Interplay between Hypoxia and Hydrodynamic Force in Three-Dimensional Cultivation of Articular Cartilage

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Abstract— **Mimicking the hypoxic environment (1%~7% O_2) of native cartilage results in improved secretion of extracellular matrix (ECM) by chondrocytes in monolayer culture. However, investigations of the role of hypoxia in long-term three-dimensional (3D) development of biomaterial-based cartilage constructs are limited. The major challenge to overcome is the insufficient oxygen delivery to the inner core of cell-scaffold constructs, which may result in anoxic conditions (<<1%) that are linked to nitric oxide (NO)-induced damage to ECM and is associated with diseases such as osteoarthritis. To evaluate the interaction between hypoxia and hydrodynamic stimuli, chondrocyte-seeded PGA scaffolds were cultured in a petri dish (static) or a wavy-walled bioreactor (dynamic) under ambient (21%) or hypoxic (5%) conditions for 28 days, followed by biochemical and immunohistochemical evaluation. We found that sulfated glycosaminoglycan (GAG) production increased under 5% O_2 in the presence of hydrodynamic forces, whereas total collagen content was down-regulated by hypoxia in both static and dynamic groups. Greater amounts of soluble GAG were released into media under hypoxia and under mixing. In addition, expression of inducible nitric oxide synthase (iNOS) increased under static and under hypoxic conditions, revealing that NO production was elevated. Significantly decreased collagen content of Hypoxic-Static constructs may indicate that suboptimal oxygen and NO levels might be reached. These findings suggest that hydrodynamic forces are required for 3D regeneration of articular cartilage under hypoxia, but further study is required to enhance retention of soluble GAG within the construct and to minimize NO damage to collagen fibrils.**

Keywords— **Hypoxia, Hydrodynamic, Wavy-walled bioreactor, Nitric oxide, Cartilage.**

I. Introduction

Articular cartilage lacks a direct blood supply, thus nutrients and oxygen are transported by diffusion from the synovial fluid into cartilage tissue. Chondrocytes are exposed to ~7% oxygen tension near the articular surface, and oxygen levels decline in the deeper zone of cartilage (~1% O_2) due to oxygen consumption by cells in the upper layers [1]. Therefore, articular cartilage resides in a hypoxic environment when compared to vascularized tissues, and chondrocytes are thought to be able to adapt to low oxygen concentration. In vitro, it has been shown that chondrocytes survive under the same oxygen conditions they are thought to experience in vivo and maintain normal metabolic activities [2]. However, the normal oxygen gradient within cartilage is disturbed in various articular diseases. For example, oxygen transfer from the capillaries of the synovial membrane to the synovial fluid is reduced in osteoarthritic joints, resulting in anoxic conditions (<<1% O_2) [3].

Oxygen concentrations of 1%~5% have been shown to promote matrix synthesis and to protect cells from apoptosis when chondrocytes are cultured in monolayers [4-5] and aggregates [6-7]. However, their function is severely compromised when chondrocytes are cultured in alginate beads under anoxic conditions [7], suggesting that a relatively narrow range of oxygenation is critical for chondrocyte cultivation. It is important to note that these studies were conducted in the absence of a biomaterial which is required for long-term 3D development of tissue-engineered cartilage. In biomaterial-based tissue engineering, a major challenge to overcome is the inefficiency of oxygen delivery to the inner core of cell-scaffold constructs as the porosity of the material decreases as cell-secreted matrices gradually occupy the spaces. Although one group has previously reported the benefits of low oxygen tension to engineered cartilaginous constructs cultured within a concentric cylinder bioreactor [8], investigations of the role of hypoxia in long-term 3D cartilage regeneration are still limited.

In the present study, we employed a wavy-walled bioreactor (WWB) which provides a unique and well characterized hydrodynamic environment to evaluate the interplay between oxygen tension and hydrodynamic force in cartilage construct development. We hypothesize that oxygen distribution throughout the chondrocyte-polymer complex is at equilibrium with the dissolved oxygen in the culture medium only under fluid-induced agitation, and thus hydrodynamic stimuli is required for hypoxic cultivation of engineered constructs to prevent detrimental anoxic conditions.

II. Materials and mthods

A. Materials

Unless otherwise specified, all materials were purchased from VWR International (West Chester, PA).

