Photopolymerizable Injectable Cartilage Mimetic Hydrogel for the Treatment of Focal Chondral Lesions

A Proof of Concept Study in a Rabbit Animal Model

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Background: In this study, we investigate the in vitro and in vivo chondrogenic capacity of a novel photopolymerizable cartilage mimetic hydrogel, enhanced with extracellular matrix analogs, for cartilage regeneration.

Purpose: To (1) determine whether mesenchymal stem cells (MSCs) embedded in a novel cartilage mimetic hydrogel support in vitro chondrogenesis, (2) demonstrate that the proposed hydrogel can be delivered in situ in a critical chondral defect in a rabbit model, and (3) determine whether the hydrogel with or without MSCs supports in vivo chondrogenesis in a critical chondral defect.

Study Design: Controlled laboratory study.

Methods: Rabbit bone marrow–derived MSCs were isolated, expanded, encapsulated in the hydrogel, and cultured in chondrogenic differentiation medium for 9 weeks. Compressive modulus was evaluated at day 1 and at weeks 3, 6, and 9. Chondrogenic differentiation was investigated via quantitative polymerase reaction, safranin-O staining, and immunofluorescence. In vivo, a 3 mm–wide × 2-mm-deep chondral defect was created bilaterally on the knee trochlea of 10 rabbits. Each animal had 1 defect randomly assigned to be treated with hydrogel with or without MSCs, and the contralateral knee was left untreated. Hence, each rabbit served as its own matched control. Three groups were established: group A, hydrogel (n = 5); group B, hydrogel with MSCs (n = 5); and group C, control (n = 10). Repair tissue was evaluated at 6 months after intervention.

Results: In vitro, chondrogenesis and the degradable behavior of the hydrogel by MSCs were confirmed. In vivo, the hydrogel could be delivered intraoperatively in a sterile manner. Overall, the hydrogel group had the highest scores on the modified O'Driscoll scoring system (group A, 17.4 ± 4.7; group B, 13 ± 3; group C, 16.7 ± 2.9) (P = .11) and showed higher safranin-O staining (group A, 49.4% ± 20%; group B, 25.8% ± 16.4%; group C, 36.9% ± 25.2%) (P = .27), although significance was not detected for either parameter.

Conclusion: This study provides the first evidence of the ability to photopolymerize this novel hydrogel in situ and assess its ability to provide chondrogenic cues for cartilage repair in a small animal model. In vitro chondrogenesis was evident when MSCs were encapsulated in the hydrogel.

Clinical Relevance: Cartilage mimetic hydrogel may offer a tissue engineering approach for the treatment of osteochondral lesions.

Keywords: cartilage lesions; tissue engineering; stem cells; hydrogel

Osteoarthritis is a serious clinical and economic burden for the orthopaedic community and the public health system.[18] To date, surgical restoration techniques used for cartilage repair do not regenerate articular hyaline cartilage. Although patients' symptoms improve after surgery, this is temporary and a high percentage of patients progress to failure after the procedure.[13] Tissue engineering approaches represent a promising therapeutic tool for the treatment of articular cartilage defects. These approaches provide biomaterial scaffolds as a template on which cells can be situated to locally produce a new matrix that replicates the biomechanical properties of the surrounding cartilage.[14] Among these approaches, injectable hydrogels have attracted much attention because of their performance characteristics. Hydrogels can form 3-dimensional networks that can be fine-tuned for their biocompatibility,

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bioadhesiveness, and biodegradability.[48] In addition, injectable hydrogels can be delivered as a liquid solution and then polymerized in vivo, which allows a perfect fit between the hydrogel and the surrounding native tissue.[30] Controlled delivery of peptides and proteins (eg, growth factors) can be achieved by directly tethering them to the hydrogel for presentation to the embedded or surrounding cells.[47]

Synthetic crosslinked polymers such as polyethylene glycol (PEG) have been used to develop cartilage mimetic hydrogels. To improve the chondrogenic capacity of synthetic hydrogels, cartilage-related extracellular matrix (ECM) analogs and matrix metalloproteinase 2 (MMP-2)–sensitive peptide crosslinks can be incorporated. Among them, chondroitin sulfate (ChS) and the cell adhesion peptide arginyl-glycyl-aspartic acid (RGD) have been introduced to improve chondrogenesis.[34] ChS imitates the native ECM cartilage by introducing a high density of fixed negative charges into the hydrogel, which mimics the aggrecan and elevates the local osmolarity[2] and can interact with growth factors.[27] Studies with fully differentiated chondrocytes have shown that osmolarities within the range of native cartilage support ECM synthesis.[19] The incorporation of ChS into PEG hydrogels has been shown to support chondrogenesis of mesenchymal stem cells (MSCs).[2,45,46] The addition of RGD, which is found in many ECM proteins including fibronectin, provides a mechanism by which cells may interact with the hydrogel; this process improves MSC viability[23] and supports chondrogenesis.[2,37] The use of MMP-2-sensitive peptide crosslinks allows the polymer to be degradable and promote chondrogenesis by MSCs.[3]

In this study, we used a novel cartilage mimetic hydrogel that allows cells to be encapsulated during the hydrogel formation process, with the final combination to be formed in situ within the articular chondral defect. This system was developed to be photopolymerizable, whereby the different components (ie, PEG, ChS, peptide crosslinker, and RGD) are modified with polymerizable groups and, upon exposure to light, react to form a crosslinked polymer network. Advantages of this photopolymerizable hydrogel include spatial and temporal control during formation, the ability to polymerize at physiological pH and temperature, and rapid polymerization (seconds to minutes). Data from in vitro studies have shown that this PEG-based hydrogel enhanced with ECM analogs supports chondrogenesis of human MSCs, but it has yet to be tested in an in vivo animal model.[2] Delivery of a photopolymerizable hydrogel can be surgically challenging. In addition, cartilage healing depends on multiple variables that cannot be tested during in vitro experiments. These include the effect of the in vivo environment on degradation, inflammatory response, and integration to the surrounding tissue. Using a rabbit model, we set out to (1) determine whether rabbit MSCs embedded in a photopolymerizable and degradable cartilage mimetic hydrogel support in vitro chondrogenesis, (2) demonstrate that the proposed hydrogel can be delivered in situ in a chondral focal defect, and (3) determine whether the hydrogel with or without MSCs supports in vivo chondrogenesis. We hypothesized that the proposed hydrogel would support in vitro and in vivo chondrogenesis and could be delivered in a sterile manner intraoperatively.

