) to determine the degree of alignment and mean fiber diameter (Fig. 2). Degree of alignment is reported using an established alignment index (AI) measure (Bowles et al., 2010; Chaudhuri et al., 1987; Ng et al., 2005). An initial FFT was performed to determine the maximum angle of alignment by summing the intensity of the FFT at 5° increments from 0° to 180°. This angle was further confirmed using the MATLAB radon transform function. The AI was determined by summing the intensity ±20° from the maximum alignment angle as previously described (Bowles et al., 2010). AI values range from 1(unaligned) to 4.5(completely aligned).

$$AI = \frac{\int_{\theta_m - 20^\circ}^{\theta_m + 20^\circ} I\partial\theta}{(40^\circ/180^\circ) * \int_{0^\circ}^{180^\circ} I\partial\theta}$$

To determine the diameter of fibers, the original image was rotated according to the radon transform to fix the maximum angle of alignment at 90°, ensuring that fibers are primarily vertical in the image (Fig. 2). The image was cropped and resized to avoid border effects and another FFT was performed, providing information on the frequency of sinusoidal patterns along the *x*-axis. To determine average fiber size a histogram of intensity along the *x*-axis was created and averaged. The histogram span corresponding to the average intensity was converted to pixels and further microns using the image size. The code was validated using control sinusoidal phantom images and by relating results to ImageJ calculated fiber diameters.

2.3.2. Histological and biochemical analysis

Histological and biochemical analysis were performed as previously reported (Ballyns et al., 2008; Puetzer and Bonassar, 2013). Histologically, radial and circumferential cross-sections of the menisci were fixed, embedded into paraffin, sectioned, and stained with picrosirius red. They were imaged under polarized light to observe large-scale collagen organization.

For biochemical analysis 4 samples from each meniscus were weighed wet (WW), frozen, lyophilized, and weighed dry (DW). The samples were digested in 1.25 mg/ml papain solution and analyzed for DNA, GAG and collagen content via the Hoechst DNA assay (Kim et al., 1988), a modified 1,9-dimethylmethylene blue assay at pH 1.5 (Enobakhare et al., 1996), and a hydroxyproline assay (Neuman and Logan, 1950), respectively. Media samples taken every 2–3 days were analyzed with the same assays to track the release of biochemicals to the media.

2.3.3. Mechanical analysis

The equilibrium and tensile moduli were determined as previously described (Ballyns et al., 2010; Gleghorn et al., 2007; Puetzer and Bonassar, 2013). Briefly, 2–4 4×1 mm^2 plugs from each construct were obtained for compression testing, while radial and circumferential dogbone punches were taken to determine directional tensile properties. The equilibrium modulus was determined in confined compression via a stress relaxation test of 10×50 μm steps fit to a poroelastic model using MATLAB (Gleghorn et al., 2007). The tensile modulus was determined at a strain rate of 0.75%/s and calculated as the slope of the linear region of the stress–strain curve (Ballyns et al., 2010). Quasi-static loading was assumed and it was ensured failure was not near clamps. Circumferential and radial punches from neonatal bovine menisci were tested using the same procedure for native comparison.

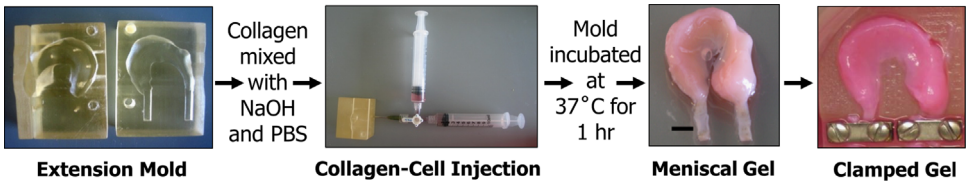

Fig. 1. Injection molding process and clamping of meniscal constructs. Ovine based meniscal molds had 12 mm long extension added to the horns, high density collagen meniscal constructs were created using injection molding techniques and constructs were clamped at the extensions throughout culture.