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B. Bioreactor Configuration and Preparation

The WWB (Fig. 1A) was designed by modifying the circular wall of a traditional spinner flask (Fig. 1B) into a six-wave sinusoidal contour. The internal radius, r, of the WWB is a function of the radial angle θ, and is governed by the equation: $r(\theta) = R_{avg} + A\sin(N\theta)$ where r and θ are the cylindrical coordinates, R_{avg} is the average internal radius, A is the magnitude of peak amplitude, and N is the number of lobes. The WWB was scaled down to a capacity of 120 mL with an average radius (R_{avg}) of 3.35 cm and a wave amplitude (A) of 0.45 cm. Each WWB is fitted with two Teflon side-arm caps, a magnetic stir bar (0.8 cm diameter x 4 cm long), and a silicone stopper. Four 6-in. long, 21 gauge needles were placed at equidistant positions (1.6 cm from the center of the WWB) and anchored in the stopper. The interiors of bioreactors, needles, and stir bars were treated with Sigmacote® to prevent cell adherence. The assembled bioreactors were washed and steam-sterilized.

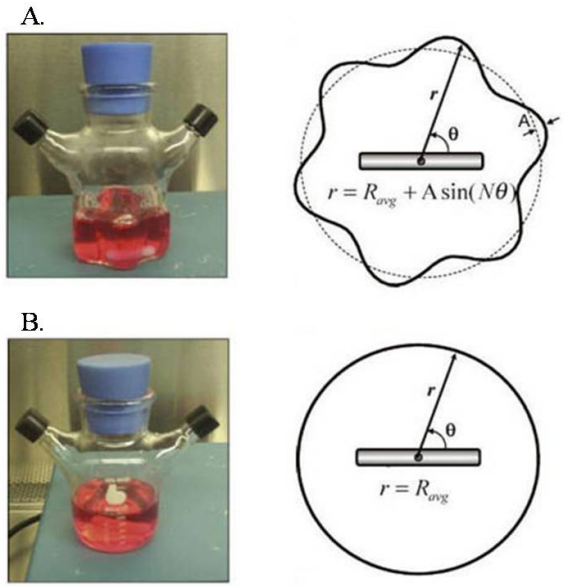

Fig. 1 Snapshot and schematic top view of (A) wavy-walled bioreactor (WWB) and (B) spinner flask

C. Cell Seeding and Tissue Culture

Two sterilized 10mm (D) x 2mm (T) PGA discs (Concordia Medical, Warwick, RI) were threaded onto each needle and separated by a silicone spacer, resulting in a total of eight PGA discs per bioreactor. The WWBs were filled with 120 mL of DMEM supplemented with 2% FBS, 1% ITS, 1X pen/strep, 10 mM HEPES, 0.4 mM L-proline, 0.1 mM NEAA, 3.6 mg/mL sodium bicarbonate, 2.5 µg/mL fungizone and 50 µg/mL ascorbic acid, and allowed to incubate overnight, with a stir bar spinning at 50 rpm. Two loose side-arm caps allowed gas exchange. After stabilization, fresh chondrocytes isolated from the femoropatellar groove of freshly slaughtered bovine calves were added to each bioreactor at a density of five million live cells per scaffold, and cell seeding was allowed to proceed for 3-4 days until the attachment rate reached 95%.

Four experimental groups were created: Normoxic (21%)-Static, Hypoxic(5%)-Static, Normoxic-Dynamic and Hypoxic-Dynamic. At the end of seeding, cell-scaffold constructs were randomly assigned to each group and cultured with fresh medium in a petri dish (static) or a WWB (dynamic) for an additional 28 days. The ratio of medium volume to number of constructs remained identical in all groups. Culture medium was renewed 100% every 3 days thereafter. Constructs were harvested at the end of cultivation for biochemical and immunohistochemical analyses.

D. Biochemistry

Engineered cartilage constructs were weighed (wet weight), frozen, lyophilized and digested with papain enzyme (Worthington, Lakewood, NJ). DNA was quantified using a PicoGreen dsDNA kit (Gibco, Carlsbad, CA) and the number of chondrocytes per construct was calculated by assuming 7.7 pg of DNA per chondrocyte. The sulfated GAG contents retained in the construct and released into media were assessed spectrophotometrically using a 1,9-dimethylmethylene blue dye-binding assay. The total collagen content was determined using orthohydroxyproline (OHP) colorimetric assay, assuming a 1 to 10 OHP-to-collagen concentration ratio. The GAG and collagen data were presented in values normalized to the wet weight of constructs.

