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Subchondral chitosan/blood implant-guided bone plate resorption and woven bone repair is coupled to hyaline cartilage regeneration from microdrill holes in aged rabbit knees

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SUMMARY

Objective: Little is known of how to routinely elicit hyaline cartilage repair tissue in middle-aged patients. We tested the hypothesis that in skeletally aged rabbit knees, microdrill holes can be stimulated to remodel the bone plate and induce a more integrated, voluminous and hyaline cartilage repair tissue when treated by subchondral chitosan/blood implants.

Design: New Zealand White rabbits (13 or 32 months old, $N = 7$) received two 1.5 mm diameter, 2 mm depth drill holes in each knee, either left to bleed as surgical controls or press-fit with a 10 kDa (distal hole: 10K) or 40 kDa (proximal hole: 40K) chitosan/blood implant with fluorescent chitosan tracer. Post-operative knee effusion was documented. Repair tissues at day 0 ($N = 1$) and day 70 post-surgery ($N = 6$) were analyzed by micro-computed tomography, and by histological scoring and histomorphometry (SafO, Col-2, and Col-1) at day 70.

Results: All chitosan implants were completely cleared after 70 days, without increasing transient post-operative knee effusion compared to controls. Proximal control holes had worse osteochondral repair than distal holes. Both implant formulations induced bone remodeling and improved lateral integration of the bone plate at the hole edge. The 40K implant inhibited further bone repair inside 50% of the proximal holes, while the 10K implant specifically induced a "wound bloom" reaction, characterized by decreased bone plate density in a limited zone beyond the initial hole edge, and increased woven bone (WB) plate repair inside the initial hole ($P = 0.016$), which was accompanied by a more voluminous and hyaline cartilage repair ($P < 0.05$ vs control defects).

Conclusion: In a challenging aged rabbit model, bone marrow-derived hyaline cartilage repair can be promoted by treating acute drill holes with a biodegradable subchondral implant that elicits bone plate resorption followed by anabolic WB repair within a 70-day repair period.

Introduction

Many patients needing cartilage repair therapy aged 40–65 years old, are considered too young for a joint replacement, and too old to respond well to standard therapies[1,2]. Treatments that work in skeletally immature rabbits (2–6 months old), which have a high spontaneous rate of regeneration compared to rabbits over 8 months old[3–6], may prove to be ineffective in middle-aged patient knees, which have low remodeling rates, declining mesenchymal stem cell populations, and low intrinsic capacity for chondrocytes to synthesize collagen type II[7]. Rabbits attain skeletal maturity with closed epiphyses at 7 months, but rabbits over 8 months old are needed to analyze osteochondral repair reactions in fully mature epiphyses[8].

In rabbit knees, drilled osteochondral defects of different diameter (0.9–3 mm) will spontaneously resurface with a soft fibrocartilage repair tissue, or occasionally form a depressed bone cyst[6,9–12]. Fibrocartilage contains mesenchymal stromal cells and chondrocytes embedded in an extracellular matrix of mixed collagen type I (Col-1) and collagen type II (Col-2) with low levels of glycosaminoglycan (GAG). A more hyaline cartilage can be elicited from subchondral bone defects, when a chitosan-glycerol phosphate/blood implant is flooded over the osteochondral defect surface, and solidified *in situ*[10,13].

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However in rabbits >12 months old, the anabolic response to bone marrow stimulation is significantly attenuated, even with a bioactive chitosan-GP/blood implant[4].

To obtain a more potent cartilage repair response in skeletally aged knees, we previously developed a solid blood clot implant incorporated with chitosan particles, that is press-fit directly into a subchondral bone defect to attain a closer proximity between the implant and bone marrow-derived repair cells[14,15]. In a previous rabbit study carried to 3 weeks, it was shown that three pre-solidified implant formulations (10, 40, and 150 kDa chitosan) press-fit into adjacent trochlear drill holes induced local bone remodeling and delayed fibrocartilage deposition compared to control drill holes[14]. However, the 150 kDa chitosan/blood implant elicited significantly more apoptotic neutrophils that could potentially lead to a tissue void, and treated drill holes were encroaching upon each other[14]. Therefore, in this study, we used a two-hole drill model to test the hypothesis that pre-solidified blood implants containing rapidly degrading chitosan particles (80% degree of deacetylation (DDA), 10 kDa or 40 kDa) press-fit into microdrill holes, proceed to a phase of bone plate remodeling coupled to hyaline cartilage regeneration after 70 days, in rabbits >12 months old. This end-point was chosen based on the observation that microdrilled cartilage defects treated with an *in situ*-solidified chitosan/blood implant regenerate the subchondral bone to the osteochondral junction at 56 days post-operative[4,10]. In addition, given the anti-inflammatory properties of chitosan[16–18], post-operative knee effusion was documented.