METHODS

In Vitro Studies

Isolation of MSCs. Bone marrow–derived MSCs were isolated and culture expanded using density separation gradient from the humerus of 1 New Zealand White male rabbit (8 months old) as previously described.[51] When the cells reached 60% confluency, Accumax (Millipore Sigma) was used to passage them, and then they were reseeded at 1×10^3 cells/cm^2 up to passage 3.

Cell Encapsulation. MSCs were encapsulated in the photopolymerizable hydrogel consisting of 9% (wt/vol) PEG (8 arm, 10 kDa) norbornene, 1.4% (wt/vol) of an MMP-2 degradable peptide crosslinker (CVPLSLYSGC), 1% (wt/vol) thiolated ChS, and 0.1 mM CRGDS in sterile phosphate-buffered saline (PBS) at 50 million cells per milliliter of precursor solution containing 0.05 wt% (wt/vol) of the photoinitiator Irgacure 2959 via photopolymerization with 352 nm light at 5 mW/cm or for 8 minutes. The cell-laden hydrogels were cultured in chondrogenic differentiation medium: Dulbecco's modified Eagle medium, 10% fetal bovine serum, L-ascorbic acid 0.05 mg/mL, L-proline 0.0004 M, nonessential amino acids 0.1 nM, HEPES buffer 0.01 M, L-alanyl/glutamin 4 mM, and 1% PSA for 9 weeks with medium changed every other day. Samples were taken at weeks 3, 6, and 9 and analyzed for differentiation via quantitative polymerase chain reaction (qPCR) and immunofluorescence (IF).

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TABLE 1
Primer Sequences and Efficiency for Quantitative Polymerase Chain Reaction Analysis[a]

Gene	Forward Sequence	Reverse Sequence	Efficiency
GAPDH	5′-TCACCATCTTCCAGGAGCGA-3′	5′-CACATTGCCGAAGTGGTCGT-3′	99%
Sox9	5′-GGTGCTCAAGGGCTACGACT-3′	5′-GGGTGGTCTTTCTTGTGCTG-3′	99%
Aggrecan	5′-AGGTCGTGGTGAAAGGTGTTG-3′	5′-GTAGGTTCTCACGCCAGGGA-3′	101%
Collagen II	5′-AAGAGCGGTGACTACTGGATAG-3′	5′-TGCTGTCTCCATAGCTGAAGT-3′	99%
Runx2	5′-CCTTCCACTCTCAGTAAGAAGA-3′	5′-TAAGTAAAGGTGGCTGGATAGT-3′	100%
Collagen I	5′-ATCAAGGAAGGGCAAACGAG-3′	5′-GGCAACAGCAGGTTCACTTACA-3′	
Collagen X	5′-GAAAACCAGGCTATGGAACC-3′	5′-GCTCCTGTAAGTCCCTGTTGTC-3′	99%

[a]GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Mechanical Testing. Hydrogels were assessed for compressive modulus after 1 day (initial) and at weeks 3, 6, and 9 post formation (n = 3). Hydrogels were compressed to 15% strain, at a rate of 0.1 mm/min (MTS Synergy 100, 10 N). The compressive modulus was determined by the slope tangential to the linear region of the stress-strain curve between 10% and 15% strain.

Quantitative Polymerase Chain Reaction. Gene expression was analyzed via qPCR for the chondrogenic markers Sox9, aggrecan, and collagen II and for the hypertrophic markers Runx2 and collagen X. Hydrogels (n = 3) were lysed with Tissuelyser (Qiagen), and RNA was extracted by use of a MicroElute Total RNA Kit (Omega Bio-Tek). RNA was transcribed to cDNA following the manufacturer's procedure for a high-capacity reverse transcription kit (Applied Biosystems). All gene expression data were calculated from delta cycle threshold values and reported as relative to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Table 1).

Immunofluorescence and Safranin-O. Cell-laden hydrogels (n = 3) were fixed overnight in 4% paraformaldehyde in PBS at 4°C (n = 3). After a series of dehydration steps, the constructs were embedded in paraffin. Hydrogel sections (10 μm) were stained with IF for the presence of PEG, collagen II, and collagen X and with safranin-O (Sigma-Aldrich) for sulfated glycosaminoglycans (sGAGs). Enzyme pretreatments of 2000 U/mL hyaluronidase for PEG and collagen II and 1 mg/mL protease followed by 1 mg/mL pepsin for collagen X were performed. After permeabilization and blocking, the sections were treated with primary antibodies overnight at 4°C: 1:50 anti-PEG (courtesy of Steve Roffler at Anti-PEG) and 1:50 anticollagen II and 1:50 anticollagen X (both from University of Iowa, Developmental Studies Hybridoma Bank) in 1% BSA blocking solution. Sections were then treated with secondary antibody for 2 hours with goat antimouse or antirabbit IgG-labeled AlexaFluor 488 (1:100) and counterstained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) for 10 minutes at room temperature.

