Effects of Low Oxygen Tension on the Biomechanical Properties, Composition and mRNA Expression of Engineered Nucleus Pulposus

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Introduction

The central nucleus pulposus (NP) is implicated in the initiation of disc degeneration: decreasing NP proteoglycan content impairs the ability of the NP to transfer and distribute compressive loads leading to progressive structural degradation. NP tissue engineering is currently being explored as a potential therapy for disc degeneration.[1] Previous studies aimed at functional NP tissue engineering have largely been undertaken in normoxic (21% oxygen) conditions; however, due to the avascular nature of the native NP tissue, NP cells reside in a hypoxic tissue niche.[2] The objective of this study, therefore, was to investigate the effects of a physiologically appropriate low oxygen tension on the functional and biosynthetic response of NP cells using an established 3D agarose culture model.[3]

Methods

Cell Isolation and Culture: NP cells were isolated from adult bovine caudal discs and expanded in monolayer. Passage 2 cells were suspended in 2% agarose at 20x106/ml. Constructs 4 mm diam. x 2.25 mm thick were cultured for 2 weeks in chemically defined media with (CM+) or without (CM−) 10 ng/ml TGF-β3, in either 21% oxygen or 2% oxygen in an environmental workstation (HypOxygen, Frederick, MD).

Histology: Samples (n=3) were processed into paraffin and sections were stained with alcian blue or picosirius red to evaluate GAG and collagen deposition.

Mechanical Testing: Constructs (n=5) were tested in confined compression as described previously [3]. Constructs were subjected to a 0.02 N preload for 500 sec, followed by a stress relaxation test of 10% strain applied at 0.05%/sec followed by relaxation to equilibrium for 10 min.Aggregate modulus was calculated from the equilibrium stress/applied strain.

Biochemical Composition: After mechanical testing, constructs (n=5) were digested in papain, and GAG content determined using the DMMB assay and normalized to wet weight. DNA content was determined using the PicoGreen assay.

mRNA Levels: RNA was isolated from the constructs (n=3), and quantitative PCR performed to determine mRNA levels of matrix proteins: aggrecan, collagen I and collagen II; the transcription factor SOX9; TGF signaling genes TGF-β1, TGF-βR1 and TGF-βR2; and cell stress markers inducible nitrix oxide sythase (INOS), p53 and Bcl-2-associated X protein (BAX), with expression normalized to GAPDH and presented as a ratio to initial (day 0 of culture).

Cell Viability: Viability and cell number was assessed using the Live/Dead® staining kit (n=3). Cells from 10X fluorescent images from three regions on each sample were analyzed using a custom MATLAB program.[4]

Statistics: Effects of oxygen tension (21% or 2%) and media (CM+ or CM−) for each outcome measure were established using 2-way ANOVAs with Bonferroni post hoc tests ($p<0.05$).

Results

After 2 weeks of culture NP cells deposited both GAG and collagen in a uniform manner throughout the construct (Fig.1). Matrix deposition was more robust for the CM+ groups. As expected, modulus and GAG content were both significantly higher for CM+ than CM− for both oxemic states (Fig 2). Constructs cultured in CM− had greater modulus (not significant) in 2% O2 than in 21% O2; however, for constructs cultured in CM+, the 21% O2 group had a significantly higher modulus (Fig. 2A). These same trends were reflected in the GAG content (Fig.2B). Neither the media formulation nor oxemic state had a significant effect on cell proliferation or cell viability. mRNA levels (Fig. 3) of BAX, p53, INOS,TGF-β1,TGF-βR1,TGF-βR2, collagen I, and SOX9 for constructs cultured in 2% O2 were lower than those cultured in 21% O2 for both media conditions. In CM−, mRNA levels for aggrecan and collagen II were significantly lower and higher, respectively, in 2% O2 than 21% O2, and not significantly different between 2% and 21% O2 in CM+ (Fig 3).

