Chitosan–glycerol phosphate/blood implants elicit hyaline cartilage repair integrated with porous subchondral bone in microdrilled rabbit defects


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Summary

Objective: We have previously shown that microfractured ovine defects are repaired with more hyaline cartilage when the defect is treated with in situ-solidified implants of chitosan–glycerol phosphate (chitosan–GP) mixed with autologous whole blood. The objectives of this study were (1) to characterize chitosan–GP/blood clots in vitro, and (2) to develop a rabbit marrow stimulation model in order to determine the effects of the chitosan–GP/blood implant and of debridement on the formation of incipient cartilage repair tissue.

Methods: Blood clots were characterized by histology and in vitro clot retraction tests. Bilateral 3.5 × 4 mm trochlear defects debrided into the calcified layer were pierced with four microdrill holes and filled with a chitosan–GP/blood implant or allowed to bleed freely as a control. At 1 day post-surgery, initial defects were characterized by histomorphometry (n = 3). After 8 weeks of repair, osteochondral repair tissues between or through the drill holes were evaluated by histology, histomorphometry, collagen type II expression, and stereology (n = 16).

Results: Chitosan–GP solutions structurally stabilized the blood clots by inhibiting clot retraction. Treatment of drilled defects with chitosan–GP/blood implants led to the formation of a more integrated and hyaline repair tissue above a more porous and vascularized subchondral bone plate compared to drilling alone. Correlation analysis of repair tissue between the drill holes revealed that the absence of calcified cartilage and the presence of a porous subchondral bone plate were predictors of greater repair tissue integration with subchondral bone (P < 0.005), and of a higher total O’Driscoll score (P < 0.005 and P < 0.01, respectively).

Conclusions: Chitosan–GP/blood implants applied in conjunction with drilling, compared to drilling alone, elicited a more hyaline and integrated repair tissue associated with a porous subchondral bone replete with blood vessels. Concomitant regression of a vascularized bone plate during cartilage repair could provide progenitors, anabolic factors and nutrients that aid in the formation of hyaline cartilage.

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Key words: Cartilage repair, Marrow stimulation, Chitosan, Animal model, Blood clot, Collagen type II, Bone repair, Histomorphometry.

Introduction

Partial thickness cartilage lesions fail to heal and often lead to further degeneration and joint disease1. Focal cartilage lesions can be resurfaced in human patients2 and in experimental defects in animals3,4 when residual cartilage is removed, and access to the vascularized bone marrow provided through abrasion, arthroplasty, or debridement and microfracture or drilling5–7. However, in both patients11 and animal models5–10, the resulting repair tissue is typically fibrocartilaginous or fibrous, tissue types known to have weak biomechanical properties and reduced wear capacity compared to hyaline cartilage, which contains high levels of collagen type II and glycosaminoglycans (GAG)5–12. Bone marrow-derived repair cells can give rise to a degree of cartilaginous repair tissue in young rabbits12–14, however, this repair occurs only sporadically in adult rabbits15–7. Repair processes, which reliably lead to hyaline cartilage regeneration and integration in skeletally mature defects have yet to be elucidated.

Since bleeding has been identified as an initiating event in post-surgical repair2–4, we hypothesized that microfracture-based repair could be improved by stabilizing the clot formed in the lesion with a polymer that is thrombogenic and actively stimulates the wound repair process. Chitosan is a positively charged polymer composed of (1 → 4)-β-linked glucosamine and acetyl-glucosamine residues. Previous studies
have shown that chitosan is hemostatic\textsuperscript{14–17} and when left in the wound site, stimulates revascularization of the wound and connective tissue repair\textsuperscript{14–18,20–25}. Chitosan is also biodegradable\textsuperscript{7,13,21,22}, non-immunogenic\textsuperscript{7,13,21,22}, and adheres to connective tissues including cornea, bone, and cartilage\textsuperscript{25,26}. We discovered a means of solubilizing chitosan under physiological conditions by combining it with glycerol phosphate to form chitosan—glycerol phosphate (chitosan—GP) solutions\textsuperscript{27–29}. And in a recent study we showed that mixtures of whole blood with chitosan—GP could form solid implants above microfractured cartilage defects\textsuperscript{26}. These implants adhered more to the defect than normal clots, and elicited significantly more hyaline repair compared to microfracture alone in an ovine model at 6 months\textsuperscript{26}.

