Preconditioning Stem Cells to Maximize Regenerative Potential in the Challenging Microenvironment of the Intervertebral Disc

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Introduction

Nearly 85% of adults will experience low back pain in their lifetime. Disc degeneration, an inflammatory cascade resulting in structural and mechanical failure, is strongly implicated as a cause. Current therapies, both palliative and surgical, do not restore disc structure and mechanical function[1]. Current research is focused on the use of stem cell-based therapies for disc regeneration. Previous studies have demonstrated that mesenchymal stem cells (MSCs) are capable of undergoing differentiation to a nucleus pulposus (NP)-like phenotype following implantation into prototype hydrogels[2]. However, it is likely that the specialized environment of low oxygen and nutrient supply in the NP will profoundly affect MSC survival and growth. To address this challenge, we sought to develop a method by which MSCs could be "primed" prior to implantation to maximize survival and extracellular matrix biosynthesis in vivo. Specifically, we evaluated MSC matrix production and proliferation in high-density, low oxygen pellet cultures following monolayer preconditioning in atmospheric (21%) and physiological low (2%) oxygen environments, and with or without TGF-β3 supplementation.

Methods

Cell Isolation and Preconditioning: MSCs from juvenile bovine hind legs were isolated and expanded to confluence in basal media (high glucose DMEM, 10% FBS, and 1% PSF) at atmospheric oxygen. Cells were then trypsinized and expanded through one additional passage in each of the six conditions: 21% O_2 ± TGF-β3 (10 ng/ml), 2% O_2 ± TGF-β3 and Late Addition (LA) of TGF-β3 (added 1 day prior to pelleting) in both 21% and 2% O_2. Further, to investigate effects of preconditioning on proliferation during expansion, 5,000 cells/well were also plated into 6-well plates using four of the previously described conditions (excluding LA 21% and 2% O_2), and the number of live cells were quantified using the MTT assay at time points up to 7 days.

Pellet Culture: At 80% confluency, cells from each preconditioning group were pelleted at a density of 200,000 cells/well in 96-well plates and cultured in chemically defined media with (CM+) or without (CM-) TGF-β3 (10 ng/ml) under three different conditions: High Glucose CM+, High Glucose CM-, and Low Glucose CM-. All groups were cultured for 14 days in 2% O_2. Glycosaminoglycan (GAG, DMMB assay) and DNA content (PicoGreen assay) per pellet were determined following Proteinase K digestion ($n = 3$ per condition). Statistical significance between preconditioning methods was established by one-way ANOVA with Bonferonni post hoc tests ($p < 0.05$).

Results

Effects of Preconditioning on Proliferation during Expansion: Cells expanded in 2% O_2 without TGF-β3 exhibited significantly, though only moderately higher rates of proliferation 1 and 3 days after plating compared to both 21% O_2 groups (Figure 1). Cells expanded in 2% O_2 with TGF-β3 exhibited significantly higher rates of proliferation 3 and 5 days after plating compared to both 21% O_2 groups. By 7 days, cells approached confluence for all groups and differences were no longer apparent.

Effects of Preconditioning on Pellet Composition: GAG content of pellets cultured in Low Glucose CM- (representative of the challenging in vivo conditions of the NP) was significantly greater for cells preconditioned in 2% O_2 with continuous TGF-β3 compared to all other conditions except 2% O_2 LA (Fig. 2). For pellets cultured in High Glucose CM−, cells preconditioned in 2% O_2 (with or without TGF-β3) resulted in GAG content that was significantly greater than all 21% preconditioning groups. For pellets cultured in High Glucose CM+, cells that were preconditioned in 2% O_2 with LA TGF-β3 had significantly greater GAG content than cells preconditioned in 21% O_2 with continuous TGF-β3, but there were no other differences. While there was lower GAG content in all 21% O_2 preconditioning groups in High Glucose CM pellet media compared to CM+ as expected, interestingly those preconditioned in 2% O_2 had similar GAG content as found in the High Glucose CM+ pellet groups. DNA content in the Low Glucose CM− condition was significantly higher in cells preconditioned in 2% O_2 LA TGF-β3 compared to all other expansion

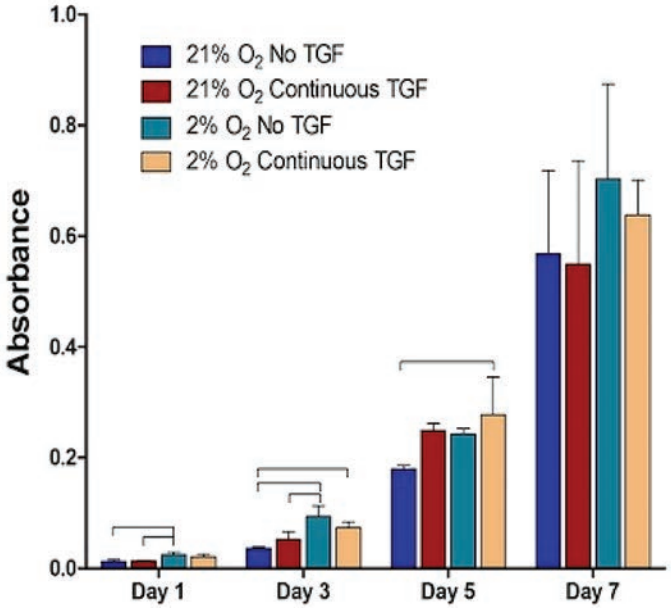

Figure 1. Proliferation during expansion [MTT Assay]. Hypoxic (2%) environment significantly enhanced cell growth until confluency (n = 3)

