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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 clots 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 regeneration of a vascularized bone plate during cartilage repair could provide progenitors, anabolic factors and nutrients that aid in the formation of hyaline cartilage.

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 disease[1]. Focal cartilage lesions can be resurfaced in human patients[2–4] and in experimental defects in animals[5–10] when residual cartilage is removed, and access to the vascularized bone marrow provided through abrasion arthroplasty, or debridement and microfracture or drilling[2–4]. However, in both patients[11] and animal models[5–10], 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 rabbits[7,12,13], however, this repair occurs only sporadically in adult rabbits[5–7]. 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 repair[2–4], 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

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have shown that chitosan is hemostatic[14–17], and when left in the wound site, stimulates revascularization of the wound[14] and connective tissue repair[14,18–20]. Chitosan is also biodegradable[17,21,22], non-immunogenic[23,24], and adheres to connective tissues including cornea, bone, and cartilage[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[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[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[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[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[13,30,31] which do not mimic human lesions. The generation of rectangular trochlear 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 MilliQ-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®), 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 atravet 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[32], however, whole blood clotting times from anesthetized animals and unanesthetized humans were similar to published values[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 screw-caps 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, Salem 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[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_{530} using an enzyme-linked immunosorbent assay (ELISA) plate reader, modified from a previous method[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 into, 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.