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Chitosan-Glycerol Phosphate/Blood Implants Improve Hyaline Cartilage Repair in Ovine Microfracture Defects

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Background: Microfracture is a surgical procedure that is used to treat focal articular cartilage defects. Although joint function improves following microfracture, the procedure elicits incomplete repair. As blood clot formation in the microfracture defect is an essential initiating event in microfracture therapy, we hypothesized that the repair would be improved if the microfracture defect were filled with a blood clot that was stabilized by the incorporation of a thrombogenic and adhesive polymer, specifically, chitosan. The objectives of the present study were to evaluate (1) blood clot adhesion in fresh microfracture defects and (2) the quality of the repair, at six months postoperatively, of microfracture defects that had been treated with or without chitosan-glycerol phosphate/blood clot implants, using a sheep model.

Methods: In eighteen sheep, two 1-cm² full-thickness chondral defects were created in the distal part of the femur and treated with microfracture; one defect was made in the medial femoral condyle, and the other defect was made in the trochlea. In four sheep, microfracture defects were created bilaterally; the microfracture defects in one knee received no further treatment, and the microfracture defects in the contralateral knee were filled with chitosan-glycerol phosphate/autologous whole blood and the implants were allowed to solidify. Fresh defects in these four sheep were collected at one hour postoperatively to compare the retention of the chitosan-glycerol phosphate/blood clot with that of the normal clot and to define the histologic characteristics of these fresh defects. In the other fourteen sheep, microfracture defects were made in only one knee and either were left untreated (control group; six sheep) or were treated with chitosan-glycerol phosphate/blood implant (treatment group; eight sheep), and the quality of repair was assessed histologically, histomorphometrically, and biochemically at six months postoperatively.

Results: In the defects that were examined one hour postoperatively, chitosan-glycerol phosphate/blood clots showed increased adhesion to the walls of the defects as compared with the blood clots in the untreated microfracture defects. After histological processing, all blood clots in the control microfracture defects had been lost, whereas chitosan-glycerol phosphate/blood clot adhered to and was partly retained on the surfaces of the defect. At six months, defects that had been treated with chitosan-glycerol phosphate/blood were filled with significantly more hyaline repair tissue (p < 0.05) compared with control defects. Repair tissue from medial femoral condyle defects that had been treated with chitosan-glycerol phosphate/blood contained more cells and more collagen compared with control defects and showed complete restoration of glycosaminoglycan levels.

Conclusions: Solidification of a chitosan-glycerol phosphate/blood implant in microfracture defects improved cartilage repair compared with microfracture alone by increasing the amount of tissue and improving its biochemical composition and cellular organization.

Clinical Relevance: The use of chitosan-glycerol phosphate/blood implants in conjunction with microfracture can improve the structural and compositional properties of repaired cartilage. These effects may result in better integration, improved biomechanical properties, and longer durability of the repair tissue.
Surgical resurfacing techniques have been used for the treatment of focal articular lesions, osteochondritis dissecans, and degenerative cartilage lesions. For most patients, pain and function scores improve for several years after microfracture or drilling, but full recovery of joint function and complete relief of pain are not always attained. Furthermore, some patients simply fail to respond to this approach. This may be because these procedures create repair tissue that consists of a mixture of fibrocartilage and hyaline cartilage that is not attached or integrated with the surrounding cartilage and thus is subject to reinjury and rapid degeneration. Despite promising results in previous studies, some patients are obliged to undergo additional surgical treatment, suggesting that improvements in standard surgical resurfacing therapies are needed.

During the microfracture procedure, a physical conduit is created to connect vascularized subchondral bone marrow with the debridged cartilage lesion. Animal models involving rabbits, dogs, and horses have been developed to study the repair tissue that is formed following resurfacing procedures. Taken together, these studies have indicated that, over the course of three to twelve months, there is partial filling of these defects with mainly fibrous repair tissue, with corresponding control defects demonstrating considerably less filling. All of these procedures require the formation of a blood clot and an inflammatory response that stimulates bone marrow cells to migrate, proliferate, and form a granulation tissue, which is gradually replaced with fibrocartilage or fibrous repair tissue. The repair tissue should become integrated with the surrounding cartilage and should form a seamless junction with the articular surface in order to persist under load.