In the study presented here, we describe the development of a bilateral rabbit cartilage repair model to efficiently screen implants for their ability to improve repair of marrow-stimulated defects as well as to elucidate mechanisms of action. This model was used to test the hypothesis that hyaline cartilage repair after marrow stimulation depends upon the surgical approach and the use of chitosan—GP/blood implants. Chitosan—GP stabilized blood clots were made by combining chitosan—GP solutions with peripheral whole blood from human volunteers, or with blood from two distinct animal species used in cartilage repair studies, rabbit and sheep. Our surgical model was based on a rabbit model previously published by Mitchell and Shepard\textsuperscript{6}, representing a scaled-down version of human lesions that are treated by microfracture or drilling. This is in contrast to other rabbit models where repair was observed over single, large osteochondral drill holes\textsuperscript{13,30,31} which do not model human lesions. The generation of rectangular drill-leaf defects with precisely placed drill holes also permitted us to analyze the effect of treatment on repair tissue formed directly over the drill holes, compared to repair tissue formed over the defect surface between the drill holes.

**Methods**

**MATERIALS**

Ultrapure chitosan was obtained from BioSyntech (77.1% degree of deacetylation (DDA), <375 endotoxin units (EU)/g; 79% DDA, <3000 EU/g; 83.4% DDA, <500 EU/g free base from Laval, QC, Canada) or Pronova (84.5% DDA, 130 EU/g, HCl salt from Oslo, Norway). BioSyntech chitosan was dissolved at 1.66% w/v or at 1.89% w/v (taking into account loss on drying) in 60 mM to 83 mM HCl and autoclave-sterilized. Pronova chitosan was lyophilized, weighed, sterilized by exposing the powder to UV light for 90 min, and then dissolved in sterile Milli-Q-filtered water at 1.66% w/v, taking into account HCl salt content. Sterile chitosan solutions were combined with filter-sterilized disodium beta-glycerol phosphate (Tissue Culture grade, Sigma, St. Louis, MO) to yield transparent solutions with 1.5% w/v or 1.7% w/v chitosan with 135 mM glycerol phosphate (also called BST-CarGel\textsuperscript{8}), pH 6.8, osmolality of 470 mOsm, and viscosity of 250–1100 mPa s at 25°C, that were stored at ~80°C.

**BLOOD COLLECTION AND CHITOSAN—GP/BLOOD MIXTURES**

All protocols involving animals were approved by institutional animal care committees and all protocols involving human subjects were approved by an Internal Review Board Ethical Committee. Peripheral whole blood was collected aseptically from the central ear artery from 15 New Zealand White rabbits with atravel or ketamine/xylazine/buprenorphine and isoflurane gas anesthesia and the jugular vein of 10 sheep with or without ketamine/diazepam and halothane gas anesthesia. Anesthetics can affect platelet function\textsuperscript{32}, however, whole blood clotting times from anesthetized animals and unanesthetized humans were similar to published values\textsuperscript{33}. Venous blood was collected aseptically from non-fasting healthy volunteers (n = 7, 23–43 years old). Non-activated whole blood was drawn into plastic syringes after a 2 mL first discard, with a 21-gauge 3/4 inch butterfly needle with female luer and outlet needle. Homogeneous aseptic mixtures were obtained by filling 4.0 mL Wheaton glass vials with plastic screwcaps to capacity with 1.1 mL chitosan—GP solution, 3.3 mL fresh blood, and six surgical 316 stainless steel mixing beads (4.5 mm diameter, Saiem Specialty, Canton, CT), closing the vials, and shaking vigorously for 10 s.

