Hypoxic Culture of Chondrocytes Induces Production of Type Collagen VI in the Pericellular Matrix

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INTRODUCTION: Osteoarthritis (OA) is a degenerative joint disease affecting more than 32.5 million adults in the US (1). A hallmark of OA is the progressive deterioration of the load bearing articular cartilage. Articular cartilage is avascular, which limits its nutrient supply and therefore repair capacity. Chondrocytes, the sole cell type in cartilage, rely on diffusion for nutrients and oxygen, resulting in a hypoxic microenvironment that varies by tissue depth: from ~5% O_2 in the superficial zone to as low as 1% in the deep zone (2).The hypoxic nature of cartilage has implications for chondrocyte biology and OA pathogenesis where subsequent changes in metabolism play a critical role in chondrocyte homeostasis and dysfunction (3,6). Under low oxygen tension, chondrocytes increase levels of glycolytic metabolites. This shift is largely regulated by hypoxia-inducible factor 1-alpha (HIF-1α), which can promote the expression of glycolytic enzymes and reduces mitochondrial oxygen consumption. This glycolytic reprogramming is closely tied to cell matrix production, as glycolytic intermediates serve as precursors for protein biosynthesis(7). However, because tricarboxylic acid (TCA) cycle metabolites are also required for matrix production, the relative contributions of glycolysis and the TCA cycle to chondrocyte matrix synthesis remain unknown. The pericellular matrix (PCM) is a thin tissue immediately surrounding each chondrocyte composed primarily of collagen type VI. The PCM plays a crucial role in mechanotransduction and regulating cell–matrix interactions (5). Ascorbate (vitamin C) is a known cofactor in collagen synthesis and has been widely used to enhance matrix production in vitro (4). However, the regulatory impact of hypoxia on type collagen VI synthesis remains understudied. It is unclear whether hypoxic conditions modulate type collagen VI production independently of, or in synergy with, ascorbate, and through which metabolic pathways these effects are mediated. This study aims to investigate the effects of hypoxic culture and ascorbate supplementation on collagen type VI synthesis in primary bovine chondrocytes. We also evaluate associated changes in central energy metabolism by quantifying concentrations of bovine cell TCA metabolites using Liquid Chromatography Mass Spectrometry (LCMS) to better understand how oxygen tension shapes PCM composition and overall cellular function.

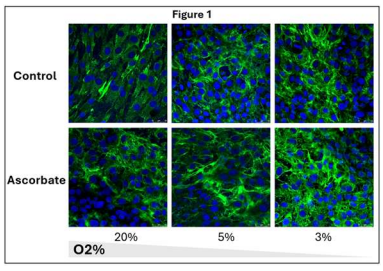
METHODS: Cell Culture Preparation: Primary bovine chondrocytes (Passage 1) were seeded at a density of ~3,158 cells/cm² in 6-well plates. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Bio-Techne), 10,000 I.U./mL penicillin, 10,000 µg/mL streptomycin, 4.5 g/L glucose, 2 mM glutamine, 110 mg/L sodium pyruvate, and 50 µg/mL sodium L-ascorbate (Sigma-Aldrich). Control cultures were maintained in identical media lacking sodium L-ascorbate. Media were changed every other day. Plates were incubated at 37 °C in 20% O_2 and 5% CO_2 overnight for acclimatization, then transferred to normoxic (20% O_2), intermediate (5% O_2), or hypoxic (3% O_2) conditions for 72 hours prior to metabolic analysis by targeted TCA cycle liquid chromatography–mass spectrometry (LC-MS). Metabolomics**:** Cells were harvested, and metabolites were extracted using 100% methanol with four freeze–vortex cycles at –80 °C, followed by overnight incubation at –80 °C. Lysates were centrifuged at 4,000 rpm for 10 minutes, and supernatants containing extracted metabolites were dried using a SpeedVac concentrator (Eppendorf, Hamburg, Germany) at 30 °C. The resulting dried metabolite pellet was reconstituted in a 50:50 (v/v) solution of HPLC-grade acetonitrile and water. Aliquots were submitted for targeted TCA cycle metabolite profiling using LC-MS (Waters Synapt XS).Normoxic and Hypoxic Immunocytochemistry (ICC): Bovine chondrocytes were seeded at ~16,000 cells/cm² onto 25 mm × 25 mm Fisher brand microscope coverslips and cultured to confluence. Coverslips were placed in 60 mm × 15 mm tissue culture dishes containing complete media with 50 µg/mL sodium L-ascorbate (Sigma), with media changed every other day. Plates were incubated at 37 °C in 5% CO_2 under either 20%, 5%, or 3% O_2 for 72 hours. Control cultures were maintained in complete media lacking sodium L-ascorbate. Cells grown on coverslips were fixed in 4% paraformaldehyde in 1× phosphate-buffered saline (PBS) for 10 minutes at room temperature, followed by three 5-minute washes with 1× PBS. Cells were permeabilized using 0.1% Triton X-100 and blocked with 10% normal goat serum in PBS for 30 minutes at room temperature. Primary antibody staining was performed using rabbit polyclonal anti-collagen VI antibody (ab6588, Abcam) diluted in 0.1% Triton X-100/1% bovine serum albumin (BSA)/1× PBS for 1 hour at room temperature. After washing three times with PBS, cells were incubated with donkey anti-rabbit IgG H&L Alexa Fluor 488 (Abcam) and Vibrant™ DyeCycle™ Violet nuclear stain (Invitrogen) for 1 hour in 0.1% Triton X-100/1% BSA/PBS. Following three final PBS washes, coverslips were mounted using ProLong™ Diamond Antifade Mountant (Invitrogen). Digital images were acquired using a Leica TCS SP8 confocal microscope with Leica Application Suite Advanced Fluorescence (LAS AF) software.

