Effects of Micronized Cartilage Matrix on Cartilage Repair in Osteochondral Lesions of the Talus

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ABSTRACT INTRODUCTION: A promising new technique in the treatment of osteochondral lesions of the talus (OLT) involves the use of an acellular micronized cartilage matrix (MCM), BioCartilage® to fill the lesions. The micronized cartilage matrix is thought to improve the production of hyaline-like cartilage by resident cells in a cartilage defect. In clinical use, the micronized cartilage powder is reconstituted with about 0.8ml of plasma to a putty-like consistency, which is then applied to the debrided lesion and sealed in place with fibrin glue. Marrow cells from microfracture are thought to repopulate the micronized cartilage and ultimately form hyaline-like cartilage. However, microfracture is not always performed, and the micronized cartilage itself does not contain viable cells. Furthermore, the effect of the BioCartilage on bone marrow cells remains untested. Here we hypothesized that adding bone-marrow derived stem cells to the BioCartilage would result in the chondrogenic differentiation of the stem cells. We designed an in-vitro model to mimic the clinical situation, and test the hypothesis that the combination of human bone marrow derived mesenchymal stem cells (MSCs) and micronized cartilage matrix would produce hyaline-like cartilage construct in-vitro. Verifying that this combination of matrix and cells results in cartilage regeneration would provide a reliable, one-step treatment for osteochondral lesions in the talus.

METHODS: **Cells:** Human bone marrow-derived stem cells were obtained from consented patients, with IRB approval, and expanded in monolayer culture using standard protocols, to a maximum passage of 4. **Viability:** viability was measured using Live/Dead cell viability assays (Thermofisher), and imaged on a Nikon TE2000 inverted fluorescent microscope. To establish initial viability, stem cells were mixed with BioCartilage at the same ratio recommended by the manufacturer for reconstitution of the desiccated product with plasma; namely 0.8ml to 1cc of BioCartilage. **Chondrogenic Culture:** To mimic the clinical use of micronized cartilage in an osteochondral lesion of the talus (Figure 1A), we custom-manufactured a polysulfone device to create four 6mm diameter 3mm deep indentations in agarose, in each well of standard 6-well culture plates (Figure 1B, C). In each well, we placed cells+micronized matrix to a depth of 2mm, which was "sealed" with 1mm depth of TISSEEL fibrin glue as is done clinically. Each well contained approximately 200,000 viable cells. Control groups had either no cells, or no MCM. 6 ml of chondrogenic media (αMEM with dexamethasone (0.1 μM), TGFβ (10 ng/ml), L-proline, BMP2 (50 ng/ml) and ascorbate-2-phosphate (50 ng/ml)) was added to a height of 6 mm on top of each well, and replenished every other day. **Analysis:** At the end of 3 weeks, cartilage constructs were extracted and divided into 3 sections, each directed to a separated analysis (Figure 1D). A 300um slice of each defect was set aside for viability, while the two larger sections were dedicated to Histology and Gene Expression analysis, respectively. Gene Expression Analysis: Total RNA was isolated from each defect according to the QIAGEN miRNeasy kits according to protocol RT-PCR was subsequently used to evaluate mRNA expression levels of the following chondrocyte-specific genes: Collagen Type II, Aggrecan, Link Protease, Collagen Type I and Versican. Histology: Upon harvesting, tissue samples were immersed in 4mL 4% Paraformaldehyde for 24-40 hours at room temperature, then paraffin embedded, sectioned, and stained with H&E or Safranin-O/Fastgreen. Experiments were performed with 4 technical replicates, and repeated at least 3 times. Statistical comparisons were made to control groups using ANOVA with Dunnett's test, in JMP Pro 12.1 software.