**BLOOD SMEARS, CLOTTING TIME, CLOT RETRACTION, AND HEMOLYSIS MEASUREMENTS**

Blood smears were generated with blood or chitosan—GP/blood mixtures within 3 min after mixing and stained with May Grunwald Geimsa (Sigma, St. Louis, MO). Clotting time was measured using the Lee—White clotting test in dry, sterile, non-siliconized glass tubes with vented steel caps and 0.5 mL sample volumes\textsuperscript{34}. Tubes containing 0.5 mL solid clot samples were transferred to a humidified 37°C incubator for 4 h to determine percent retraction by weighing separately the excluded serum and mass of the solid clot. Hemolysis was determined by serum hemoglobin absorbance at OD\textsubscript{540} using an enzyme-linked immunosorbent assay (ELISA) plate reader, modified from a previous method\textsuperscript{35}.

**FEASIBILITY STUDIES**

To test the effect of removal of the calcified cartilage layer on repair with or without implant, bilateral defects were created identically in each trochlear groove in the knees of skeletally mature rabbits. In three rabbits, defects were debrided, but not beyond, the calcified cartilage while in another three rabbits, bilateral defects were completely debrided of the calcified layer to bleeding cancellous bone. All defects were then pierced with four or five, 0.9 mm diameter 3 mm deep holes with either a drill bit or a custom-made pick and hammer. Chitosan—GP/blood implant was solidified in one defect while the contralateral defect was allowed to bleed as a surgical control. Defects were allowed to repair for 7–11 weeks then fixed, decalcified, processed in LR White plastic resin, stained with Toluidine Blue and analyzed histologically.

**RABBIT CARTILAGE REPAIR MODEL**

Thirty-eight bilateral defects were created in the knees of 19 skeletally mature New Zealand White rabbits (9–15 month old females. 4.6 ± 0.8 kg, Table I). Three of these rabbits were used to evaluate the initially debrided defects at 1 day post-op while defects in the remaining 16 rabbits were evaluated at 8 weeks post-op. Rabbits were anesthetized by an intramuscular injection of ketamine/xylazine/buprenorphine, and maintained on 3% isoflurane/oxygen. The ears (phlebotomy site) and legs were shaved and disinfected with Baxedin, poviodine, and 70% ethanol.
Cartilage defects were created in each trochlear groove using sequential bilateral arthroto- mies with a medial parapatellar incision followed by patellar luxation. Joint surfaces were kept moist with sterile phosphate buffered saline (PBS) irrigation every 30–60 s to maintain viability of cells in the exposed articular and calcified cartilages. Chondral defects (3.5 × 4 mm) were made in the center of the trochlear groove to a depth of around 200 μm, 3–5 mm above the lateral collateral ligament insertion, by scraping with 1.5–2.75 mm flat surgical blades (Fine Science Tools, North Vancouver, B.C., Canada) into the calcified cartilage. Four, 3–4 mm deep microdrill holes were then generated in each of the corners of the defect using a high-speed, hand-held microdrill and 0.9 mm diameter drill bit (Fine Science Tools, North Vancouver, B.C., Canada) into the calcified cartilage. The patella was repositioned and the knee closed in supraspinatus fibers within 7 min of mixing where the implant solidified. Histologically identified 8 (left knee) 1 day, 3 (left knee) 1 day, 8 (left knee) 1 day, and 8 (right knee) 8 weeks, percent of integrated repair tissue area was determined as the cross-sectional area of the defect (Table I) to measure bone porosity. Histologically identified pores were traced with a line tool to render them more visible. A grid with 19.75 μm thick sections were generated from the middle of each defect and stained with Safranin O/Fast Green. Defects after 8 weeks of repair were decalcified in 0.5 M HCl/0.1% glutaraldehyde for 7–10 days then trimmed and bisected transversely with a razor between the drill holes. The proximal defect half was embedded in LR White plastic resin, while the distal half was embedded in paraffin. Five to 20, 1 μm-thick plastic sections were generated in the proximal half of the repaired defects, including between the drill holes, within the drill holes, and proximal to the drill holes and stained with Toluidine Blue. Paraffin sections 6 μm thick were generated from the distal half within the drill holes, then dewaxed, digested with 2.5% w/v hyaluronidase and processed by immunohistochemistry as previously described for collagen type II using monoclonal II-II6B3 (DISH, Iowa, USA) at 1:10 dilution, biotinylated goat anti-mouse diluted to 22 μg/mL, and avidin-Alkaline Phosphatase-red substrate detection (Vector Laboratories Inc., Burlington, Ontario, Canada), followed by light Iron Hematoxylin counterstain. Control isotype antibody or omission of primary antibody showed no staining.