In Vivo Studies

Generation of Critical Chondral Defect and Treatment. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee. Ten skeletally mature male New Zealand White rabbits, 8 months old, were used. After general anesthesia, a medial parapatellar arthrotomy approach was performed, followed by lateral patellar dislocation. A critically sized defect, 3 mm wide × 2 mm deep, was created bilaterally with a 3 × 2–mm drill, under cooled irrigation, in the central region of the femoral trochlear groove (20 knees) through use of a previously reported technique.[10,25]

Each animal had 1 defect randomly assigned to be treated with hydrogel with or without MSCs, and the contralateral knee was left untreated. Hence, each rabbit served as its own matched control. Animals were allocated to 3 groups: group A (n = 5), hydrogel; group B (n = 5), hydrogel + MSCs; and group C (n = 10, contralateral knees), controls. Before the application of the treatment, the defect was dried with CO_2 to improve hydrogel adhesion to the lesion. After the defect was generated, lights in the operating room were turned off to avoid premature photopolymerization. A red light was used to allow defect visualization and polymer delivery. Approximately 30 to 40 μL of polymer solution containing 0.05% (wt/vol) of the photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (3×10^6 MSCs per defect, for group B) was injected onto the chondral defect until it was full and level with the articular surface. Then, photopolymerization was performed in situ by use of a 405-nm blue light for 40 seconds (Figure 1). The patella was then reduced, and a Monocryl suture was used to perform closure through different layers. Postoperatively, animals were able to freely ambulate and bear weight, and water and food were provided ad libitum.

Macroscopic and Microscopic Scoring. At 6 months postoperatively, the rabbits were euthanized. Defects were evaluated and graded by 2 blinded evaluators, who were experienced professionals in the cartilage field (C.P.G., L.R.G.). The International Cartilage Repair Society (ICRS) score was collected and averaged for each of the groups.[47] The ICRS evaluation consists of different categories including degree of defect repair, integration with border zone, and macroscopic appearance. Tissues were graded from 1 to 4 (1, normal cartilage; 2, nearly normal; 3, abnormal; 4, severely abnormal). The repaired tissue, including the center of the defect, with surrounding native tissue was fixed in neutral-buffered 10% formalin for 24 hours; the samples were dehydrated with increasing concentrations of ethanol and embedded in paraffin. Tissue quality analysis was performed in 5-μm-thick sections

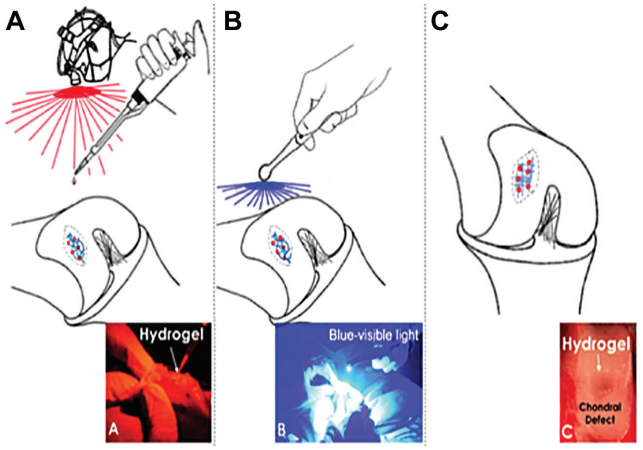

Figure 1. (A) In situ injection of hydrogel precursor in a dark operating room under red light. (B) Photopolymerization of the hydrogel in situ using a 405-nm blue-visible light in a dark operating room. (C) Defect filled with polymerized hydrogel.

with safranin-O and IF for collagen II staining. Images were taken through use of a Nikon Eclipse 80i microscope. Histological sections were graded according to the modified O'Driscoll score (MODS), where the total point value ranged from 0 (no signs of cartilage repair) to 28 (complete regeneration).[24,30] To assess subchondral bonding, to enhance our scoring, we designed subchondral bone bonding (SBB) criteria grading ranging from 0 to 3 depending on the bonding percentage (grade 3, 100%-76% of subchondral bonding; grade 2, 75%-50%; grade 1, 49%-25%; grade 0, <24%). In addition, safranin-O-stained slides were randomly selected by an individual who was not one of the authors and were blindly quantified for proteoglycan content (stained red) by 3 of the authors (C.P.G., F.R.F., and L.R.G.). The NIS-Elements AR (Nikon Instruments Inc) was used for such purpose by demarking the defect area and quantifying the red stain percentage per total defect area.

Statistical Analysis

In vitro and in vivo intergroup-intragroup comparisons were performed with paired *t* tests and 1-way analysis of variance. Tukey post hoc correction and the Holm-Sidak test were used to adjust for multiple group comparisons. The tests were performed with SigmaPlot 11.0 statistical software (Systat Software). Data are reported as mean ± SD. P values of .05 or lower were considered significant, and values from $P > .05$ to $P < .1$ were considered borderline significant.

RESULTS

In Vitro Chondrogenesis and ECM Deposition

MSC chondrogenic differentiation was evaluated by qPCR for gene expression of chondrogenic markers Sox9, aggrecan, and collagen II and for hypertrophy markers Runx2 and collagen X. Mean mRNA levels of Sox9 were upregulated from week 3 to week 9 ($P = .094$). Similarly, aggrecan mean mRNA levels were elevated throughout the study ($P = .159$), indicating chondrogenic differentiation of encapsulated MSCs. Concomitantly, mean levels of the hypertrophic markers were elevated over 9 weeks, with both Runx2 ($P = .066$) and collagen X ($P = .058$) increasing 10-fold. However, at week 6 the Sox9 expression was higher than Runx2 ($P = .045$), and by week 9 collagen II was higher than collagen X ($P = .029$) (Figure 2).

Histological images of sGAGs stained by safranin-O indicated the presence of sGAGs over 9 weeks. Positive staining at early time points was expected given the incorporation of ChS in the hydrogel formulation; however, after 9 weeks, the positive sGAG stain was retained due to sGAG synthesis despite hydrogel degradation. At the protein level, the encapsulated MSCs expressed collagen II after 3 weeks, and by week 9 the ECM production of collagen II was prevalent throughout the hydrogel (Figure 2B). The IF stain for PEG showed significant degradation of the polymer matrix in the hydrogel over the course of the 9-week study. On comparison of the collagen II and PEG staining, it appeared that collagen II protein production correlated with the reduction in positive PEG staining; and by week 9 there appeared to be more collagen II predominance over PEG (Figure 2).