Since the first step in marrow stimulation is the formation of a blood clot, stabilization of the clot by incorporating a thrombogenic polymer scaffold might improve the ensuing repair response. Chitosan is a particularly promising scaffolding material because this polysaccharide, which is composed predominantly of polyglucosamine, is charged and thrombogenic. Chitosan is prepared by deacetylation of chitin, a natural polymer composed of acetylated glucosamine. The polycationic nature of chitosan facilitates adhesion to negatively charged surfaces of tissues and cells, including cartilage. A wide range of medical uses for chitosan have been discovered in the past fifteen years, including accelerated skin and fracture repair. Other studies have demonstrated biocompatibility, low toxicity, and negligible immunogenicity. A cytocompatible chitosan solution, chitosan-glycerol phosphate, has also been generated by using glycerol phosphate as a buffer to titrate the polyionic surface in order to persist under load.

In the present study, we aimed to remove as much calcified cartilage as possible without damaging the subchondral bone. In all eighteen sheep, cartilage defects were created in either one knee or both knees (as described below). In all knees with operatively created defects, one defect (9 × 10.5 mm) was made in the central load-bearing region of the medial femoral condyle and one defect (7.6 × 12 mm) was made in the lateral distal facet of the trochlea. In fourteen sheep, cartilage defects were created in only one knee and the contralateral knee was left intact in order to allow for the comparative measurement of thickness, safranin-O staining, and biochemical composition between the repaired cartilage and normal cartilage in the regions of the defects (see schematic, Table IV). In each knee
that had been operated on, both defects were treated either with microfracture only (control group; six sheep) or with microfracture and chitosan-glycerol phosphate/blood (treatment group; eight sheep), followed by a six-month repair period (Table I). Two animals from each six-month repair group were administered a contusive impact injury (30 MPa; impulse duration, 10 msec) in the medial femoral condyle three weeks before creation of the defects. Such impact injuries induce immediate cartilage degeneration and eventually progress to osteoarthritis over six months, as reported previously.

With use of an arthroscopic microfracture awl and mallet, fourteen to twenty evenly spaced microfracture holes (measuring 1.5 mm in diameter and 3 mm deep) were made in each defect, starting with a ring of holes spaced 2 to 3 mm apart at the defect rim and followed by five to eight holes in the center. The open joint and soft-tissue surfaces were continually rinsed with Ringer lactate solution. The knee was closed in three separate layers with resorbable suture material. Postoperatively, all animals received non-steroidal antiinflammatory pain medication (75 mg of intravenous flunixin meglumine; Bimed-MTC Animal Health, Cambridge, Ontario, Canada) and antibiotics (20,000 IU/kg of penicillin G; Novopharm, Toronto, Ontario, Canada) for three days, followed by intermittent administration of anti-inflammatory medication for as long as two weeks. All animals were walking within an hour after recovery from anesthesia. Postoperative mobility was partly limited by reducing pen size for one week. Temporary postoperative lameness was observed in all sheep for one to three weeks after the arthrotomy.

In the cases of the remaining four sheep, bilateral cartilage defects were created, the joints were sutured, and the animals were killed immediately postoperatively in order to characterize the initial cartilage defects and the retention of implant in the defects. Defects in alternating left or right knees were treated with chitosan-glycerol phosphate/blood implants (Table I). These four sheep were killed on the operating table within one hour after the creation of the defects on the side without implants and within 2.5 hours after the creation of the defects on the side with implants.

At the time when the animals were euthanized, lameness, joint diameter (as an indication of effusion), hematological studies, and synovial fluid analysis were used to evaluate safety and biocompatibility. During necropsy, all joints were kept moist with Ringer lactate solution. Defect sites were harvested with a band saw and were photographed with a digital camera and dissection microscope. Full-thickness biopsy specimens of repair tissue weighing 1 to 10 mg were recovered from the distal medial corner of each trochlear and condylar defect, from proximal sites outside of the defects, and from four matching sites from the intact, contralateral knee (see schematic, Table IV). Biopsy specimens were weighed, frozen in liquid nitrogen, and stored at 80°C for biochemical analysis.

**TABLE I Animal Assignment and Treatment**

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Operated Stiffle</th>
<th>3-Week Prior Impact</th>
<th>Chitosan-Glycerol Phosphate/Blood Clot Treatment</th>
<th>Repair Period (wk)</th>
<th>Condylar Defect Subchondral Cyst Score*†</th>
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*Scale = 0 (no cyst) to 4 (severe cyst). †NA = not applicable.
Sample Processing, Histomorphometric Analysis, and Immunohistochemical Analysis