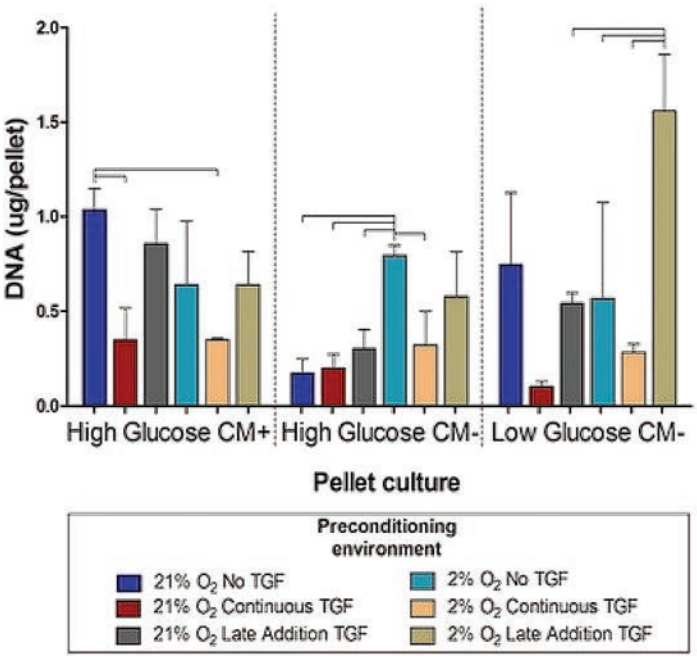

Figure 3. DNA content in the Low Glucose CM− pellet culture was significantly enhanced in cells preconditioned in 2% O_2 Late Addition TGF (n = 3). bar = $p < 0.05$

conditions except 21% O_2 without TGF-β3 (Figure 3). Pellets cultured in High Glucose CM− had significantly higher DNA for cells preconditioned in 2% O_2 without TGF-β3 compared to those preconditioned in 21% O_2 (all variations) and those preconditioned in 2% O_2 with continuous TGF-β3. For pellets cultured in High Glucose CM+, cells preconditioned in 21% O_2 without TGF-β3 had higher DNA content compared to cells preconditioned in 21% or 2% O_2 with continuous TGF-β3.

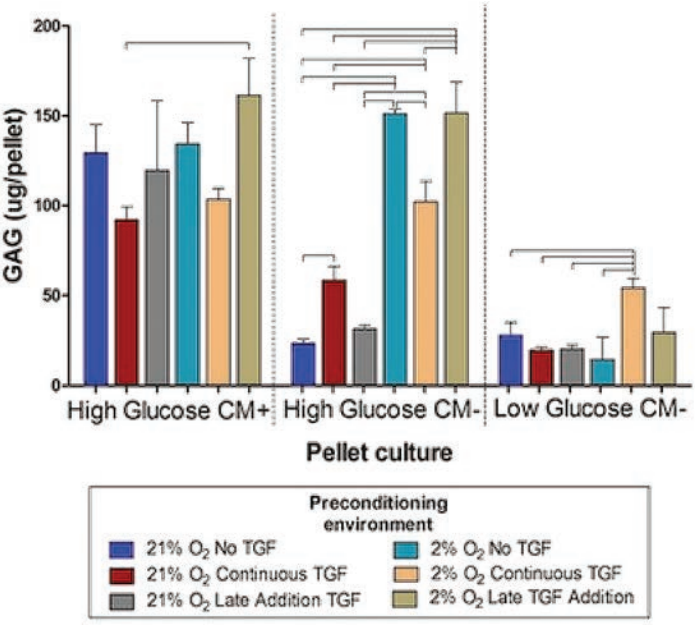

Figure 2. GAG content in the Low Glucose CM− pellet Culture was signivicantly enhanced in cells preconditioned in 2% O_2 with continuous TGF. Pellets preconditioned in 2% O_2 had a significantly greater GAG content than 21% O_2 when pelleted in High Glucose CM− (n = 3). bar = $p < 0.05$

Discussion

In this study, we investigated the efficacy of monolayer preconditioning using low oxygen and TGF-β3 in order to improve the biosynthetic performance of MSCs in the challenging in vivo biochemical environment of the NP. Low glucose media and 2% O_2 were used to simulate a nutrient- and oxygen- deficient environment. Increased proliferation during monolayer expansion in 2% O_2 is consistent with recent work [3]. Cells preconditioned in 2% O_2 with continuous TGF-β3 supplementation resulted in the greatest GAG content in the Low Glucose CM− environment, which is consistent with previous findings involving MSCs cultured on 3-D scaffolds. By contrast, DNA production for pellets in the Low Glucose CM− media was greater in cells preconditioned in 2% O_2 with late addition of TGF-β3. Since DNA content is indicative of cell proliferation, 2% O_2 LA TGF-β3 may be the best preconditioning environment to promote cell growth after implantation in the disc. While increased cell proliferation may enhance regeneration potential, this must be balanced against the limited nutritional supply in the in vivo space. Interestingly, preconditioning in 2% O_2 resulted in significantly improved GAG synthesis for pellets cultured in High Glucose CM−, almost equivalent to TGF-β3 supplementation. This suggests that glucose supplementation combined with stem cell delivery may be an alternative to growth factor therapy. In conclusion, this study suggests that monolayer preconditioning in 2% O_2 with TGF-β3 may lead to enhanced proliferation rates during monolayer expansion and enhanced GAG production following delivery to the in vivo space.

Significance

Stem cells represent a novel therapeutic approach for disc degeneration and associated low back pain. This study investigates the conditions required to optimize these cells for successful growth and survival in the challenging in vivo environment, and is a critical step toward pre-clinical studies.

Acknowledgements

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