The compressive modulus was evaluated to determine hydrogel degradation and matrix deposition. At day 1, the compressive modulus was determined to be ~65 kPa. The compressive modulus decreased to ~30% of its initial value after 3 weeks ($P < .0001$) and to ~15% after 6 weeks ($P = .029$), where it was maintained up to 9 weeks (Figure 2).

In Vivo Delivery and In Situ Photopolymerization of the Hydrogel

No signs of premature polymerization during delivery were detected. In all cases, the hydrogel was readily photopolymerized in the defect with no loosening or dislocation of the hydrogel from the chondral defect.

Macroscopic Scoring

At 6 months, no synovial reaction or inflammation was apparent. The control group showed better scoring with normal tissue in most of the defects (7/10), whereas the treatment groups showed nearly normal repair tissue. The ICRS score indicated no intergroup statistical difference: group A (hydrogel alone), 10 ± 1.7 (nearly normal); group B (hydrogel with MSCs), 10 ± 1.4 (nearly normal); group C (control), 11.3 ± 1.06 (normal) ($P = .088$). One knee that was treated with the hydrogel alone (rabbit No. 3) had a lateral subluxation of the patella that resulted in a macroscopic reactive fibrocartilage-type tissue over the lateral aspect of the trochlear ridge. The chondral defect showed abnormal cartilage repair tissue.

Microscopic Scoring

Overall, the hydrogel had the highest average total MODS, but no significant intergroup difference was found (group

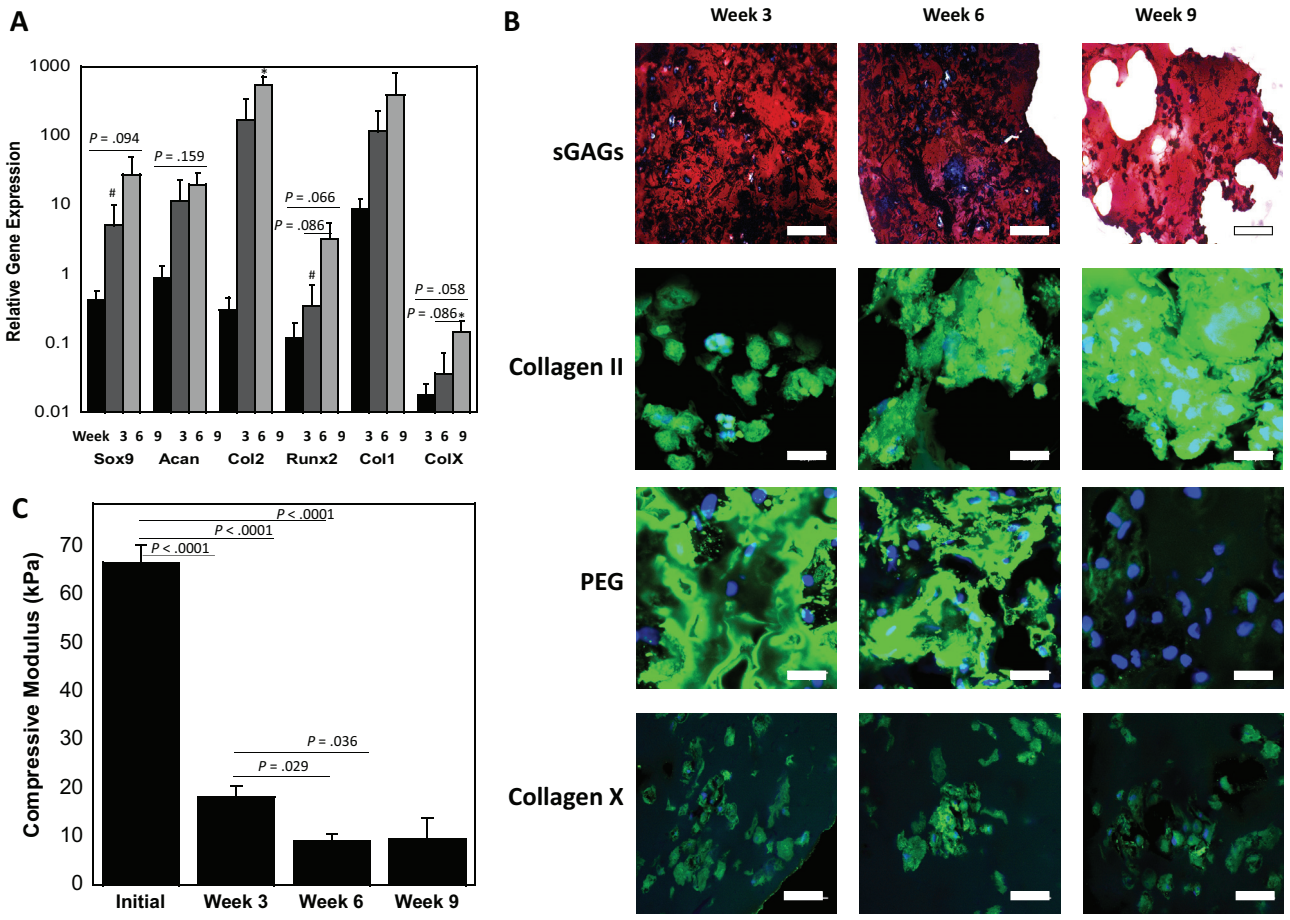

Figure 2. (A) The relative gene expression data of chondrogenic and hypertrophic markers at weeks 3, 6, and 9 (n = 3). Data are represented as mean, and error bars are SD. #Higher Sox9 expression than Runx2 (P = .045). *Higher collagen II expression than collagen X (P = .029). (B) Representative immunofluorescence (IF) and safranin-O images (n = 3) of sulfated glycosaminoglycans (sGAGs) (red), collagen II (green), polyethylene glycol (PEG) (green), and collagen X (green) at weeks 3, 6, and 9. The nuclei in the histology images of sGAGs (safranin-O) were counterstained with Fast Green (black), scale bar = 100 μm. In IF images of collagen II and PEG, nuclei were counterstained with DAPI (blue), scale bar = 20 μm. (C) The bulk compressive modulus initially (day 1) and at weeks 3, 6, and 9 (n = 3).