### Table I

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### Rabbit cartilage repair study design

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**QUALITATIVE HISTOLOGICAL SCORING AND QUANTITATIVE HISTOMORPHOMETRY**

Four to 12 sections from distinct areas within the proximal half of each defect were graded by a blinded observer using the O’Driscoll score, with the exception that staining intensity was determined with Toluidine Blue instead of Alcian Blue. The score for normal cartilage is 24. This evaluation revealed that the edge of the “tufts” of repair tissue covering the drill holes contained less differentiated cells than the center of the tufts, as previously reported by Mitchell and Shepard. Therefore, one section in the middle of the holes and one section between the holes were selected from each defect to compare O’Driscoll scores. All histomorphometric and stereologic analyses were performed on these same two plastic-embedded sections by a blinded observer, using a Zeiss Axiom microscope, digital camera, and histomorphometric software (Northern Eclipse, Empix Imaging, Mississauga, ON) to measure the width of the cross-section of each defect, the percent of different tissues covering the defect (repair tissue, bone or calcified cartilage), and the percent of repair tissue distinctly detached from the base of the defect. The same histomorphometric analysis for percent of different tissues covering the initial defects was performed on one section from the middle of each defect after 1 day of repair (n = 3 sections per condition). In sections from defects repaired for 8 weeks, percent of integrated repair tissue was determined as the total length of the repair tissue attached to the defect base divided by the defect width. Repair tissue area was determined as the cross-sectional area of tissues above subchondral bone, excluding bone, and excluding soft tissues occasionally present in the drill holes. Collagen type II staining in repair tissue above the subchondral bone was quantified by threshold analysis as the percent of total repair tissue area that stained pink and light red, vs a dark red hue that matched the staining intensity of normal cartilage.

**HISTOPROCESSING AND IMMUNOHISTOCHEMISTRY**

All clot samples were fixed after 1–4 h post-solidification in 4% glutaraldehyde/PBS or 10% buffered formalin and then either processed in LR White plastic resin and stained with Toluidine Blue, or processed in paraffin and stained with Safranin O/Fast Green/Iron Hematoxylin to identify chitosan by specific Fast Green-specific staining characteristics. Immediately after sacrifice, distal femora were recovered, dissected of all soft tissues and fixed for 3 days in 4% paraformaldehyde/1% glutaraldehyde/100 mM sodium cacodylate, pH 7.2. Defects from 1 day post-op were decalcified in ethylenediaminetetraacetic acid (EDTA), and cryosections generated from the middle of each defect and stained with Safranin O/Fast Green. Defects after 8 weeks of repair were decalcified in 0.5 M HCl/0.1% glutaraldehyde for 7–10 days then trimmed and bisected transversely with a razor between the drill holes. The proximal defect half was embedded in LR White plastic resin, while the distal half was embedded in paraffin. Five to 20, 1 μm-thick plastic sections were generated in the proximal half of the repaired defects, including between the drill holes, within the drill holes, and proximal to the drill holes and stained with Toluidine Blue. Paraffin sections 6 μm thick were generated from the distal half within the drill holes, then dewaxed, digested with 2.5% w/v hyaluronidase and processed by immunohistochemistry as previously described for collagen type II using monoclonal II-II6B3 (DISH, Iowa, USA) at 1:10 dilution, biotinylated goat anti-mouse diluted to 22 μg/mL, and avidin-Alkaline Phosphatase-red substrate detection (Vector Laboratories Inc., Burlington, Ontario, Canada), followed by light Iron Hematoxylin counterstain. Control isotype antibody or omission of primary antibody showed no staining.