E. Immunohistochemistry

Harvested constructs were fixed in formalin, embedded in paraffin, and sectioned in 5 µm thick slices. Immunohistochemical detection of iNOS expression within constructs was performed using a standard avidin-biotin peroxidase complex method (ABC, Vector Lab, Burlingame, CA). Briefly, after deparafinization, tissue sections were treated with 0.3% hydrogen peroxide solution for 30 minutes followed by 20-min incubation with blocking serum buffer. The sections were then incubated with rabbit anti-bovine iNOS polyclonal antibody (Millipore, Billerica, MA), overnight at 4˚C. Biotin-conjugated to goat anti-rabbit IgG was subsequently added for 30 minutes, followed by 30-min incubation with ABC. Diaminobenzidine reagent (DAB) was added to the sections until optimal staining developed. Color images were captured using a standard microscope.

F. Statistical Analyses

Six constructs were harvested from each group (n = 6) and three samples of spent media were collected during each medium change (n = 3). Statistical data are presented as mean ± one standard deviation, and were analyzed using Student's t test with significant comparisons generating a *p* value less than 0.05.

III. Results

A. Biochemical Composition

Assessed at day 28 of cultivation, chondrocyte proliferation (Fig. 2A) and ECM deposition (Fig. 2B and 2C) were significantly enhanced by hydrodynamic forces (*p<0.05). However, cell number was not influenced by oxygen concentration. Interestingly, GAG and collagen content were differentially modulated by hypoxia. Specifically, the GAG content of hypoxic constructs was significantly higher than that of normoxic constructs in the presence of hydrodynamic stimuli (^p<0.05); whereas, constructs under static culture had similar amounts of GAG in both normoxic and hypoxic groups. With respect to collagen production, the collagen content of both static and dynamic groups decreased significantly when cultivated under 5% O_2 (^{+}p<0.05). Culture medium was sampled every three days and analyzed for soluble GAG and released collagen. Soluble GAG was significantly elevated by hypoxia and by dynamic mixing, while collagen in the culture medium was extremely low or undetectable (data not shown).

B. Immunohistochemistry of iNOS

Immunohistochemical analysis of cartilaginous constructs (Fig. 3) revealed that iNOS production was higher in static cultures than in dynamic samples. Specifically, intensive staining of iNOS was observed throughout static constructs, whereas iNOS expressed by chondrocytes mainly located close to the center of constructs. Moreover, 5% O_2 increased iNOS staining intensity in both static and dynamic groups.

IV. Discussion

The role of hypoxia as an important modulator of chondrocyte growth is well recognized; however, its function on 3D cartilage development is still unclear, and merits further study. Herein, we examined the influence of hypoxia on construct development and matrix synthesis, and employed dynamic shear forces to improve efficiency of oxygen

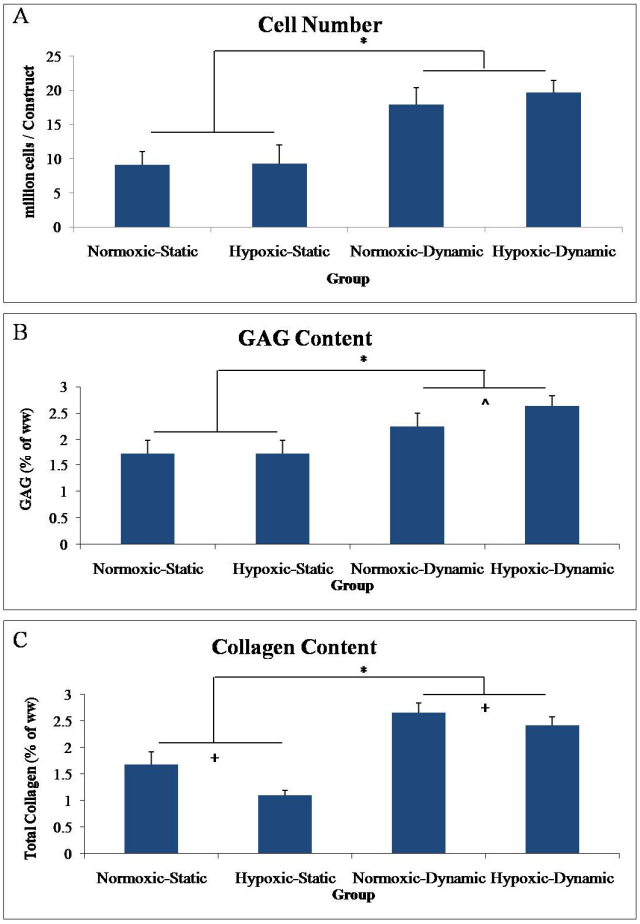

Fig. 2 Dynamic culture promotes increased cell number (A), GAG (B), and total collagen (C) of 28-day constructs. Maximum GAG deposition was detected in Hypoxic-Dynamic cultures (B). Collagen production was enhanced under normoxia (C). *, ^ and $^{+}$ indicate significance (p<0.05)

