Chondrogenic Preconditioning of Equine Bone Marrow-Derived Mesenchymal Stem Cells in Self-Assembling Peptide Hydrogel

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Disclosures: J.D. Kisiday: None. S. Tangtrongsup: None.

Introduction: Undifferentiated mesenchymal stem cells (MSCs) that have received no chondrogenic cues prior to implantation have not proven capable of healing cartilage defects. It has been postulated that the induction of MSC chondrogenesis prior to implantation, hereafter referred to as ‘chondrogenic preconditioning’, is necessary to result in a stable, chondrocyte-like phenotype that secretes and maintains cartilage-like repair tissue in vivo [1]. In this study, we evaluated protocols for chondrogenic preconditioning in vitro by seeding undifferentiated MSCs into self-assembling peptide hydrogel, and then culturing in chondrogenic medium containing dexamethasone (Dex) and transforming growth factor beta (TGFβ) [2] for up to three days. Following preconditioning culture, commitment to chondrogenesis was evaluated by isolating the MSCs from the peptide hydrogel, seeding into agarose hydrogel, and culturing in the absence of TGFβ.

Methods: Equine MSCs were isolated from bone marrow aspirates taken from 5 young adult horses and culture-expanded through ~8 population doublings in αMEM + 10% fetal bovine serum and 2 ng/ml FGF2. After expansion, MSCs were encapsulated in the self-assembling peptide hydrogel KLD12 using previous established techniques [3] at 10x106 cells/ml and cultured in chondrogenic medium (high-glucose DMEM, 1% ITS+, 37.5µg/ml ascorbate-2-phosphate, 100 nM dexamethasone, 10 ng/ml TGF-β1). After 1, 2, or 3 days of culture, MSCs were released from the peptide by disrupted the hydrogel with micropipetting. The cell/peptide suspension was treated with 0.25% trypsin/EDTA for 5 minutes, and then expansion medium plus 0.1% collagenase for 45 minutes. The cell-peptide suspension was incubated for 30 minutes in tissue culture flasks to allow the released cells to adhere, and the adherent cell population was collected as an individual cell suspension and seeded into 1.5% agarose at 10x106 cells/ml. MSCs from preconditioning culture were cultured in chondrogenic medium without TGFβ. Agarose control cultures were created in a similar manner using undifferentiated MSC at the start of priming cultures and maintained in TGFβ-free (abbreviated as TGFβ-) or 10 ng/ml TGFβ medium (abbreviated as TGFβ+). All cultures were evaluated for GAG and hydroxyproline accumulation, and type II collagen immunohistochemistry (IHC) 17 days after the start of priming culture. GAG and hydroxyproline accumulation was analyzed using a mixed model analysis of variance using the donor animal as the random effect (n=5) with individual comparisons using least square means procedure. p-values< 0.05 were considered significant.

Results: Approximately half the cells seeded into the priming were recovered into an individual cell suspension. Preliminary testing of control samples after three days of culture indicated minimal GAG accumulation; therefore, extracellular matrix accumulation on day 17 was directly compared among all groups (Fig. 1). In control cultures, GAG and hydroxyproline accumulation in TGFβ+ cultures was ~15-fold higher than TGFβ- cultures (p<0.005). GAG: 1 day of KLD preconditioning did not significantly stimulate GAG accumulation over TGFβ- cultures (p=0.76). GAG accumulation following 2 days of KLD preconditioning was ~12-fold higher than TGFβ- cultures (p<0.01), and not significantly different than TGFβ+ cultures (p=0.57). Three days of KLD preconditioning resulted in the highest GAG accumulation (p<0.05), ~80% greater than that in TGFβ+ cultures. Hydroxyproline: Hydroxyproline accumulation closely mirrored GAG accumulation, with comparable magnitude of changes and the same pattern of significant differences among groups except that hydroxyproline accumulation with 2 days of KLD preconditioning was 62% of TGFβ+ cultures (p<0.05). Type II collagen IHC: Staining for type II collagen was largely absence in TGFβ- cultures (Fig. 2). In general, ECM accumulation coincided with positive type II collagen staining that was largely localized to pericellular regions. For MSCs subjected to 1 day of KLD preconditioning, the modest extracellular matrix accumulation coincided with strong type II collagen staining around a subset of the cell population, with the majority of the cells showing no type II collagen staining.

Discussion: Chondrogenic preconditioning in KLD12 peptide hydrogel for a period of days was sufficient to stimulate robust chondrogenesis without additional exposure to TGFβ. Further, these data are the first to demonstrate that 3 days of preconditioning is capable of stimulating subsequent extracellular matrix synthesis without TGFβ to a greater degree than the gold standard of multiweek culture in medium containing TGFβ. These results differed from our previous work in which preconditioning conducted in scaffold-free suspension cultures did not stimulate GAG accumulation over TGFβ- cultures [4]. Additional work is needed to explore mechanisms by which KLD12 preconditioning induces rapid and robust chondrogenesis. One possibility is that the 50% recovery rate from preconditioning culture selects for MSCs that are especially chondrogenic,
although a comparable recovery rate was also noted from our previous work with suspension preconditioning [4]. A second area of future investigation is temporal heterogeneity in which MSCs commit to chondrogenesis, which is suggested by IHC for MSCs subjected to 1 day of KLD preconditioning in which type II staining is either strong or absent across the cell population. Future studies will also include a more detailed exploration of whether preconditioning results in a phenotype which resembles that of articular chondrocytes.

**Significance:** Chondrogenic preconditioning represents a next-step in characterizing the ability of MSCs to resurface cartilage defects. We have chosen an approach that emphasizes ease of clinical translation, resulting in a suspension of chondrogenic MSCs that may be cryopreserved, and/or implanted using minimally-invasive, injectable techniques.

**Acknowledgments:**


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**Fig. 1** — GAG and hydroxyproline accumulation

**Fig. 2** — Type II collagen IHC

**ORS 2014 Annual Meeting**

**Poster No:** 0404