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of all pores was manually counted. In addition, we counted the number of points (grid intersections) that fell within all bone pores ($P$) and within the entire bone sample ($B$), the latter serving as a reference volume. Pore surface area per reference volume ($S_v$ in $\mu m^2/\mu m^3$) was calculated according to the equation $S_v = I/(B \times 0.01975 \, mm)$. Pore volume per reference volume ($V_v$, in percent $m^3/m^3$) was obtained according to the equation $V_v = P/B$. Some degree of sampling bias was present in these measurements, due to the unavoidable anisotropy and non-random orientation of the osteochondral sections.

**STATISTICAL ANALYSIS**

To test the hypothesis that treatment affected repair, differences in O’Driscoll histological scores were analyzed by the Mann–Whitney $U$ test for one section per defect over the holes ($n = 16$) and one section per defect between the
holes (n = 16) (Statistica version 6.1, Tulsa, OK). Differences were considered significant for P exact values, P < 0.05. The Student's t test was used to determine whether clot retraction was significantly different between chitosan–GP and normal blood clots 4 h post-solidification, and whether chitosan–GP/blood implants significantly improved histomorphometric and stereological variables compared to blood clots alone. Correlation coefficients were obtained by multivariate analysis of covariate scatterplots (Statistica version 6.1, Tulsa, OK) with significance determined when P < 0.05.

Results

CHITOSAN–GP SOLUTIONS STABILIZE BLOOD CLOTS

Mixing experiments were performed in order to create hybrid chitosan blood clots that solidified within 10 min. Trials with different ratios of blood and chitosan–GP solutions revealed that homogeneous chitosan–GP/blood clots formed most rapidly using a ratio of 1 volume chitosan–GP to 3 volumes whole blood from rabbit, sheep, or human. After 1 h at 37°C, whole blood clots retracted up to 40% of their original mass, were stiff and non-elastic [Fig. 1(A,B)]. In contrast, hybrid chitosan–GP/blood clots containing final concentrations of 0.4% w/v chitosan and 34 mM glycerol phosphate were firm and elastic [Fig. 1(C,D)]. Mixture of chitosan without glycerol phosphate (pH 4.7) into whole blood resulted in unstable and brittle clots with substantial hemolysis that was not seen in normal clots or in chitosan–GP/blood clots (data not shown). Compared to normal clots [Fig. 1(E,G)], chitosan–GP/blood clots were comprised of a chitosan scaffold homogeneously dispersed among clumps of red blood cells (RBCs) with histologically visible fibrin-like fibers [Fig. 1(F,H)]. In blood smears generated from fresh chitosan–GP/blood mixtures, chitosan specifically mediated adhesion between RBCs (Fig. 1, compare panels I and J). Chitosan–GP/blood clots retracted significantly less than the normal clots [Fig. 1(A–D)] in three different species (P < 0.0001, Fig. 2).