A, 17.4 ± 4.7; group B, 13 ± 3; group C, 16.7 ± 2.9) (P = .11) (Figure 3). As for the SBB criteria designed for the study, the group treated with hydrogel + MSCs tended to have better integration (group A, 0.8 ± 1.3; group B, 2.2 ± 0.84; group C, 1.5 ± 1.35), although the result was not significant (P = .23). Of note, the SBB was compromised in group A, with overall less than 25% bonding to the subchondral bone. The percentage of proteoglycans (red stain) was calculated from randomly selected slides in each group. Although not significantly different, the hydrogel alone showed a trend of more matrix safranin-O staining (group A, 49.4% ± 20%; group B, 25.8% ± 16.4%; group C, 36.9% ± 25.2%) (P = .27). Three of 5 chondral defects treated with hydrogel alone showed moderate staining, whereas 2 of the 5 chondral defects showed minimal staining. One of these defects was from the rabbit that had a patellar subluxation (Figure 4).

The collagen II expression was qualitatively evaluated via IF (Figure 5). A range in collagen II expression was found in all groups. Collagen II prevalence in both the treated and control groups appeared to be highly variable from rabbit to rabbit. Collagen II was found along the surface of the repaired tissue in most controls. In the hydrogel group, compared with the controls, collagen II presence was similar, or even better in 3 cases. A range of collagen II expression was also found in those treated with hydrogel + MSCs. When compared with their controls, only 2 cases expressed higher collagen II expression.

DISCUSSION

The study demonstrates, first, that MSCs undergo in vitro chondrogenesis in the cartilage mimetic hydrogel; second,

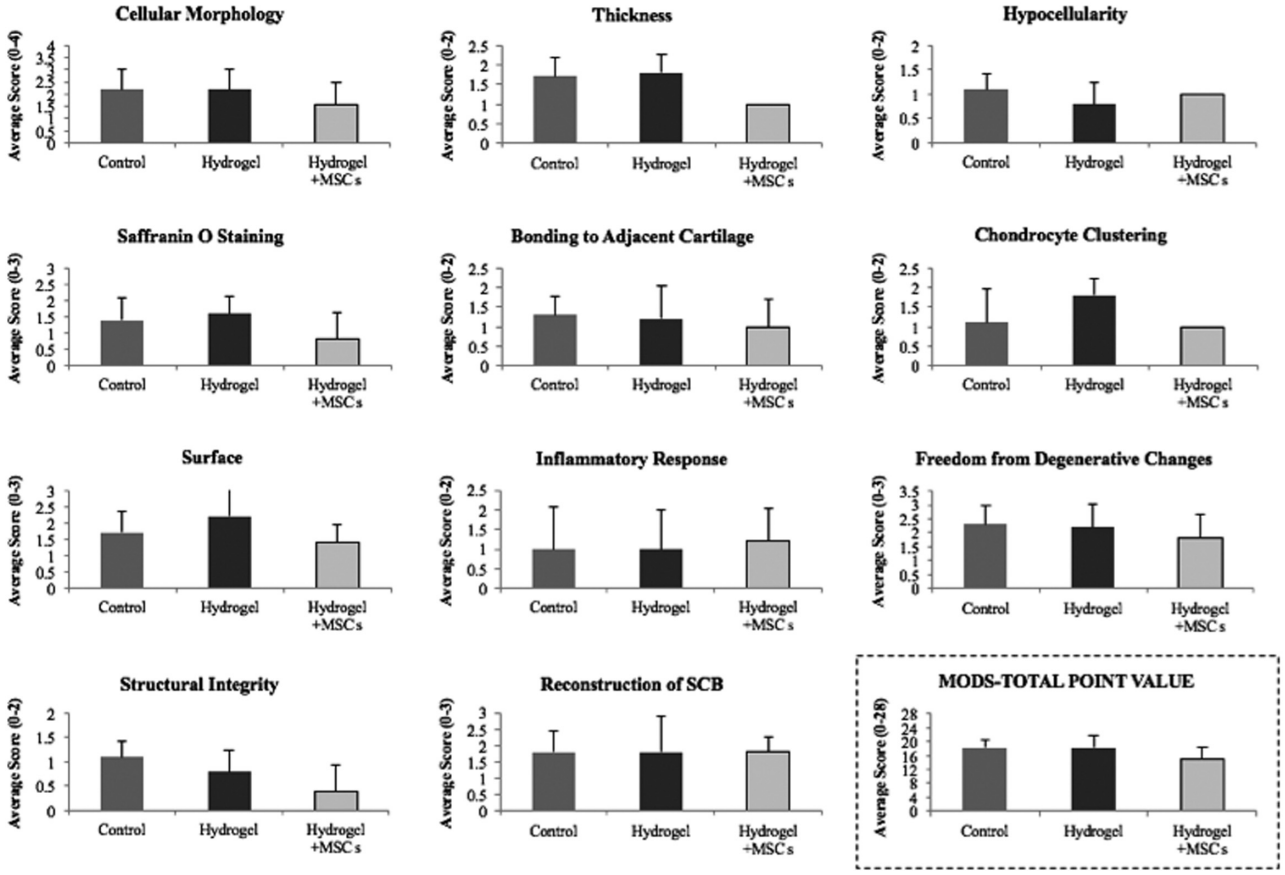

Figure 3. Histological results of the different parameters assessed with the modified O'Driscoll score (MODS) for each group. MSC, mesenchymal stem cell; SCB, subchondral bone.

that hydrogel can be delivered in vivo and photopolymerized intraoperatively in situ; and third, that hydrogel supports in vivo chondrogenesis. Implantation of the hydrogel did not generate inflammation or any observable adverse events in the animal model. Based on these findings, the proposed photopolymerized system can be inoculated intraoperatively and supports cartilage repair. However, contrary to our hypothesis, the inclusion of MSCs did not enhance the hydrogel chondrogenic potential.