RESULTS: We found that stem cells were almost immediately killed when added directly to the dry micronized cartilage powder. Rehydrating the micronized cartilage prior to addition of cells was required to maintain the viability of the added stem cells, with no statistically significant difference between rehydration with serum or saline (Figure 2A). After 3 weeks of culture in chondrogenic media, we observed that the combination of stem cells and micronized cartilage produced a cohesive structures that were easily handled, suggesting chondrogenic differentiation of the stem cells (Figure 3A). Without the micronized matrix, the stem cells did not form viable constructs (not shown). In constructs that contained both cells and micronized cartilage, the 3-week cell viability was over 98%, with no dead cells visible in many constructs.

DISCUSSION: Our study demonstrates that the micronized cartilage matrix is a suitable scaffold for the chondrogenic differentiation of bone marrow-derived stem cells, given that the matrix is first rehydrated before adding cells. Technical observations include that the MCM itself generated a "dead cell" signal initially, therefore the normalized total number of live cells in each condition was used for statistical comparisons (Figure 2A). After 3 weeks of culturing under chondrogenic media conditions, we observed robust cell survival with nearly 100% viability. Preliminary results suggest cartilage matrix deposition occurred surrounding the cells after 3 weeks of chondrogenic culture.

SIGNIFICANCE: The findings of this study indicate that micronized cartilage matrix (BioCartilage®) has supports cartilage differentiation of MSC's *in vitro* as long as it is first reconstituted with either saline (DPBS) or FBS. These results are promising and provide strong evidence for the use of this product and technique in the surgical repair of OLT.

ACKNOWLEDGEMENTS: Human bone marrow derived mesenchymal stem cells were generously donated from the Institute of Regenerative Cures (IRC) at the University of California, Davis (Jan Nolta PhD and Fernando Fierro PhD). Micronized cartilage matrix, BioCartialge® was donated from Arthrex (Arthrex INC, Naples FL). Fibrin sealant was donated by TISSEEL (Baxter, Deerfield IL).

IMAGES AND TABLES:

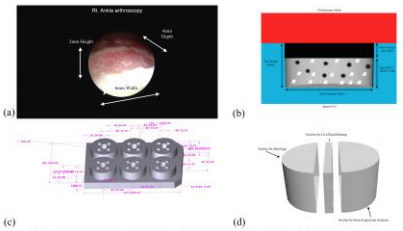

Figure 1. (a) Osteochondral lesion of the talus in vivo with implanted Biocartilage. (b) Schematic of defect replication in agarose including Mesenchymal stem cells, micronized cartilage matrix, Fibrin sealant and chondrogenic media. (c) Graphic design of custom-made polysulfone defect mold. (d) Illustration of defect harvesting technique yielding three sections.

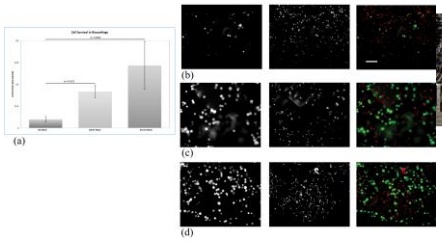

Figure 2. (a) Washing micronized cartilage matrix significantly increases cell survival. Cell number has been normalized by calculating the ratio of each viability experiment to its corresponding control group average. (b) Viability in No Wash condition demonstrating near-zero viability. (c-d) Viablity in Saline Wash (c) and Serum Wash (d) conditions results in a larger number of live cells due to decreased dessication. However, dead cell signal native to Biocartilage still skews results low. Scale bar equals 1um.

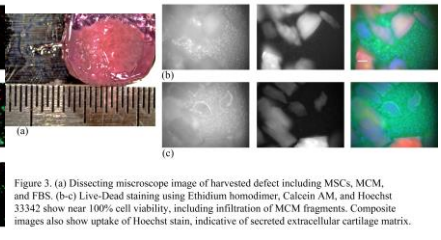

Figure 3. (a) Dissecting miscroscope image of harvested defect including MSCs, MCM, and FBS. (b-c) Live-Dead staining using Ethidium homodimer, Calcein AM, and Hoechst 33342 show near 100% cell viability, including infiltration of MCM fragments. Composite images also show uptake of Hoechst stain, indicative of secreted extracellular cartilage matrix.