EFFECT OF SURGICAL DEBRIDEMENT ON CARTILAGE REPAIR

In skeletally mature rabbits, the trochlear calcified cartilage layer had an average thickness of 77 ± 20 μm, sometimes attaining half the thickness of the non-mineralized articular cartilage, which in the area of the defects had an average thickness of 160 μm ± 41 μm (n = 6). A preliminary study was performed to examine the effect of debridement of the calcified layer on cartilage repair. Complete debridement of the calcified layer to the bleeding cancellous bone followed by marrow stimulation led to severe subchondral bone resorption and fibrous repair [Fig. 3(A,C,E)]. By comparison, defects that were debrided into the calcified layer, drilled, and treated with chitosan–GP/blood implant developed variable amounts of hyaline repair tissue [Fig. 3(B,D,F)]. Based on these results, our study was conducted using drilled defects that had been debrided into, but not beyond, the calcified layer [Fig. 4(A)]. Control defects were allowed to fill with bone-derived blood [Fig. 4(A), inset], while treated defects were filled with chitosan–GP/blood which formed a solid implant after 7–10 min [Fig. 4(B), Table I]. Histomorphometry of initial defects showed that an average 40% of the cross-section of the defect between the drill holes was covered by residual calcified cartilage, the remainder of the defect consisting of exposed subchondral bone [n = 3, Fig. 5(A)].
Treatment led to repair tissue with more intense staining for collagen type II over the drill holes \( P < 0.005 \), Figs. 5(D), 7. Treatment also led to greater hyaline cartilage morphology, Toluidine Blue staining intensity, slightly more cellularity, and higher total O'Driscoll scores \( (P \text{ values: } P < 0.05 \text{ to } P < 0.0005, \text{ Table II}) \). More remarkable histological differences between treated and control repair tissues were observed between the drill holes and these differences are summarized in Table II.

Fig. 3. Development of fibrous or hyaline repair following marrow stimulation depends on the extent of debridement. Bilateral trochlear defects were generated in the knees of skeletally mature rabbits by debriding the calcified cartilage layer completely and administering five microfracture holes (A) or by debriding into the calcified cartilage layer and piercing four microdrill holes (B). In each rabbit, one defect was allowed to fill with bone marrow-derived blood (for example, A, inset), while the contralateral defect was filled with chitosan–GP:blood which was allowed to clot \textit{in situ} (for example, B, inset). Repair at 7–11 weeks showed that defects debrided of the calcified layer had extensive subchondral bone resorption and fibrous tissue repair (C and E), while more hyaline repair was seen in defects debrided slightly into the mineralized zone with no visible punctuate bleeding that were then drilled, and treated with chitosan–GP:blood implant (D and F). Arrows: resorbed bone. AC: articular cartilage layer.

[Fig. 6(A,B,E,F); Table II, parameter VI]. Treatment led to repair tissue with more intense staining for collagen type II over the drill holes \( (P < 0.005, \text{ Figs. 5(D), 7}) \). Treatment also led to greater hyaline cartilage morphology, Toluidine Blue staining intensity, slightly more cellularity, and higher total O'Driscoll scores \( (P \text{ values: } P < 0.05 \text{ to } P < 0.0005, \text{ Table II}) \). More remarkable histological differences between treated and control repair tissues were observed between the drill holes and these differences are summarized in Table II.
TREATMENT ELICITED HYALINE REPAIR THROUGH A MECHANISM INVOLVING BONE REPAIR AND REMODELING

Hyaline repair was frequently associated with porous, vascularized subchondral bone [Fig. 6(D,H), open arrows; Fig. 7(E), dotted black circles]. In the superficial 250 μm of the repaired subchondral bone plate, treatment was found to increase the pore surface area (Sv) and pore volume (Vv) over the holes \( P < 0.005 \), [Fig. 5(E,F)]. This effect was partly due to the fact that in many controls, the microdrill holes had repaired with fibrous tissue instead of bone [Fig. 7(B)]. A lack of bone repair at 8 weeks in the microdrill holes was associated with fibrous or fibrocartilage repair [Fig. 7(C)]. Treatment also increased Sv and Vv between the holes \( P < 0.005 \), consistent with the observation that treatment elicited remodeling of the subchondral bone plate between the drill holes at earlier time points, between 2 and 5 weeks post-surgery. Sv correlated positively with Vv for all 32 defects \( P < 0.0001 \), approximately doubling in...
both cases, thereby maintaining a similar pore volume to surface area ratio. These data suggest that gross pore morphology was not altered by treatment, but rather that the pore number was increased by treatment throughout the bone immediately below the defect. The average Sv of the subchondral bone plate below control vs treated defects [6 vs 10 mm²/mm³, Fig. 5(E)] was in-between Sv values previously obtained for cortical bone vs cancellous bone (2.5 vs 20 mm²/mm³).40

A covariate correlation analysis of all defects (n = 32) revealed that the absence of calcified cartilage was a significant predictor of integration of repair tissue with the bone pore surface area per reference volume (Sv), or (F) bone pore volume per reference volume (Vv). Symbols: $\# = samples from day 1 post-operative; *P < 0.05, **P < 0.005, and ***P < 0.0005, compared to control; $# < 0.05 for detached repair tissue compared to treated, and $###P < 0.005 for integrated repair tissue compared to control.

