Biofabrication of Ligament Constructs by Physioxic Preconditioning and Maturation of hBM-MSC-laden Dense Aligned Collagen Gels

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INTRODUCTION: Ligament injuries are among the most prevalent musculoskeletal pathologies, characterized by limited intrinsic healing capacity due to hypovascularization, hypocellularity, and suboptimal biomechanical environments [1]. The current gold standard of ligament reconstruction surgery suffers high rates of graft failure, complications, and frequent need for revision [1]. Implantable bioengineered ligament grafts offer a promising alternative solution. Tissue-engineered ligament constructs seeded with cells show greater regenerative potential compared to cell-free scaffolds, but the optimal combination of cell source, scaffold architecture, and culture microenvironment for engineering functional ligament grafts remains an unresolved challenge. Human bone marrow-derived mesenchymal stem cells (hBM-MSCs) have been shown to exhibit a promising ligamentogenic potential. Moreover, dense aligned collagen gels mimic the native tissue architecture and composition and can be tuned to optimize their mechanical properties. One critical limitation is the sizeable mismatch between standard *in vitro* culture conditions and the physiological environment of ligament tissues, which are inherently hypovascularized and characterized by relative hypoxia and reduced nutrient supply. Therefore, reproducing the physiological oxygen (O2) and glucose concentrations experienced in vivo within joint spaces is critical for optimizing the biofabrication of optimal bioengineered ligament grafts. This study aims to develop bioengineered ligament grafts by maturing hBM-MSC-seeded dense aligned collagen gels (DACGs) under near physiological oxygen tensions and glucose concentrations, mimicking the intraarticular environment, and to evaluate the impact of these culture conditions on graft development and functionality.

METHODS: hBM-MSCs (RoosterBio, n=3, male and female) were differentiated and primed for 10 days at 37℃ in ligamentogenic media (1.0 g/L Glucose DMEM supplemented with 10% Fetal Bovine Serum, 1% Antibiotic/Antimycotic, 5ng/mL TGF-β, 1ng/mL bFGF, and 50µg/mL L-ascorbic acid) under three different oxygen tensions: atmospheric and standard cell culture oxygen tension (20% O_2), intravenous (*IV*, 5% O_2), intraarticular (*IA*, 2% O_2). Cell-seeded culture plates were placed in a standard incubator with 5% CO2 at 37℃ for atmospheric oxygen tension. For IV and IA, hypoxia incubator chambers (StemCell Technologies) were sealed, flushed with 5% O_2 and 2% O_2 gas premixes, respectively, and placed inside an incubator at 37°C for the duration of the experiment. After the 10-day priming preconditioning period, the cells were trypsinized and seeded in highly hydrated collagen gels. The gels were then compacted and densified using the gel aspiration-ejection technique using an automated biofrabrication platform (Gesim Bioscaffolder, Gesim Robotics, Germany). Post-bioprinting, the gels were matured for 21 days in a custom tethering device under 20% O_2, 5% O_2, or 2% O_2. Cell viability and distribution were assessed using Live/Dead™ staining, and metabolic activity was examined using Alamar Blue™ assays. Scaffold structure and collagen fiber alignment were observed and characterized using scanning electron microscopy (SEM). Mechanical functionality was evaluated by uniaxial tensile testing to failure. All datasets were tested for normality, and appropriate statistical tests were performed accordingly.

RESULTS SECTION: Live/Dead assays indicate high cell viability in the constructs across culture conditions, both at day 1 and day 21 in culture, while gels on day 21 exhibited more cell-spreading and alignment along the long axis of the gel (Figure 1A). hBM-MSC-seeded dense aligned collagen gels exhibited contraction over 21 days, with the greatest contraction under 20% O_2, moderate contraction at 5% O_2, and minimal contraction at 2% O_2 (Figure 1A). SEM imaging revealed high collagen fiber alignment across all three culture conditions, with the 2% O_2 group showing features of matrix deposition and remodeling over the collagen scaffold (Figure A). Preliminary uniaxial tensile testing results indicate significant improvements in mechanical performance under hypoxic conditions, with 2% O_2 constructs showing the highest ultimate tensile stress and apparent modulus (Figures 1B and 1C), followed by 5% O_2, and with 20% O_2 constructs being significantly weaker.

DISCUSSION: The reduced contraction observed at 5% and 2% O_2 suggests that hypoxia limits excessive cytoskeletal tension and matrix reorganization, helping maintain construct dimensions during maturation. Sustained cell viability and alignment under all oxygen levels indicate that the DACG architecture provides a supportive microenvironment; however, only hypoxic groups translated this organization into improved mechanical strength. The superior tensile properties at 2% O_2 imply enhanced matrix deposition, crosslinking, and/or collagen fiber compaction under more physiologically relevant oxygen levels. In contrast, constructs at 20% O_2 showed lower strength and modulus, consistent with higher contraction and potentially less organized or less dense matrix assembly. Overall, these findings highlight the critical role of oxygen tension in regulating both structural stability and functional mechanical maturation during ligament engineering. Future work will include varied histological studies to investigate the ligamentogenic cellular phenotype and matrix deposition in the matured cell-laden dense aligned collagen gels.

SIGNIFICANCE/CLINICAL RELEVANCE: This study addresses the critical challenge of engineering cell-based ligament grafts by integrating physiologically relevant O_2 and glucose conditions throughout construct maturation. Insights gained have the potential to improve the preclinical efficacy of bioengineered ligament grafts, contributing to better clinical outcomes in reconstructive surgery and translational tissue engineering.

REFERENCES: [1] Sanders et al. 2016, PMID: 26920430

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IMAGES AND TABLES:

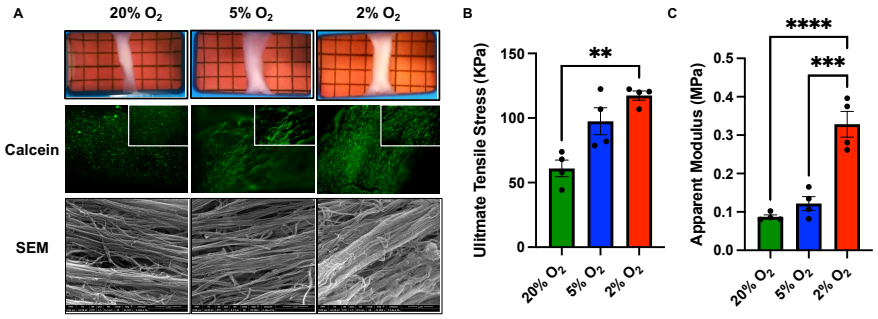

Figure 1: *Microscopic and mechanical characterization of hBM-MSC-laden DACGs. Data= Mean ± SEM, *p-value<0.05, **p-value<0.01, ###p-value<0.001.*