Cartilage regeneration continues to be a clinical challenge. Animal studies are critical translational models to help develop effective treatments for cartilage injuries.[10] This is our first experiment probing the delivery of a photopolymerizable hydrogel intraoperatively for the treatment of chondral defects in a small animal model. Before testing in vivo, the hydrogel was tested for its ability to support chondrogenesis of rabbit MSCs and macroscopic neocartilage growth. Previous studies using human MSCs have shown that a PEG hydrogel with RGD and ChS creates a cartilage mimetic environment that supports in vitro chondrogenesis.[2] Previously we have shown that chondroitin sulfate is important both in maintaining a stable chondrogenic phenotype within a dynamic loading environment and in preventing MSC hypertrophy.[49] Similarly, rabbit MSCs cultured in this hydrogel successfully underwent chondrogenic differentiation. Since MSCs are notorious for undergoing hypertrophy and terminal differentiation,[7,27] we analyzed expression of RunX2 and collagen X hypertrophic markers. Their levels were significantly lower than levels of the chondrogenic genes Sox9 and collagen II. The increase in the hypertrophic markers may raise the concern of potential mineralization of the construct. However, immunohistochemical staining for collagen X was observed in the hydrogel construct at week 3 and remained localized to the pericellular space throughout the 9-week culture period. Conversely, the collagen II deposition was found throughout all of the construct during the 9-week period. Since the in vitro studies were performed in the absence of loading, it is not surprising that some collagen X was evident, which is consistent with our previous findings.[49] Mineralization was not evaluated due to the lack of a phosphate ion source in the medium, which is necessary to induce mineralization in vitro.

The cell seeding density in these constructs was not investigated. We chose a cell seeding density (50 million cells per milliliter of precursor solution) based on our prior published studies.[2] The goal of the in vitro study was to confirm that rabbit MSCs undergo chondrogenesis in the cartilage mimetic hydrogel and that the encapsulated cells are able to degrade the hydrogel. For the latter, it was

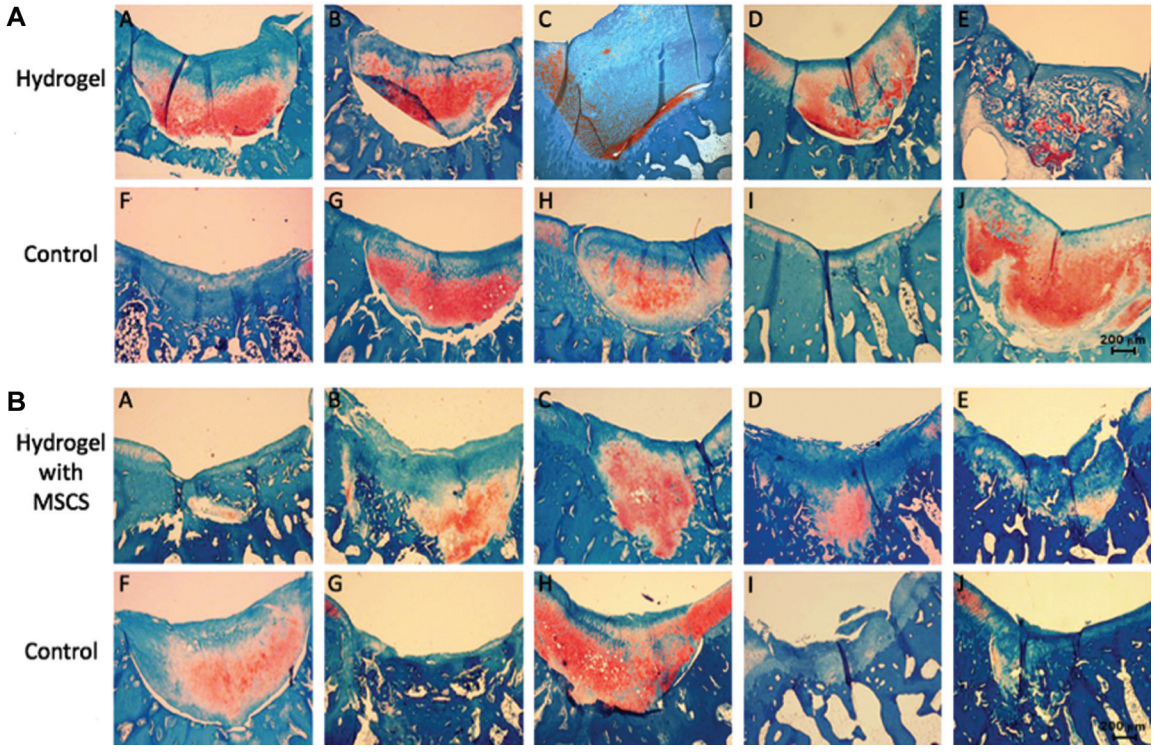

Figure 4. Safranin-O staining of sections from (A) the hydrogel group and (B) the hydrogel + mesenchymal stem cell (MSC) group with corresponding matched contralateral controls. Original magnification ×4 and scale as represented on panels *A-J*. (A) *A*, 48.6%; *B*, 54.7%; *C*, 34.7%; *D*, 80%; *E*, 28.9%; *F*, 11.4%; *G*, 53.7%; *H*, 48.7%; *I*, 11.8%, *J*, 68.3%. *C* belonged to a rabbit that experienced patellar subluxation. (B) *A*, 17%; *B*, 24.4%; *C*, 54.1%; *D*, 20.4%; *E*, 12.9%; *F*, 55.9%; *G*, 11.9%; *H*, 72.5%; *I*, 16.3%; *J*, 18.6%.