### Fig. 5. Histomorphometric and stereological analyses. Initial defects repaired for 1 day ($n = 3$) were analyzed only for residual calcified cartilage (A) while defects repaired for 8 weeks ($n = 16$) were submitted to all of the analyses (A–F). All data are given as the average ± standard deviation. (A) Percent of defect width covered by residual calcified cartilage was quantified between the drill holes of initial defects, and between or through the drill holes of 8 week repaired defects. (B) Cross-sectional repair tissue area over and between the holes. (C) Percent of repair tissue covering the cross-sectional area of the defect that was integrated (white and black bars) or detached (hatched bars) from the base of the defect. (D) Percent collagen type II staining tissue (light staining, grey or black bars; intense staining, striped bars) detected in the total repair tissue area over the distal drill holes. Stereological analysis of the superficial 250 μm region of the subchondral bone plate for (E) bone pore surface area per reference volume (Sv), or (F) bone pore volume per reference volume (Vv). Symbols: $\# = samples from day 1 post-operative; *P < 0.05, **P < 0.005, and ***P < 0.0005, compared to control; $# < 0.05 for detached repair tissue compared to treated, and $###P < 0.005 for integrated repair tissue compared to control.

### Fig. 6. Histology of Toluidine Blue-stained plastic sections taken from between the drill holes of bilateral defects at 8 weeks. Control defects (A, C, E, and G) and contralateral treated defects (B, D, F, and H) are shown for the bilateral defects with tufts of repair tissue shown in Fig. 4(C, D) (panels A through D), or the completely resurfaced bilateral defects from Fig. 4(E, F) (panels E through F). Hyaline repair was defined according to O’Driscoll31 as a repair tissue containing cells with a chondrocyte morphology surrounded by distinct lacunae, and more intense Toluidine Blue staining. Subchondral bone pores associated with hyaline repair (D and H) contained blood vessels (open arrowheads).
subchondral bone both over \((P < 0.005)\) and between microdrill holes \((P < 0.01)\). Other predictive relationships between variables were only observed between the drill holes and not over the drill holes. Between drill holes, the absence of calcified cartilage predicted a higher total O'Driscoll score \((P < 0.005)\), while a higher bone pore surface area (Sv) in the subchondral plate was a predictor of better repair tissue integration \((P < 0.005)\), and a higher total O'Driscoll score \((P < 0.01)\).

Discussion

CLOT STABILIZATION BY CYTOCOMPATIBLE CHITOSAN SOLUTIONS

Chitosan--GP/blood clots are novel implants with wound-healing properties originating both from whole blood components, and the chitosan itself. Acidic chitosan solutions and solid chitosan substrates have been previously shown to be pro-coagulatory\(^{14}\), by generating a RBC thrombus\(^{16}\), and by promoting platelet aggregation and degranulation\(^{15-17,41-43}\). Our data now extend these observations to show that liquid mixtures of whole blood and chitosan--GP can generate solid, homogeneous polymer-reinforced hybrid clots. Chitosan--GP solutions mediated erythrocyte-erythrocyte adhesion, suggesting that the surgically implanted chitosan--GP/blood clot implants had greater bonding between RBCs than normal clots. The available data\(^{31,44}\) suggest that platelets are activated in the hybrid chitosan--GP/blood clots, but are somehow inhibited from fully retracting the fibrin network. Clot retraction can be inhibited by interruption of the fibrin--platelet integrin receptor complex\(^{45}\) via blocking antibodies\(^{46}\) or genetic defects in the platelet integrin receptor.
significantly less hyaline cartilage repair than condylar could be partly related to the early time point studied, marrow stimulation alone. The repair tissue in treated cartilage in marrow-stimulated defects compared to implants elicited a more hyaline and cellular repair slightly, and contained thin fibrin-like fibers, it nonetheless blood clots solidified after a 5
with fibrin