All bone-cartilage samples were fixed in formalin for five to seven days and were decalcified in 0.5 N HCl with 0.1% glutaraldehyde for as long as two weeks. Repaired defects were transsected transversely into three blocks of equal size, postfixed, and embedded in paraffin to obtain two distinct, 6-µm-thick osteochondral sections that included the entire width of the defect (7 to 10 mm) and 2 mm of flanking cartilage. In these two sections, the cross-sectional area of the repair tissue was defined as all nonmineralized tissue above the subchondral bone plate, excluding occasional soft tissue in remnant microfracture holes. One safranin-O-stained section was also prepared from a matching region in the intact, contralateral joint and was used to measure the cartilage thickness, calcified layer thickness, safranin-O staining, and the cross-sectional area of the normal cartilage in the regions of the defects. Histomorphometric analysis was performed on two safranin O/fast green-stained sections per defect by two independent, blinded observers (C.D.H. and A.C.) with use of low-magnification digital images, generated with a Zeiss dissection microscope and a digital camera and analyzed with histomorphometry software (Northern Eclipse; Empix, Mississauga, Ontario, Canada), to determine percent fill and the percentage of hyaline cartilage in the repair tissue. Percent fill was determined by measuring the cross-sectional area of the repair tissue in a defect and then dividing this value by the cross-sectional area of the normal cartilage in the same region of the contralateral, intact joint. To estimate the amount of hyaline cartilage in the repair tissue, it was assumed that tissue that stained pink or red with safranin O was hyaline cartilage. The percentage of the repair tissue that was hyaline cartilage was then determined as the percentage of the total repair tissue area. Calibrated line measurements were used to determine the thickness of the cartilage, calcified cartilage, and repair cartilage. For fresh defects analyzed one to two hours after their creation, two adjacent sections were generated from the middle of each defect and were used to determine the percentage of the cross-section of the defect base that was composed of noncalcified cartilage, calcified cartilage, bone, or microfracture holes and to determine the thickness of noncalcified and calcified cartilage in the flanking regions outside the defects.

For immunohistochemical analysis, sections were dewaxed and treated successively with trypsin for collagen type I or pronase for collagen type II, followed by hyaluronidase and antigen retrieval. Sections were then blocked with 20% goat serum in PBS/0.1% Triton X-100, followed by successive incubations with 1:50 dilution mouse monoclonal antibodies to human collagen type II (II-4C11) or type I (I-8H5) (ICN Biochemicals, Costa Mesa, California), biotin-conjugated goat anti-mouse, and ABC-Alkaline Phosphatase Red Substrate Detection kit (Vectastain; Vector Laboratories, Burlingame, California). Controls, with secondary antibody only, showed no background staining.

International Cartilage Repair Society (ICRS) histological profiles were generated with use of a modification of the system described by Mainil-Varlet et al.35 The repair tissues contained within the defects were scored as four contiguous, approximately 2-mm segments. Each 2-mm segment was individually scored for six different parameters, including (I) surface smoothness (with 3 indicating smooth and 0 indicating irregular), (II) the type of matrix (with 3 indicating hyaline and 0 indicating fibrous), (III) cell organization (with 3 indicating columnar and 0 indicating random), (IV) cell viability (with 3 indicating predominantly viable cells and 0 indicating few viable cells), (V) the absence of subchondral bone pathology (with 3 indicating normal, 2 indicating remodeled, and 1 or 0 indicating detached and/or necrotic), and (VI) calcified cartilage (with 3 indicating normal and 0 indicating abnormal [formed >100 µm higher or lower than the adjacent tidemark] ). The final scores for each parameter were the collective average from two sections per defect and four segments per section as evaluated by two independent blinded observers (A.C. and E.R.).

Determination of Collagen Content, Glycosaminoglycan Content, and Cell Density

Thawed cartilage samples were weighed and subjected to papaain digestion at pH 6.5 to determine the DNA content (by means of Hoechst fluorometric assay), glycosaminoglycan content (by means of DMB assay), and collagen content (by means of hydroxyproline assay), normalized to wet weight.38 Samples for biochemical analysis were not retrieved from the defect when repair tissue was absent from the distal medial corner. Cell density was determined with use of 7.7 pg DNA/cell, and collagen content was determined with use of 7.6 mg collagen/mg hydroxyproline.39 Standards included sheared calf thymus DNA, 4-trans-hydroxyproline, and shark cartilage chondroitin sulfate B (Sigma-Aldrich, Oakville, Ontario, Canada).

Safety Outcome Measures

Peripheral EDTA-anticoagulated blood and synovial fluid were collected at the time of surgery and at the time of necropsy for hematological analysis and for synovial fluid analysis of total serum protein, mucin, and leukocyte counts (University of Guelph Animal Health Laboratory, Guelph, Ontario, Canada). Lameness was evaluated on a scale of 0 (no lameness) to 5 (severe lameness). Joint effusion was evaluated by comparing the diameters of stifles with and without operatively created defects and by measuring the total synovial fluid aspirated at the time of necropsy. Subchondral cysts were scored histomorphometrically for both sections taken from within each defect, and the scores were averaged. Cyst severity, as indicated by the diameter of fibrous white tissue or tissue voids, was evaluated on a scale of 0 (<1 mm) to 4 (>6 mm).