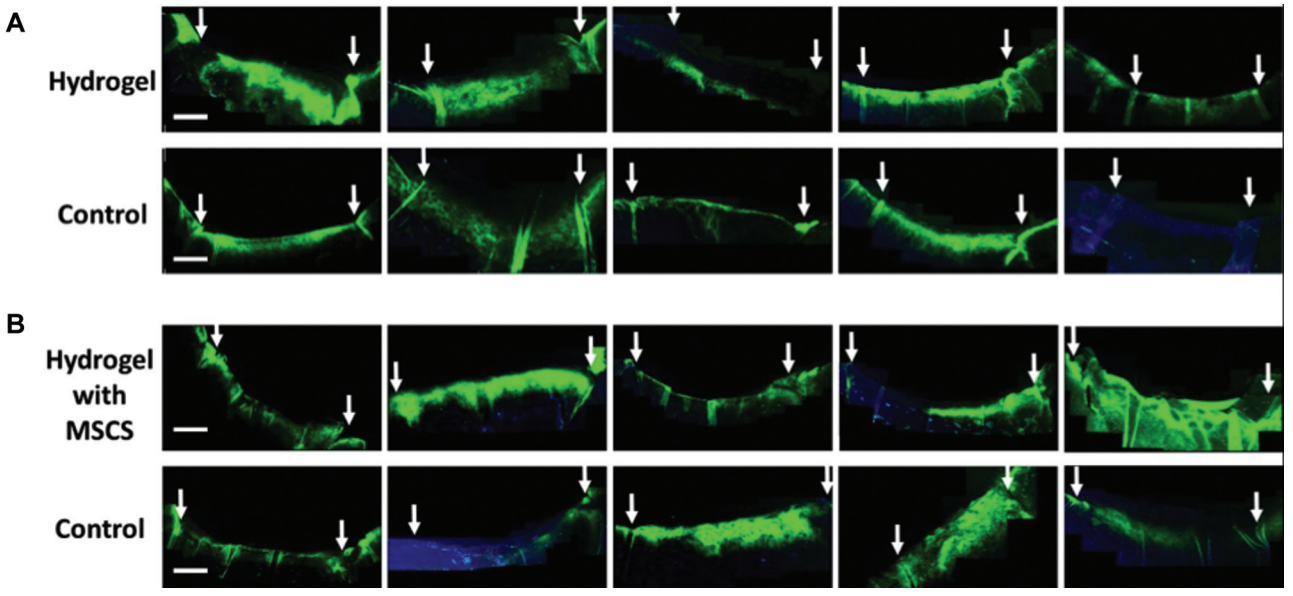

Figure 5. Immunofluorescence images of collagen II (green) at the chondral defect (indicated between white arrows). (A) Group treated with hydrogel and corresponding controls; (B) group treated with hydrogel + mesenchymal stem cells (MSCs) and corresponding controls. Nuclei are counterstained with DAPI (blue), scale bar = 500 μm.

important that the cell concentration was not too dense so that it was possible to observe the evolution from single cells producing a localized ECM into regions of macroscopic tissue where multiple cells and their tissue form a connected matrix. In future studies, investigators will need to optimize the cell seeding density for the in vivo joint environment.

Degradation of the hydrogel is critical to support neocartilage growth. The proposed hydrogel is MMP-2 sensitive, allowing neocartilaginous tissue formation, which was composed of the 2 main ECM molecules of cartilage, sGAG and collagen II. The initial and significant decrease in the compressive modulus confirms that MSCs are able to degrade the hydrogel, which is supported by the fact that rabbits are known to secrete MMP-2 during healing and remodeling.[5,9,34] Despite the hydrogel degradation, the compressive modulus was maintained after week 6. This observation indicates that the neocartilage matrix deposited by the chondrogenically differentiated MSCs is forming a suitable matrix, which is important to support mechanical loads, albeit lower than that of native cartilage. Importantly, the formation of neocartilage by encapsulated rabbit MSCs in vitro is an improvement over other studies using MMP-sensitive hydrogel that have shown limited deposition of ECM molecules to the pericellular space with little to no connectivity.[29,43,44] Overall, the promising in vitro results motivated the advancement to testing this novel hydrogel in a small animal model.

For successful in vivo treatment of focal osteochondral defects, the in situ delivery of the hydrogel must be achieved. Injectable, photopolymerizable hydrogels hold great promise for cartilage tissue engineering as they can act as a vehicle to deliver cells, ECM analogs, and other biological factors to the site of injury while maintaining cell viability and allowing for chondrogenic differentiation.[¶] Unlike prefabricated scaffolds that are physically placed into the defect, the liquid precursor solution of the PEG hydrogel can be injected directly into the defect to fully fill the shape of the defect. This approach is advantageous for clinical practice and for treatment of challenging chondral defects located in specific anatomic areas such as the hip or femoral condyle, where the natural curvature and thin cartilage make resurfacing cartilage techniques especially challenging.[12] Additionally, the hydrogel is photopolymerizable by visible light, which minimizes the possibility of any damage to the surrounding tissue.[38]

The aim of our in vivo study was to provide a proof of concept that the hydrogel alone or combined with MSCs could be delivered intraoperatively in a sterile fashion and to determine whether cartilage healing could be improved when the cells were added to the hydrogel. In addition, any possible inflammation or adverse effect that the inoculation of the hydrogel may have produced was investigated. In this study, in situ delivery of the hydrogel to the chondral defect was successfully performed aseptically and with no complications. The delivery method was simple and reproducible and added minimal additional time to the surgery itself.[33] We demonstrated that the hydrogel prepolymer could be injected and polymerized through use of a 405-nm visible blue light for 40 seconds.