GPⅡb, GPⅢa)45,47, suggesting that chitosan may interfere with fibrin–platelet interactions. Given that chitosan—GP/blood clots solidified after a 5–10 min delay, retracted slightly, and contained thin fibrin-like fibers, it nonetheless appears that the clotting cascade and generation of a fibrin network are part of the structural stabilization of the hybrid chitosan—GP/blood clot.

CHITOSAN—GP/BLOOD CLOTS STIMULATE CHANGES IN THE SUBCHONDRAL BONE PLATE THAT LEAD TO HYALINE CARTILAGE REPAIR

A bilateral rabbit cartilage repair model was developed to study the effect of chitosan—GP/blood implants on cartilage repair following marrow stimulation. After 8 weeks of repair, a similar average amount of repair tissue was formed in control and treated defects although the repair tissue integration and quality were significantly improved by treatment. In this rabbit study, as well as in our previous ovine cartilage repair study46, chitosan—GP/blood implants elicited a more hyaline and cellular repair cartilage in marrow-stimulated defects compared to marrow stimulation alone. The repair tissue in treated rabbit defects was still somewhat heterogeneous and was not entirely composed of hyaline cartilage, which could be partly related to the early time point studied, and also to the use of trocheal defects which developed significantly less hyaline cartilage repair than condylar defects in sheep56. We have found that an 8-week repair period in this particular rabbit model was a minimal yet sufficient time period needed to measure a significant improvement in repair over controls. Longer repair periods in this rabbit model will allow us to characterize repair tissue durability.

The aim of marrow stimulation is to generate access channels for repair cells, including pluripotent bone marrow stromal cells, to migrate into the chondral defect and possibly give rise to chondrocytes and hyaline cartilage matrix. In all drilled defects, hyaline cartilage repair was frequently detected in areas where bone repair or remodeling gave rise to a porous bone plate [Figs. 5(E,F), 6(B), 7(E)], while fibrous or fiброcartilage tissue was principally observed in regions of failed bone repair [Figs. 3(C), 7(B)] and in regions of failed repair tissue—bone integration [Figs. 5(C), 6(G), 7(B), Table II, between holes]. Taken together, these observations are consistent with the notion that chondral precursors can gain access to the defect by regenerating or remodeling bone. The failure of integrated repair tissue to form over large areas of calcified cartilage in this study [Fig. 6(A)], and in other studies3–10,48,49 could be partly explained by the inability of marrow-derived repair cells to adhere to, and migrate over, large areas of mineralized tissue containing negatively charged GAG. Repair tissue was able to form over debrided bone in controls [Fig. 5(B), 6(G), 7(B)], however, the tissue frequently failed to adhere [Fig. 5(C)], as previously observed after resurfacing procedures in rabbit6 and human patients2.

The formation of more integrated and hyaline tissue by chitosan clot implants could be related to several mechanisms. Residual chitosan clot implant adhering to trocheal calcified cartilage and bone26 could potentially provide an adhesive surface onto which repair tissues growing from the marrow holes could attach. Stimulation by chitosan clots of bone remodeling between the drill holes39, and remodeling and resorption of the calcified cartilage layer [Figs. 5(A), 6(B)] could give direct access to the defect by marrow cells in the bone plate, including blood microvessel pericyte chondral progenitors56. Finally, chitosan clots stimulated cell recruitment and transient subchondral angiogenesis during acute repair39, which in this study, and also in our ovine model26, led to the generation of a porous, revascularized subchondral bone plate [Figs. 5(E,F), 6(D,H), 7(E)]. Cartilage repair tissue integrated with a local vascular supply protected by bone could serve to provide progenitors, oxygen and nutrients, or other marrow-derived factors that stimulate chondrogenic proliferation, differentiation, and hyaline matrix production.

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