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In Vitro Maturation and In Vivo Delivery of Cartilage Repair Composites Composed of Minced Cartilage in a Photopolymerizable Hyaluronic Acid Hydrogel

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Introduction

Damage to the articulating cartilages of joints causes focal lesions that are recalcitrant to repair and provoke more widespread osteoarthritic changes over time. As such, a number of surgical repair strategies have been tested, including microfracture, osteochondral grafting, and autologous chondrocyte implantation. Fragments generated by rim preparation and/or loose bodies contain viable chondrocytes and well organized cartilage matrix.[1,2,3] Indeed, in vitro studies have shown that chondrocytes can migrate out from such cartilage[4,5] and the tissue can be maintained by mechanical loading.[6] More recently, a novel treatment option has been described wherein allogeneic (juvenile) cartilage fragments are secured within defects using fibrin glue as a vehicle.[7,8,9] Likewise, several studies in goats[4,10] and rabbits[11] have evaluated the possibility of using autologous cartilage fragments in a one step re-insertion repair of the lesion site. The purpose of this work was to determine whether a photo-crosslinkable hydrogel based on methacrylated hyaluronic acid (HA), which has been shown to enhance chondrogenic differentiation of MSCs and enhance cartilage like tissue formation,[12,13] could be used as a vehicle for the delivery of cartilage fragments. Further, we developed novel composites including both HA hydrogel, cartilage fragments, mesenchymal stem cells, and delivery microspheres for the pro-chondrogenic factor TGF beta. These composites were evaluated in vitro and in vivo.

Methods

Fabrication of HA/Minced Cartilage/MSC Constructs: Cartilage was harvested from the trochlear groove of juvenile bovine knee joints, sliced to 0.3mm thickness and minced into cubes (average fragment size 0.3x1mm). Fragments were mixed with a 1% polymerizable HA solution with or without MSCs (at 60 million cells/mL) and cast between glass plates.[12] The same process was repeated using a commercially available solution of fibrin glue. After setting, 4mm diameter constructs were punched and cultured in TGF-beta containing chemically defined medium. Constructs were harvested after 3 or 4 weeks and assessed for viability (Live/Dead staining) and histology (H&E, Alcian blue and Picrosirius Red staining of paraffin sections).

In Vivo Assessment: To test the capacity of HA/minced cartilage composites in vivo, two juvenile (6 months, male, 32.9-37.7kg) Yucatan minipigs were used. Four 4 mm diameter full thickness chondral defects were created in each trochlear groove and 25% of the harvested cartilage volume was minced and used for treatment of an adjacent defect (see Figure 2 a-d). Defects were treated by minced cartilage with HA (HAmC, n = 4), minced cartilage with HA and TGF-beta loaded microspheres (HAmC + MS, n = 4), HA only (HA, n = 4) or left as untreated chondral defects (CD, n = 4). Microspheres were produced as described.[14] After 6 weeks pigs were euthanized. Gross view images were acquired and samples were mechanically evaluated by indentation testing at the center of the defect and at the visually 'best' location using a spherical indenter (2mm diameter) and a 3 step stress-relaxation protocol. Samples then underwent micro-CT analysis (55kVp and 145μA) in 4 distinct regions of interest adjacent to the bone/cartilage interface. Analyses for bone volume per total volume (BV/TV) were evaluated. After incubation with

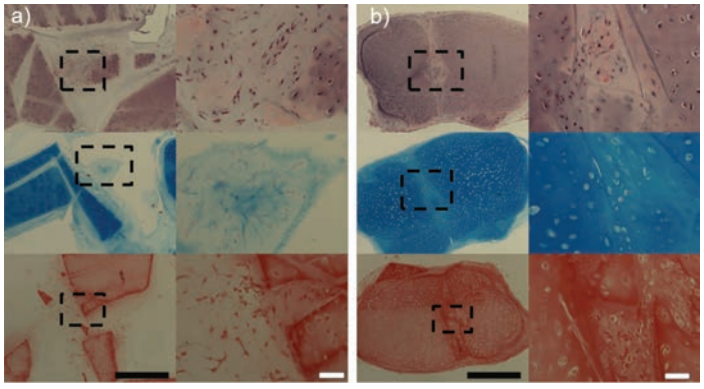

Figure 1. H&E, Alcian blue and picrosirius red staining for (A) HA embedded and (B) fibrin embedded minced cartilage fragments after 3 weeks of culture in TGF-beta containing medium (black scale bar = 500μm, white scale bar = 100μm).