Statistical Analysis

The effects of treatment (microfracture alone or microfracture combined with chitosan-glycerol phosphate/blood) and defect location on percent fill in repair cartilage were analyzed with use of analysis of variance with repeated measures, with each of the two sections analyzed by each of the two blinded observers constituting the four repeated measures (STATISTICA, version 6.1; StatSoft, Tulsa, Oklahoma). Percent hyaline con-
tent in repair tissue was statistically analyzed in an identical fashion to that used for percent fill. Averaged data from one observer differed from that of the other observer by <3% for both percent fill and percent hyaline repair for all sections, indicating the reproducibility of this histomorphometric procedure. Percent fill and percent hyaline were also analyzed together as an aggregate indicator of the overall quantity of hyaline repair cartilage by specifying two repeated-measure variables, one being either percent fill or percent hyaline and the other being the section number (1 to 4), for multivariate analysis of variance (STATISTICA GLM; StatSoft). International Cartilage Repair Society histological scores assigned by two independent observers to two sections per defect were analyzed with use of analysis of variance with four repeated measures to examine the effect of location (trochlea or condyle) as well as that of chitosan-glycerol phosphate/blood treatment on International Cartilage Repair Society repair parameters. Cyst scores and biochemical data (glycosaminoglycan content, collagen content, and cell number) were analyzed with use of the Student t test. The level of significance was set at p < 0.05.

### Results

**Characterization of the Fresh Defects and Chitosan-Glycerol Phosphate/Blood Implant Retention**

During the clinical microfracture surgical procedure, it is normally recommended that the zone of calcified carti-
lage be removed without damaging the subchondral bone.
Therefore, in the present study, we aimed to remove the calcified cartilage without damaging the subchondral bone. Histological analysis of the fresh defects collected from four sheep one hour postoperatively revealed that it is difficult to consistently remove the entire zone of calcified cartilage, even when the microfracture procedure is carefully carried out. In the fresh treated or control defects, a similar average of 48% to 60% of the cross-sectional area of the calcified cartilage still remained, and the thickness of the remaining calcified car-

Fig. 1
Photographs of initial defects (A through F) and defects collected as long as one hour (H, K, L) or as long as 2.5 hours (G, I, J) after microfracture and in situ solidification of a chitosan-glycerol phosphate/blood implant. A and D: Lateral trochlear defect (A) and medial condylar defect (D) immediately after microfracture and rinsing with Ringer lactate solution. B and E: Punctuate bleeding was observed five minutes later in trochlear (B) and condylar (E) defects. C and F: Chitosan-glycerol phosphate/blood implant solidified in situ in trochlear (C) and condylar (F) defects. G and H: Fresh defects from an animal killed 1.5 hours after implantation of chitosan-glycerol phosphate/blood (G) and one hour after generation of the control defect (H). I through L: Decalcified and trimmed specimens showing chitosan-glycerol phosphate/blood clot solidified in defects from the trochlea (I) and condyle (J) and spontaneous bleeding in control defects from the trochlea (K) and condyle (L). The small black arrows in I through L indicate microfracture holes filled with blood.
lage varied considerably (Table II). Some residual noncalcified articular cartilage also remained (Table II), mainly at the edges of the fresh defects. Microfracture holes penetrated 2.5 to 3 mm into the subchondral bone plate. Except for the microfracture holes, the subchondral bone was intact.

All microfracture holes filled slowly with blood over a period of five minutes (Fig. 1, A and D compared with B and E). Approximately fifteen minutes after the generation of mi-

![Image]

**Fig. 2**
Histological appearance of paraffin-embedded sections obtained from freshly prepared defects collected one to 2.5 hours after defect generation. A and B: Low-magnification images of a section from the intact contralateral trochlear (A) or condylar (B) defect region. C and D: The middle of an initially created defect in the trochlea (C) or condyle (D) bisecting a microfracture hole (black arrow). Note that all normal blood clot was lost during paraffin histoprocessing, as previously observed. E through H: Control defects failed to retain any clot material in paraffin-embedded sections from the trochlea (E) or condyle (G), whereas defects with chitosan-glycerol phosphate/blood implant formed an adhesive bond between calcified cartilage and the implant in the trochlea (F) and condyle (H) that remained intact even after histoprocessing. Open arrowheads indicate chitosan stained with fast-green, and black arrowheads indicate clotted erythrocytes.
Microfracture holes, a dark bruise could be seen forming underneath the defects, indicating that subchondral bleeding was occurring between the microfracture holes. This bruise also was apparent in trimmed decalcified defects as a general discoloration of subchondral tissues (Fig. 1, I through L).

