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Nutrient channels and stirring enhanced the composition and stiffness of large cartilage constructs

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ABSTRACT

A significant challenge in cartilage tissue engineering is to successfully culture functional tissues that are sufficiently large to treat osteoarthritic joints. Transport limitations due to nutrient consumption by peripheral cells produce heterogeneous constructs with matrix-deficient centers. Incorporation of nutrient channels into large constructs is a promising technique for alleviating transport limitations, in conjunction with simple yet effective methods for enhancing media flow through channels. Cultivation of cylindrical channeled constructs flat in culture dishes, with or without orbital shaking, produced asymmetric constructs with poor tissue properties. We therefore explored a method for exposing the entire construct surface to the culture media, while promoting flow through the channels. To this end, chondrocyte-seeded agarose constructs (⌀10 mm, 2.34 mm thick), with zero or three nutrient channels (⌀1 mm), were suspended on their sides in custom culture racks and subjected to three media stirring modes for 56 days: uniaxial rocking, orbital shaking, or static control. Orbital shaking led to the highest construct E_Y, sulfated glycosaminoglycan (sGAG), and collagen contents, whereas rocking had detrimental effects on sGAG and collagen versus static control. Nutrient channels increased E_Y as well as sGAG homogeneity, and the beneficial effects of channels were most marked in orbitally shaken samples. Under these conditions, the constructs developed symmetrically and reached or exceeded native levels of E_Y (~400 kPa) and sGAG (~9%/ww). These results suggest that the cultivation of channeled constructs in culture racks with orbital shaking is a promising method for engineering mechanically competent large cartilage constructs.

1. Introduction

Osteoarthritis (OA) is a debilitating disease characterized by damage to the articular cartilage layers lining diarthrodial joints. In advanced cases, lesions span the full thickness of cartilage, leading to bone-on-bone contact during joint articulation. With its relatively low cell density and avascular nature, adult cartilage lacks the ability to regenerate when injured, leading to irreversible degeneration. Currently, there is no treatment available to fully restore durable articular surfaces.

A potential approach to the treatment of OA is by culturing cells within a scaffold under suitable conditions in vitro in order to produce viable replacement cartilage (Johnstone et al., 2013; Langer and Vacanti, 1993; Vacanti and Langer, 1999). Culture of chondrocytes within agarose scaffolds is a well-established technique which stabilizes chondrocyte phenotype and encourages the elaboration of sulfated glycosaminoglycans (sGAG) which are similar to those found in native cartilage (Benya and Shaffer, 1982; Buschmann et al., 1992; Mauck et al., 2000; Mouw et al., 2005; O'Conor et al., 2014; Rahfoth et al., 1998). These chondrocyte-agarose constructs have had some success in reaching native values of compressive Young's modulus (E_Y) and sGAG content when their dimensions are small (~⌀4 mm) (Bian et al., 2009; Byers et al., 2008; Cigan et al., 2013a; Nims et al., 2014); however, constructs of this size may not be sufficiently relevant clinically, as OA symptoms often manifest themselves when cartilage defects become larger, e.g., ⌀25 mm (Curl et al., 1997; Moisio et al., 2009). Previous attempts to engineer cartilage constructs of this size have been met with transport limitations; the consumption of nutrients by cells at the construct periphery deprives its center of these nutrients, establishing concentration gradients throughout the depth of the construct. Depletion of glucose beneath a threshold level within constructs results in little to no local matrix elaboration by chondrocytes (Cigan et al., 2013a; Nims et al., 2014) and dissolved oxygen plays a prominent role in chondrocyte metabolism and forms gradients within tissue engineered cartilage (Obradovic et al., 1999;

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Zhou et al., 2008). Furthermore, interplay between glucose, oxygen, and other nutrients elicit a broad range of metabolic behaviors that vary with nutrient concentrations throughout the depth of the tissue (Heywood et al., 2006a, 2006b; Sengers et al., 2005; Zhou et al., 2008). Thus as construct scale is increased, the general result is a heterogeneous construct with a soft, matrix-deficient center with poor functional properties that would be unsuitable for implantation (Hung et al., 2003, 2004).

A number of techniques have been implemented in attempts to improve nutrient transport in large engineered cartilage constructs, such as incorporation of nutrient channels to reduce the necessary path length along which nutrients must travel to reach the construct center (Bian et al., 2009; Buckley et al., 2009), stirring or perfusion of culture media to prevent stagnation around the construct (Davisson et al., 2002; Vunjak-Novakovic et al., 1999), and dynamic loading to pump solutes into the tissue (Albro et al., 2008; Chahine et al., 2009; Mauck et al., 2003a; Mesallati et al., 2011). In statically cultured ⌀10 mm chondrocyte-agarose constructs, the incorporation of nutrient channels has yielded higher E_Y, approaching native values (Bian et al., 2009); in ⌀6 mm constructs, microchannels combined with rotational culture improved sGAG distribution, though sGAG content and E_Y fell short of native levels (Buckley et al., 2009). In chondrocyte-seeded ⌀10 mm PGA scaffolds cultured flat in Petri dishes, orbital shaking at ~0.8 Hz increased collagen but not sGAG content versus static control, though sGAG and collagen contents were below that of the native tissue (Vunjak-Novakovic et al., 1996).