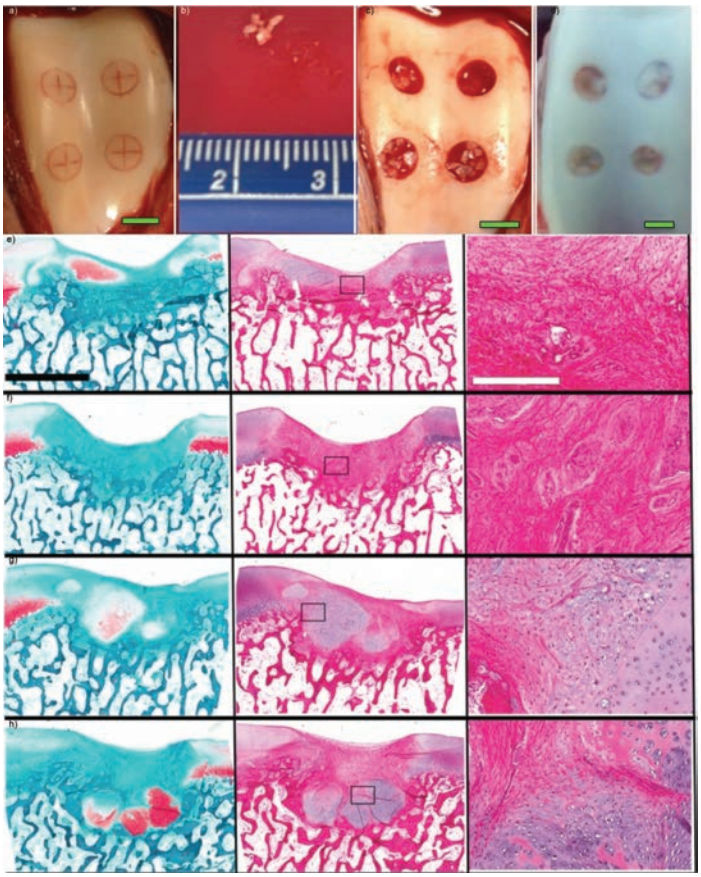

Figure 2. Intraoperative preparation of defects with (A) quartering of explants before explantation, (B) mincing of the explanted cartilage, (C) replantation of minced fragments (two bottom defects) filling 25% of the defect volume (before addition of HA) and (D) after six weeks in vivo (green scale bar = 4mm). 2e)-h) Safranin O/Fast Green (left column) and H&E (middle column) staining for representative samples from each group: (E) CD (F) HA g) HAmC h) HAmC+MS (black scale bar = 2mm). 10× magnification of H&E stains (right column) showing chondrocytes exiting from the replanted cartilage fragments (*) at 6 weeks postoperatively (white scale bar = 300μm).

Lugol's solution for 48 hours, a second scan followed to enable analysis of defect fill. Samples were decalcified, paraffin embedded, cut into 6μm sections and stained with Safranin-O/Fast-Green and H&E. One adjacent osteochondral plug per joint served as control. Statistical analysis was carried out by ANOVA with Bonferroni's post-hoc tests, as all samples were normally distributed and had equal variances. A regression analysis was performed for BV/TV and results of indentation testing in the center of the defects.

Results

Minced cartilage fragments cast in HA retained high viability, comparable to the response seen in fibrin (data not shown). Likewise, cellular outgrowth from the fragments was observed to the same extent as in fibrin (Figure 1a,b), although staining intensity was lower in HA-based composites. MSC-seeded composites showed chondrogenic differentiation of co-delivered MSCs and therefore a more homogenous distribution of cartilage specific ECM (Figure 3). The surgical model showed that the defects could be refilled (25% of the initial volume) with autologous cartilage. Histology showed that these cartilage fragments were well maintained in the defect 6 weeks postoperatively (Figure 2 e-h). Biomechanical testing revealed no significant differences amongst treatment groups, and a lower moduli compared to adjacent cartilage controls ($p < 0.001$, data not shown). Indentation testing in the visually best looking regions resulted in better mechanical properties than in the defect centers, with a small trend towards higher biomechanical properties for samples treated with minced cartilage. Micro-CT evaluation showed no significant difference in bone loss between treatment groups. Regression analysis between BV/TV and indentation modulus did not show a strong correlation ($R^2 = 0.014$). Histological sections showed the minced cartilage fragments in place, with more intense staining in the group containing TGF-beta loaded microspheres. Higher resolution images showed outgrowth of chondrocytes from the minced cartilage fragments.