Some observations from the macroscopic and histological analyses of the different treatments are worth noting. The macroscopic ICRS score showed that the control group had overall normal-like tissue (7/10 scored normal tissue). Previous studies have shown excellent correlation and reliability between macroscopic and histological results when using the ICRS score.[16,47] Thus, we decided to use it to assess macroscopic regeneration. Correlation of macroscopic ICRS scoring to histological results has been validated only for assessment of cartilage repair with microfracture technique,[16] yet it is well known that microfracture does not lead to hyaline cartilage but rather leads to fibrous tissue.[13] Evaluation of cartilage repair with more sophisticated tissue engineering therapies may require further variables to be evaluated, such as those proposed by Goebel et al.[16] In contrast with the ICRS score, Goebel's score evaluates 5 variables: defect fill and surface (also present in the ICRS score), color, presence of blood vessels, and degeneration of adjacent articular cartilage. Further analysis will be necessary to establish correlation between Goebel's score and histological scores with tissue engineering restorative procedures.[16,17]

Contrary to macroscopic results, the histological appearance of the repaired tissue at 6 months was morphologically different between groups. Regardless of the treatment, repaired tissues in all defects were scored as a mix of hyaline and fibrocartilage tissue or fibrocartilage tissue. Inflammation was not observed in any of the groups. In concordance with previous studies[26,39,41,52] (although not significant), the hydrogel alone showed better chondrogenesis and tissue quality compared with hydrogel + MSCs. One noticeable finding was that the hydrogel-only group had a higher percentage of proteoglycan content (49.4%) in 4 of 5 cases and had good bonding to the adjacent articular cartilage. Defect filling was highest in this group, observed in all 5 cases. Moreover, this group showed high cellularity in the repaired tissue. This situation can be partly explained because the chondrocytes, progenitor cells, and stem cells may migrate from the neighboring cartilage or subchondral bone and interact with the hydrogel and the ECM analogs, thereby supporting chondrogenesis. Of note, RGD is known to interact with integrins and has been demonstrated to support chondrogenesis in MSCs.[33] The presence of ChS also creates a negatively charged environment that supports chondrogenesis.[15] Notwithstanding, this phenomenon was not observed when the hydrogel was combined with MSCs. The hydrogel + MSCs group had the lowest histological score, lowest collagen II expression, and lowest proteoglycan (25.8%) but the highest bonding to the subchondral bone. Moreover, this group did not show good defect filling and presented an abnormal structure with disruption including cysts, fibrosis, and degeneration. This finding is consistent with previous studies.[26,39,52] Similarly, Guo et al[18] found that MSCs did not improve the chondrogenesis of an oligo(poly[ethylene glycol] fumarate)–based hydrogel in a osteochondral defect of a 6-month-old rabbit model, whereas Kisiday

[¶]References 8, 14, 19, 20, 32, 36, 41, 50, 53.

et al[22] showed improved chondrogenesis in a cultured, self-assembling, peptide-based hydrogel seeded with bone marrow MSCs only when supplemented with TGF-β1. These results suggest that incorporating specific chondrogenic signals into the hydrogel may improve the overall chondrogenic capacity of MSCs. Finally, the control group in the present study showed a fibrocartilaginous tissue repair type, moderate safranin-O staining (36.9%), and fair collagen II, but with overall good thickness, integrity, morphology, and reconstruction of the subchondral bone. This finding demonstrates the natural intrinsic cartilage repair capacity of the rabbit animal model, which limits the use of this animal model for cartilage repair.[10,25] Although this model has been widely used for research on cartilage regeneration based on easy handling, low cost, and ease of care, the rabbit model should not be considered useful to evaluate the translational potential of cartilage repair treatments in humans due to the rabbit's remarkable endogenous healing potential.[10,48] Nonetheless, the rabbit model provides an important means for assessing feasibility of novel scaffolds for treating focal defects before larger animal models are used.

This study had some limitations. First, the rabbit model had good intrinsic cartilage regeneration.[21,40] Second, this proof of concept study included a small sample size. Increasing the study population may provide evidence of intergroup differences, which were notable under light microscope but not statistically significant. However, high consistency was kept among the animals: all rabbits were mature, 8 months old, and male,[1] decreasing the confounding variables that could arise from these characteristics. In addition, all animals had an established paired contralateral control defect. Since chondrogenesis was observed with the cartilage mimetic hydrogel, we will continue to work on strategies to optimize it for cartilage repair. Another limitation is the use of allogenic MSCs. Using allogenic MSCs for cartilage repair has the potential to induce an immunological response and secondarily to affect cartilage healing. However, MSCs have been shown to have low immunogenicity based on the lack of expression of markers such as CD45, CD34, and HLA-DR surface molecules.[6,28] Several preclinical studies in rabbits, pigs, and goats showed effective cartilage repair after implantation of allogenic MSCs in cartilage defect without reporting any adverse events.[6,11] Furthermore, we did not find an observable, higher inflammatory response in the groups treated with MSCs and hydrogel, thus minimizing the concern of immunogenic response to the allogenic cells.[4,42] Finally, we did not track the MSCs that were implanted; thus, the contribution of implanted cells to the tissue repair, including the degree to which the cells survive and integrate into the newly formed cartilage, is uncertain. However, previous investigators performing cell tracking of the cells in defects have shown that transplanted cells survive and fill the defect.[31,35]

In conclusion, this study shows that MSCs undergo in vitro chondrogenesis when seeded in the proposed hydrogel. The hydrogel can be delivered and photopolymerized intraoperatively. The hydrogel remained in the defects, did not result in inflammation, and showed good cartilage healing capacity in 3 of 5 cases. Adding MSCs to the hydrogel did not enhance cartilage repair and in some cases led to poorer outcomes, suggesting that MSCs may orchestrate an alternative healing process, which warrants further study.

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