In all treated defects, a fresh mixture of chitosan-glycerol phosphate solution and peripheral whole blood was applied as a viscous liquid, which solidified in situ within seven to ten minutes (Fig. 1, C and F; Fig. 3, C1 and D1; and Fig. 4, C1 and D1). Fat droplets could be seen forming on the surface of chitosan-glycerol phosphate/blood before solidification. When two joints were cycled through a complete range of motion eight times, an adhesive layer of the implant remained in the defects. At the time of necropsy, a variable but higher average percentage of the defect surface was covered with chitosan-glycerol phosphate/blood implant as compared with normal clot in both condylar and trochlear defects (Table II and Fig. 1, G and H). More clot was present in fresh trochlear defects, which faced a smooth patellar surface, as compared with condylar defects facing the meniscus, tibial plateau, and fat...
pad, which is quite prominent in the sheep knee. In one control condylar defect that had been treated with microfracture only, the clot was limited to the microfracture holes (Fig. 1, H).

Evaluation of paraffin-embedded sections of these fresh defects revealed that all normal blood clot material had been lost during histoprocessing (Fig. 2, C, D, E, and G), which is consistent with the findings reported by previous investigators. However, chitosan-glycerol phosphate/blood adhered tightly to the calcified cartilage and bone and was partly retained on the surface of treated defects after histoprocessing (Fig. 2, F and H, open arrowhead).

**Histological and Biochemical Evaluation of Six-Month Repair Tissues**

After six months of repair, all condylar repair tissue was glossy, white or grey, and firmly attached (Fig. 3). Some condylar repair tissues contained a cleft communicating with a subchondral cyst (Fig. 3, B2, open arrow). Treated condylar defects were macroscopically resurfaced with more repair tissue. New

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**Fig. 4**

Representative trochlear defects and corresponding six-month repair tissues. A1 through B4: Control (microfracture-only) defects from animals 16 and 22. C1 through D4: Defects treated with microfracture and chitosan-glycerol phosphate/blood from animals 3 and 20. Trochlear defects were created in animals with an impact injury to the condyle made three weeks before this surgery (A1 through A4 and C1 through C4) or in healthy stifles (B1 through B4 and D1 through D4). A1, B1, C1, and D1: Macroscopic appearance immediately after microfracture and after delivery of chitosan-glycerol phosphate/blood (C1 and D1). A2, B2, C2, and D2: Macroscopic appearance after six months of repair showing the location of sections (arrow and double arrow). A3, B3, C3, D3, A4, B4, C4, and D4: Safranin-O/fast-green-stained sections of each trimmed plane in the defect. Arrowheads show original defect boundaries. White arrowheads indicate less safranin O-stained repair tissue in distal as compared with proximal regions in trochlear defects.
repair tissue had failed to cover residual cartilage, either calcified or noncalcified. The percentage of the defects covered with residual calcified cartilage was 9% in treated condylar defects compared with 24% in control defects (Table II). The amount of residual noncalcified cartilage in treated condylar defects was similar to that of controls (percentage covered, 6% in treated and 9% in controls) (Table II). Compared with microfracture-only defects, treated condylar defects were filled with a greater cross-sectional area of repair tissue (percent fill, 52% in treated and 31% in controls) (Table II) and demonstrated a significantly higher percentage of hyaline repair (86% in treated and 71% in controls) (Table II), which, when taken together, resulted in a significant increase in combined percent fill and percent hyaline repair over microfracture-only controls (p < 0.05). The average thickness of the repair tissue was greater in treated than in untreated defects but only attained half the thickness of the articular cartilage in the contralateral, intact joints (Table II). According to a modification of the International Cartilage Repair Society histological scoring system and control (B, D, and F) defects. More hyaline repair was seen in the condyle (A and B) than in the trochlea (C through F). Distal trochlear repair tissues closest to the intercondylar notch were consistently glycosaminoglycan-depleted (E and F). The arrow in B indicates tidemark formed below columnar repair cartilage. (Scale bar = 100 µm.)