In our studies, ⌀10 mm chondrocyte-agarose constructs cultured flat in Petri dishes with 0.8 Hz orbital shaking swelled asymmetrically due to extreme heterogeneity of sGAG deposition, rendering unconfined compression testing infeasible (Fig. 1). Peripheral regions in some specimens reached twice the thickness of the central region, and many samples experienced warping due to the occlusion of fresh media to the downward-facing sides of the constructs. In channeled constructs, the direction of media flow produced by orbital shaking was perpendicular to the nutrient channels, and channels were not found to alleviate matrix heterogeneities nor did they increase matrix content.

Therefore, we sought to overcome the matrix heterogeneities observed in large constructs by manipulating the culture system to maximize the benefits of nutrient channels. Specifically, it was hypothesized that introducing media stirring and nutrient channels aligned with the direction of media flow could produce large, symmetric, and homogeneous constructs with functional properties consistent with those of our previous studies on smaller constructs (Bian et al., 2009; Cigan et al., 2013a; Kelly et al., 2009, 2006, 2008; Lima et al., 2007; Ng et al., 2009; Nims et al., 2014). To this end, we recently developed a simple and novel technique to reorient large constructs and maximize their surface area available for nutrient transport in culture dishes, and media stirring was introduced to the culture system to improve nutrient channel efficacy (Cigan et al., 2013b). Constructs were evaluated both quantitatively for their mechanical properties and content of the major matrix constituents sGAG and collagen, and qualitatively by histological staining for distribution of these matrix components.

2. Methods

2.1. Mold fabrication

To produce constructs with precise arrangements of channels, a half-mold was fabricated (Fig. 2A). A 6.35 mm thick PTFE (polytetrafluoroethylene) sheet (McMaster-Carr) was milled to size (76 mm × 102 mm), and a CNC mill was used to drill an array of ⌀0.94 mm holes with 2.165 mm center-to-center distance in a triangular packing configuration. Stainless steel pins (⌀1 mm × 10 mm, McMaster-Carr) were press-fitted into the holes. By suppressing certain pins, several channel arrangements could be produced. For this study, equilateral triangles of pins spaced 4.33 mm center-to-center were deployed to produce three ⌀1 mm channels per construct. This triangular pattern was selected such that, when imposed upon a ⌀10 mm circle, the distance from the circle's center to the margin of the nearest channel (2 mm) matched the distance from the circle's edge to the channel margin. The PTFE half-mold was separated from a glass slide by a 2.34 mm-thick silicone rubber spacer.

2.2. Culture rack fabrication

Custom culture racks were fabricated to suspend constructs, exposing nearly all surface area to media as well as orienting channels in the direction of media stirring. The racks were comprised of PTFE as this material has low binding affinity for TGF-β, as assessed in a preliminary test. Trapezoidal pieces of PTFE (30 mm base, 8 mm height) were fashioned from a 6.35 mm thick sheet with a laser-cutter, and a drill press was used to place twelve ⌀0.99 mm holes in two rows. Twelve PTFE-coated stainless steel wires (⌀1.00 mm, 43 mm length, McMaster-Carr) were used to connect pairs of PTFE pieces. The spacing of wires allowed constructs to rest loosely upright in the culture dish without impeding media flow through the horizontally oriented nutrient channels (Fig. 2B).

2.3. Harvest and cell isolation

Chondrocytes were harvested and isolated as described previously (Cigan et al., 2013a). Briefly, slices of articular cartilage were sterilely harvested from juvenile bovine calf wrists (local abattoir) and subsequently digested with collagenase (Sigma-Aldrich); the cartilage digest was then filtered through a 70 μm mesh (McMaster-Carr) to remove debris before casting.

2.4. Construct preparation

Isolated chondrocytes were suspended in chondrogenic media (CM) (Cigan et al., 2013a) at a concentration of 120×10^6 cells/mL and combined 1:1 with molten 4% w/v type VII-A agarose (Sigma-Aldrich) to produce a 2% agarose gel with a nominal cell density of 60×10^6 cells/mL. The gel was cast into either the PTFE-glass mold (to produce channeled constructs) or a glass-glass mold

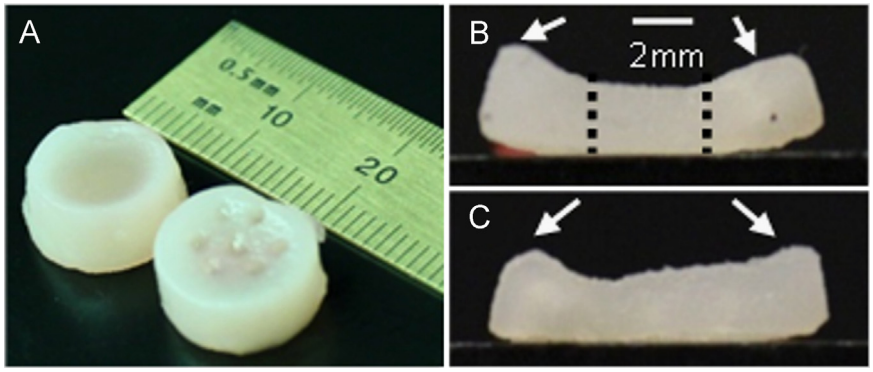

Fig. 1. Abnormal swelling of ⌀10 mm constructs cultured flat in Petri dishes for 56 days with orbital shaking; (A) perspectives of whole specimens, and (B and C) profiles of bisected specimens with or without channels (channel positions denoted by dotted lines, regions of excessive swelling denoted by arrows).