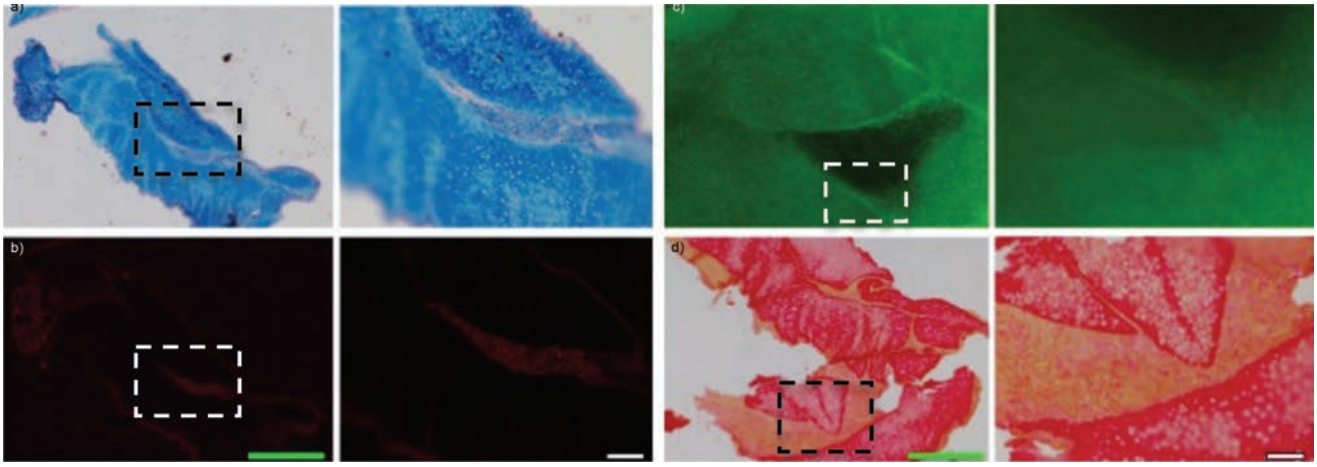

Figure 3. Composites containing minced cartilage fragments and MSCs after 4 weeks of in vitro culture. a) Alcian blue and b) Fluorescence imaging of labeled MSCs. c) Live staining and d) Picrosirius red staining at this same time point (green scale bars = 500μm, white scale bar = 100μm).

Discussion

In this study we evaluated a novel cartilage repair composite based on minced cartilage embedded in a photopolymerizable hydrogel with co-delivered MSCs. Consistent with the literature,[4-6] we found chondrocyte outgrowth from the minced cartilage fragments using both fibrin and HA as the delivery vehicle. In the case of HA, addition of MSCs to the composites led to the more rapid accumulation of extracellular matrix between minced fragments, and a higher cell density. When these composites were tested in vivo, the HA hydrogel was able to maintain the cartilage pieces in place over six weeks. However, at this early time point, functional restoration of cartilage properties had not yet been achieved, as was evidenced by our biomechanical and histological testing. Whether this was due to the relative low volume of minced fragments in the defect (only 25% of defect volume), the lack of bone marrow stimulation, or the result of the short term time points assayed here, remains to be determined. Ongoing studies are now assessing in vivo whether an increase in cell number by the addition of MSCs, the delivery of TGF-laden microspheres, and/or longer healing periods will culminate in a more functional repair.

Significance

This study demonstrates that photo-polymerizable HA is a viable delivery vehicle for situating cartilage fragments in chondral lesions. Ongoing work will establish novel composites of MSCs, cartilage fragments, and growth factor delivery and test their capacity to promote in vivo cartilage repair.

Acknowledgements

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