Discussion

In this study we examined the effects of low oxygen tension on the biomechanical

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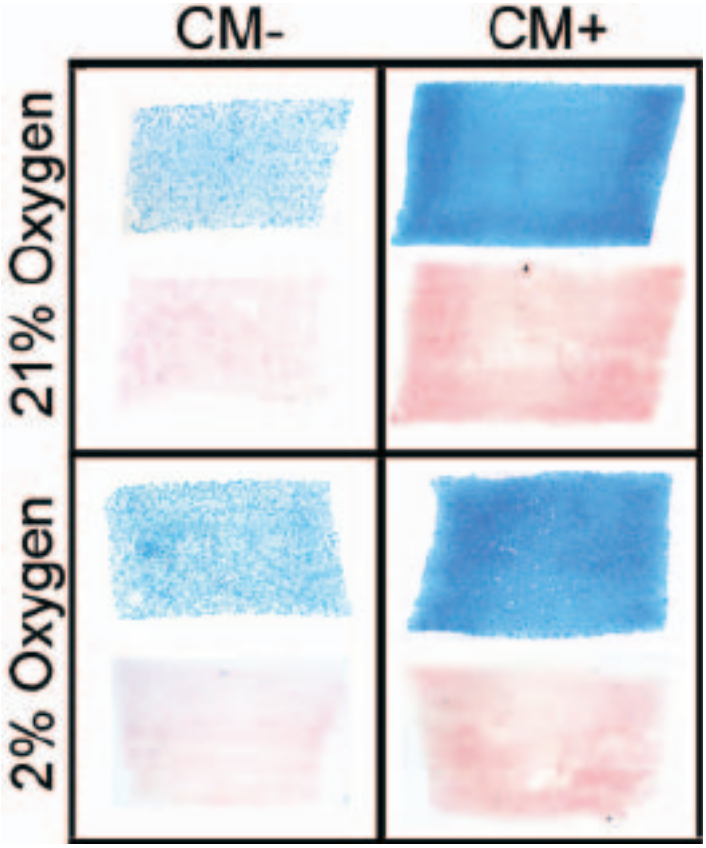

Figure 1. GAG (blue) and collagen (red) deposition was similar for both 2% and 21% O2, and in both instances more robust for CM+ than CM−.

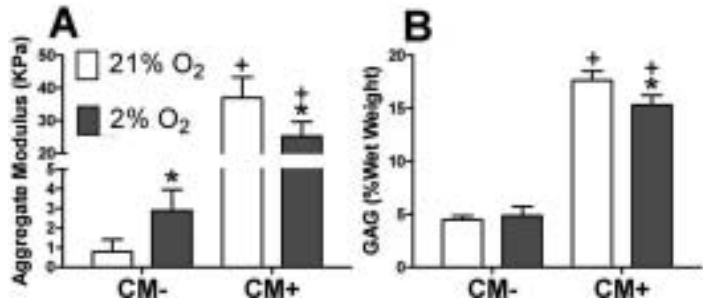

Figure 2A-B. A. Aggregate modulus. B. GAG content. *p<0.05 vs 21% O2; +p<0.05 vs CM−.

properties, biochemical composition, and mRNA expression of engineered nucleus pulposus constructs. Our results suggest that low oxygen tension attenuates the biosynthetic response of NP cells in the presence of TGF-β3 (CM+), potentially due to reduced expression of TGF receptors. This has important implications for NP tissue engineering and growth factor-based therapies, as it suggests that under physiologically appropriate oxemic conditions, NP cells are less responsive to anabolic stimulation. Importantly our results demonstrate

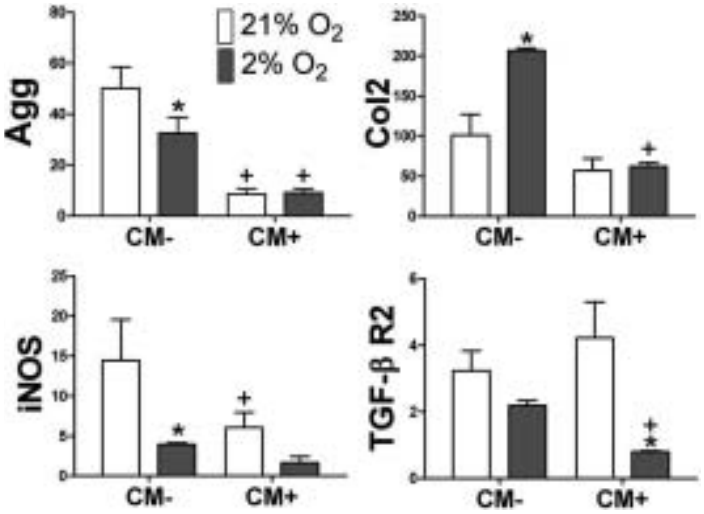

Figure 3. mRNA expression levels for aggrecan, collagen II, INOS and TGF-βR2, normalized to GAPDH and presented as ratio to day 0. *p<0.05 vs 21% O2; +p<0.05 vs CM−.

that low oxygen tension does not have a negative effect on NP cell viability, nor increase cell stress as indicated by markers of apoptosis and autophagy. Ongoing work will further examine the associated molecular pathways and examine the effect of low oxygen tension on other important anabolic and catabolic mediators of disc development and function.

Significance

NP tissue engineering shows promise as a potential therapy for disc degeneration. The work presented in this study furthers our understanding of how low oxygen tension affects the functional biosynthetic properties of NP cells to aid the development of more effective NP therapeutics.

Acknowledgements

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