Fig. 5
Histologic appearance of safranin O/fast-green-stained repair cartilage after six months of repair. Example repair tissues from treated (A, C, and E) and control (B, D, and F) defects. More hyaline repair was seen in the condyle (A and B) than in the trochlea (C through F). Distal trochlear repair tissues closest to the intercondylar notch were consistently glycosaminoglycan-depleted (E and F). The arrow in B indicates tidemark formed below columnar repair cartilage. (Scale bar = 100 µm.)
erally showed improved repair tissue integration, which also depended on percent fill (Fig. 3), although many repair tissues were still not fully integrated with the adjacent articular cartilage after six months of repair.

Repair tissue biopsy specimens from treated condylar defects contained nearly twofold more glycosaminoglycan, collagen, and cells compared with biopsy specimens from control defects treated with microfracture alone (Table IV, site 4). These treatment-dependent higher glycosaminoglycan and collagen levels were nearly significant \( (p \approx 0.06) \), despite the low sample number from the control group (three of six) due to the fact that half of the control defects were devoid of repair tissue at biopsy site 4. Both treated and control repair tissues had an identical average glycosaminoglycan/cell ratio of 0.9 ng/cell (Table IV, site 4).

In intact, contralateral joints, significant site-specific biochemical differences were found. The central part of the medial femoral condyle had the highest glycosaminoglycan/cell ratio (3.0 ng/cell), and the distal part of the trochlea had the lowest glycosaminoglycan/cell ratio (0.5 ng/cell) (Table IV, sites 4 and 2, respectively). Repair tissue from treated condylar defects contained significantly higher cell densities (57 compared with 16 million cells/g wet weight), increased collagen per wet mass (140 compared with 111 mg/g wet weight), and similar glycosaminoglycan content (49 compared with 48 mg/g wet weight) compared with articular cartilage from matching sites in intact joints (Table IV, site 4).

Trochlear repair tissue was macroscopically heterogeneous compared with condylar repair tissue, with either cobblestone tufts over the microfracture holes, a dense white or

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**Fig. 6**

Immunohistochemistry analysis of collagen type II and collagen type I in six-month repair tissue in chitosan-glycerol phosphate/blood-treated defects from animal 20 (A through F) and animal 9 (G, H, and I). A, B, C, G, and H: Condylar repair tissue. D, E, F, and I: Trochlear repair tissue. A and D: Safranin O-stained sections adjacent to those sections stained with monoclonal antibodies specific for collagen type II (B and E) and collagen type I (C and F). Positive immunostaining was revealed by red substrate, with hematoxylin counterstain, resulting in a purple hue for collagen type-I staining in bone. The arrowheads in A, B, C, D, E, F, and I delineate the original defect borders. The open arrow in F indicates red collagen type-I positive staining in trochlear repair cartilage. G: In collagen-type-II-stained condylar repair tissue, columnar repair tissue originated from porous bone. H: The collagen-type-II-stained fibers were often present as looped structures that originated from the bone and formed an acute intensely stained arc at the surface of the columnar repair tissue. I: Collagen type I immunostain showing a seamless integration of repair tissue with trochlear articular cartilage and a seamless integration of bone below the treated defect with the subchondral bone plate. The black arrowhead indicates the junction of repair cartilage (RC) and adjacent cartilage at the original tidemark, and the double-headed arrow indicates calcified cartilage thickness in adjacent cartilage.
whitish-grey covering, or a spongy white uniform covering (Fig. 4, A2, B2, C2, and D2). Histomorphometrically, treated defects had higher percent fill (94% compared with 69%), mainly because some treated defects showed modest bone resorption of the defect base, which led to some defects having >100% fill (Table II; Fig. 4, D4). In many trochlear defects, the defect base was irregular with respect to the adjacent tidemark (Table III, parameter VI). Compared with condylar repair tissue, trochlear repair tissue was significantly less hyaline on histomorphometric analysis (p < 0.05) and was significantly less hyaline (p < 0.05) and columnar (p < 0.0001) on histological analysis (Table III, parameters II and III). Consistent with histological scoring for fibrocartilage, an appreciable amount of collagen type I was detected in trochlear repair sections on immunohistochemical analysis (16% to 19% of repair tissue area) (Table II; Fig. 6, F). Fibrous trochlear repair was most frequently observed close to the intercondylar notch (Fig. 4, B4 and D4, white arrowhead). Biochemical analysis of trochlear repair tissue taken from near the notch revealed that this tissue was consistent with fibrous tissue, with very high cellularity, high levels of collagen, and significantly less glycosaminoglycan compared with condylar repair tissue (p < 0.01) (Table IV, site 2). A beneficial effect of treatment was noted for the trochlear defects; specifically, trochlear repair tissue in treated defects had a higher average percentage of type-II collagen staining (87% as compared with 71% of the repair tissue area) (Table II) and demonstrated a significant increase in combined percent fill and percent hyaline repair as compared with that in control defects that had been treated with microfracture only (p < 0.05). Trochlear repair tissue integration was generally better than condylar repair tissue integration (Fig. 6, I).