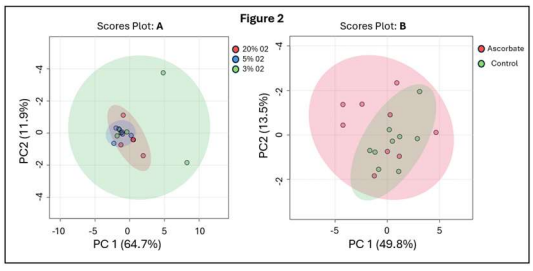
RESULTS: Cell staining found no qualitative differences between 3% and 5% hypoxic oxygen tensions, but there was increased collagen type VI deposition in hypoxic samples (3%-5%) than in normoxic samples (20%, Figure 1). Additionally, there was no visible difference between ascorbate and control groups at each oxygen tension. 13 targeted TCA metabolites were detected in the cell media and in the extracted cell pellets. There was no statistical significance observed between TCA metabolite concentrations of any experimental groups due to low sample size (n=3) and high variance.

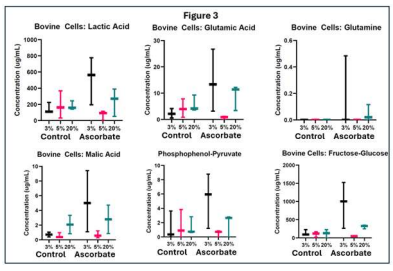
DISCUSSION: Oxygen directly influences the cell matrix output of primary bovine chondrocytes, with hypoxia increasing the production of type VI collagen. The insignificance of ascorbate on collagen VI production indicates that hypoxia, not ascorbate, is the driving factor behind the metabolic shift we see in the collagen VI production. The study is limited by the small sample size (n=3) and future studies will involve greater sample sizes as well as human OA chondrocytes.

SIGNIFICANCE/CLINICAL RELEVANCE: OA chondrocytes have shown to have a limited ability of self-repair, thought to be due to a shift or change in cell metabolism. Environmental conditions of hypoxia and ascorbate influence metabolic pathways and cell matrix remodeling which is imperative to overall tissue repair and maintaining cell homeostasis.

IMAGES AND TABLES: **Figure 1:** Hypoxia promotes collagen matrix synthesis in primary bovine chondrocytes. **Figure 2:** PCA Analysis of Bovine Cell Metabolites. **Figure 3:** Metabolite concentrations in Extracted Bovine Cell Pellet.







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