delivery to the interior of cell-seeded constructs. Not surprisingly, the data shows that dynamic cultures, regardless of hypoxia or normoxia, generated significantly improved biochemical properties than static samples, which may result from enhanced nutrition and simultaneous physical stimuli applied to constructs [9]. Conversely, low oxygen tension may have diverse effects on cell division and matrix production. Specifically, the cell doubling rate was comparable under hypoxic and normoxic conditions, which is consistent with the previous report using a concentric cylinder bioreactor [8], suggesting chondrocyte proliferation is independent of oxygen.

Hypoxia increased GAG deposition by 18% in dynamic cultures while a similar accumulation was observed in static cultures. However, when the measurement of soluble GAG in media (data not shown) is included, 5% O_2 up-regulated total synthesis of GAG in both static and dynamic groups.

One possible explanation is that low oxygen facilitates secretion of GAG but reduces synthesis of proteins that stabilize the interaction between proteoglycans and hyaluronan [10] such that their affinity decreases, promoting release of GAG. Furthermore, total collagen production of both static and dynamic constructs was significantly constrained by hypoxia, which differs from other reports in the literature [5, 8]. This may be due to dissimilar culture systems or flow patterns applied in current and previous studies.

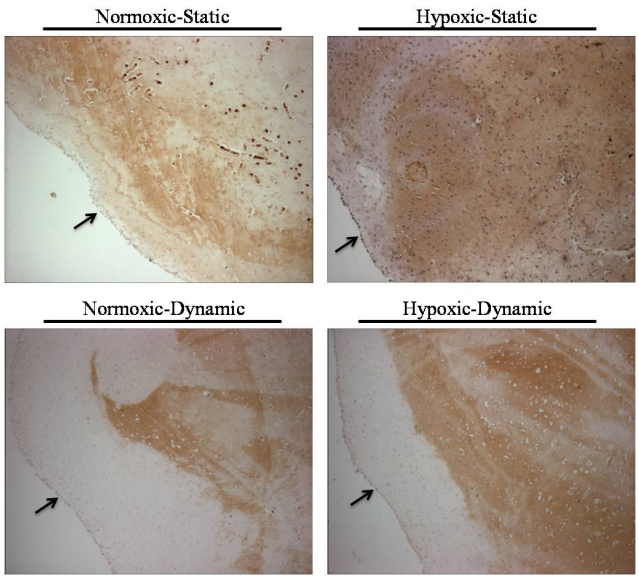

Fig. 3 iNOS (brown staining) captured at 10X reveals more intense staining under static culture, and is absent from the periphery of the constructs under dynamic culture. Arrows indicate the edge of the construct

iNOS expression increased under static and under hypoxic conditions, suggesting a higher level of NO was produced. NO is a multifunctional molecule which induces diverse biological responses including apoptosis, inflammation and muscle relaxation [11]. However, reports on its role in chondrocyte secretion of ECM vary. Cao et al. suggested that NO inhibits chondrocyte ECM production [12] whereas Hashimoto et al. indicated that hypoxia-induced NO facilitates hyaluronan synthesis [4]. Here, the reduced collagen content under hypoxia may be related to the elevated NO production. Rationally, NO rapidly converts into nitrate and nitrite, releasing hydrogen ions, producing an acidic environment that may accelerate collagen degradation. In addition, our results also suggest that hydrodynamic forces can lower NO damage to collagen fibrils. The significant decrease in collagen content of Hypoxic-Static constructs may indicate that pathological oxygen and NO levels occurred under these conditions [3, 13].

To conclude, the current study demonstrates that low oxygen tension provides a partially suitable environment for cartilage tissue engineering in the presence of fluid-induced shear stress, suggesting that hydrodynamic forces are required for long-term 3D hypoxic regeneration of articular cartilage. However, optimization of oxygen level must be further investigated to minimize damages to collagen fibrils and maximize retention of soluble GAG within constructs.

Acknowledgments

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