**Safety Outcome Measures**

At the time of necropsy at six months, safety tests indicated that treatment of defects with chitosan-glycerol phosphate/blood did not give rise to any lasting lameness, effusion, or systemic inflammation. A similar average amount of synovial fluid (approximately 0.25 mL) was found in the joints with treated or untreated defects. None of the sheep were lame at six months, with the exception of one sheep in the treatment group that had mild lameness. Subchondral cysts with varying grades of severity were observed beneath all but one control condylar defect (Fig. 3, A and B). In defects that had been treated with chitosan-glycerol phosphate/blood, cysts were absent or much smaller (Table I; Fig 3, C and D). When evaluated on a scale of 0 to 4 (with 0 indicating no cyst and 4 indicating a large cyst), the severity of cysts was attenuated 50% by treatment (median, 0.5 compared with 1.25), but, with the sample size used in the present study, this effect was not significant. In operated joints, trochlear samples proximal to the defects showed no biochemical differences compared with matching unoperated sites (Table IV, site 1), ruling out generalized joint degeneration. In contrast, condylar samples proximal to the defects showed a selective and significant (~20%) decrease in collagen content per wet weight in both treated and control joints compared with matching unoperated sites (Table IV, site 3).

**Discussion**

Previous cartilage repair studies in horses demonstrated that carpal, trochlear, or condylar defects were partly filled with repair tissues that were partly hyaline, with partial collagen type-II content and relatively low glycosaminoglycan levels, after four to twelve months of repair. Suboptimal repair in some of those studies potentially could be attributed to the use of trochlear defects; our data indicated that trochlear defects had significantly less hyaline repair and glycosaminoglycan content compared with condylar defects, results that are consistent with the findings reported by others. In the present study, condylar defects that had been treated with microfracture alone were partly filled with repair tissue (percent fill, 31%) that was partly hyaline (71%) and had low glycosaminoglycan levels (27 mg/g wet weight). Application of a chitosan-glycerol phosphate/blood clot to condylar defects resulted in a net increase in fill (52%), a modest

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**TABLE III International Cartilage Repair Society Histological Scoring of Six-Month Postoperative Repair**

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Medial Femoral Condyle</th>
<th>Lateral Trochlea</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Surface (3 = smooth, 0 = irregular)</td>
<td>1.6</td>
<td>2.3</td>
</tr>
<tr>
<td>II Matrix (3 = hyaline, 0 = fibrous)</td>
<td>1.7†</td>
<td>2.1§#</td>
</tr>
<tr>
<td>III Cell organization (3 = columnar, 0 = random)</td>
<td>1.2**</td>
<td>1.7**</td>
</tr>
<tr>
<td>IV Viability (3 = viable, 1 = partially viable, 0 = few viable)</td>
<td>2.3</td>
<td>2.5</td>
</tr>
<tr>
<td>V Bone (3 = normal, 2 = remodeled, 1 and 0 = detached and/or necrotic)</td>
<td>1.6</td>
<td>2.0</td>
</tr>
<tr>
<td>VI Calcified cartilage (3 = normal, 0 = abnormally high/low)</td>
<td>2.2</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*Control = microfracture only. †Treated = microfracture and chitosan-glycerol phosphate/blood implant. §p < 0.05 compared with trochlear defects. #p < 0.01 compared with trochlear defects. **p < 0.0001 compared with trochlear defects.
but significant increase in hyaline character (86%), and a complete restoration of normal glycosaminoglycan levels (49 mg/g wet weight) (Tables II, III, and IV). To our knowledge, this high level of glycosaminoglycan content has never been reported in cartilage repair tissues from skeletally mature adult large-animal models. Collagen type II was uniformly detected throughout both treated and control condylar repair tissues, and yet much less glycosaminoglycan was detected in repair tissue in defects that had been treated with microfracture only. This suggests that collagen type II expression is necessary, but not sufficient to retain high glycosaminoglycan levels in repair tissues. Our collective data suggest that the more cellular repair tissue (Table IV) with columnar organization (Table III) elicited by chitosan-glycerol phosphate/blood clots in the condyle led to the deposition of more cartilage repair tissue (Table IV) with greater hyaline character (Tables II, III, and IV), compared with microfracture alone.

Our biochemical analyses showed that collagen was selectively depleted in proximal condylar sites of joints with operatively created defects. High load-bearing on the defect edge in the condyle may have led to damage and swelling of the collagen network, resulting in higher water content. These same effects were not seen in the less load-bearing proximal trochlear sites. Trochlear defects were filled with more repair tissue than condylar defects were, but the repair tissue was much more fibrous (Table III).
Subchondral Cysts and Retention and Stability of the Calcified Cartilage Layer

Subchondral cyst formation has been previously observed in large-animal cartilage-repair models when the subchondral plate was violated by débridement or drilling, followed by immediate postoperative weight-bearing. A lack of bone replacement of the damaged subchondral plate has also been seen as long as five to twelve months after surgery. Thus, although much available data suggest that percent fill may be improved by a more exuberant débridement step in these surgical procedures, one potential downside could be that extensive damage to the subchondral plate, via excessive débridement or communicating microfracture holes, could cause pathological bone resorption and subchondral cyst formation. Although not previously reported in animal or clinical studies, the subchondral bruising that we observed under the microfractured defects also may be related to subchondral cyst formation. Given that subchondral cysts have only rarely been mentioned in the clinical literature as a complication of drilling or microfracture, we may assume that such cysts are asymptomatic, undetected, resolve spontaneously, or simply are not produced when controlled rehabilitation during the first eight weeks postoperatively prevents full load-bearing. In situ solidification of the chitosan-glycerol phosphate/blood implant over the microfractured defects decreased the frequency and severity of these cysts (Table I). One possible mechanism behind this treatment-induced suppression of cyst formation may involve angiogenic or osteoconductive properties of chitosan, which could facilitate a more effective repair of the subchondral bone damaged by microfracture.

Retention of the calcified layer and its persistence during cartilage repair has often proved to be mutually exclusive with resurfacing and integration of repair tissue in cartilage defects in dogs and horses. The present study in sheep demonstrated similar findings (for example, Fig. 3, A4, empty arrowhead), suggesting that residual cartilage can be a barrier to marrow-derived repair. In the present study, after six months of repair, a comparable percentage of the defects was covered with residual noncalcified cartilage in treated and control defects (6% and 9%, respectively) compared with fresh defects (10%) (Table II). This is in contrast with the residual calcified cartilage layer in the medial femoral condyle, which was detected in fresh defects (percentage covered, 54%) and to a lesser extent in control defects (24%) and treated defects (9%) (Table II). This observation, combined with the repair tissue that was seen to be uniformly adhering to bone at six months (Fig. 3, C3 and D3, and Fig. 6, G and I), suggests that the calcified cartilage can be resorbed during repair and that chitosan-glycerol phosphate/blood implants increased resorption of residual calcified cartilage. Similar observations have been previously made where implantation of slowly degrading biomaterials (such as collagen or polylactic acid fibers) into chondral defects can result in resorption of the calcified layer and subchondral bone in dog and goat. Furthermore, recently published data suggest that implantation of autologous cells also can result in a local resorption of the calcified layer and subchondral bone in dogs, goats, and human patients, thereby soliciting a marrow response.

Clinical Relevance and Limitations of the Animal Model

Our model was designed to examine clinically relevant aspects of cartilage repair, including different sites (i.e., the condyle and trochlea) and the repair of posttraumatic lesions. The entire procedure of arthroscopy, defect creation, and application of the implants took place within ninety minutes, a clinically acceptable time-frame. Terminal assessments were performed at six months after microfracture, a time at which patients typically are permitted full running and jumping activities, although many patients only experience optimal clinical improvement two years after microfracture. Large-animal chondral defects (area, 1 cm²; depth, 1.5 mm; volume, 150 µL) might be anticipated to heal in a manner closer to human defects as compared with defects in small, inbred laboratory animals such as rabbits (area, 0.02 cm²; depth, 100 µm; volume 2 µL). Also, we opted to use skeletally mature sheep because we believed that they would be more comparable to the patient population in need of treatment and because adolescent animals have a much greater potential for spontaneous repair.

All animals were allowed immediate postoperative weight-bearing, which had an unknown effect on implant retention. No chitosan was detected in any of the sixteen treated defects that were examined at six months, which is reasonable in light of previous reports indicating that chitosan is readily degraded and cleared. In blood and synovial fluid analyses carried out at six months postoperatively, there was no evidence that the chitosan-glycerol phosphate/blood implant caused any chronic inflammation. Collectively, these data suggest that chitosan-glycerol phosphate/blood implants exert an efficacious effect on cartilage repair without any toxicity at these dose levels.
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