Methods

Study design

All animal procedures were approved by institutional ethics review boards. Skeletally mature NZW rabbits (13 months old $N = 3$, 32 months old $N = 4$, 5♂, 2♀, 4.53 ± 0.67 kg, Charles River, St-Constant, QC, Canada) received two trochlear drill holes bilaterally. In one knee the holes were press-fit with a pre-solidified 40 kDa chitosan/blood (proximal) or 10 kDa chitosan/blood (distal) implant. The two contralateral drill holes were allowed to bleed as surgery-only controls. Mineralized and non-mineralized repair tissues were analyzed in initial defects ($N = 1$) and 70 days post-operative ($N = 6$).

Pre-solidified chitosan-NaCl/blood implant preparation

Chitosans (80–82% DDA, <500 EU/g, <0.2% protein, <5 ppm heavy metals, <0.2% ash) were obtained from BioSyntech (now Piramal Life Sciences, Laval, QC, Canada) and depolymerized by nitrous acid as previously described[19] to a target number-average molecular weight (M_n) of ~40 kDa, and ~10 kDa. Weight-average molecular weight (M_w), M_n and polydispersity index (PDI, M_w/M_n) were determined by size-exclusion chromatography (Table I). Chitosan powders were dissolved at 20 mg/mL in dilute HCl then autoclave-sterilized. Rhodamine-B isothiocyanate (RITC) labeled chitosans were generated at 1.0% mol RITC/mol chitosan from structurally matched 40 kDa and 10 kDa 80% DDA chitosans, dissolved in dilute HCl at 5 mg/mL and 90% protonation, 0.22 μm filter-sterilized, stored at −80°C and thawed once prior to use. Two isotonic 10 kDa or 40 kDa chitosan-NaCl formulations were prepared by combining chitosan-HCl and sterile NaCl solutions, then adding a 1:20 v:v ratio of 5 mg/mL RITC-chitosan of matching molecular mass, with a final osmolality of 292 mOsm (10 kDa chitosan-NaCl, termed "10K") and 318 mOsm (40 kDa chitosan-NaCl, termed "40K"). 250 μL aliquots of each formulation were distributed in flat-bottom 2.0 mL cryovials with three sterile 0.39 g surgical steel mixing beads (Salem Specialty Ball, Canton, CT, USA). At surgery, 0.75 mL–1.5 mL of fresh aseptic peripheral arterial autologous whole blood was added, the cryovials shaken manually for 10 s, the liquid mixtures drawn into sterile depyrognized borosilicate glass tubes (2 mm inner diameter), and coagulated for 20–60 min at 37°C. Implants were extruded onto a sterile petri dish, trimmed with a scalpel to insert in the defects, and left-over pieces fixed in formalin to document chitosan particle formation and dispersion by epi-fluorescence microscopy.

Table I
Chitosan powders used to generate pre-solidified implants

Chitosan powder	DDA (%)	M_w (kDa)	M_n (kDa)	PDI*
40K†	80.2	48.8	37.7	1.3
RITC-40K‡	81.3	61.5	46.7	1.3
10K†	81.9	23.8	12.7	1.9
RITC-10K‡	80.0	22.4	12.4	1.8

* PDI (M_w/M_n).
† Chitosan powder used to make the 20 mg/mL autoclave-sterilized solution.
‡ RITC-chitosan powder used to make the 5 mg/mL filter-sterilized solution.

Rabbit osteochondral repair model

Rabbits were anaesthetized with ketamine-xylazine-buprenorphine, had their knees shaved and disinfected, and were maintained with 3% isoflurane/8% oxygen. Small bilateral arthrotomies were made one knee at a time and the trochlea exposed by medial patellar displacement. At two equally spaced sites in each femoral trochlea, the cartilage was debrided, then microdrilled to 2 mm deep using a 1.4 mm-diameter round drill burr (Fine Sciences Tools, Foster City, CA, USA) under constant irrigation with Ringer's Lactated Saline to rinse away bone powder. Holes were left to bleed [Fig. 1(A)], or press-fit with a ~2 mm implant piece, 40K and 10K, by gently pushing with a sterile p200 pipetman tip from 10 to 25 times to fully embed the implant [Fig. 1(B)]. Knees were closed in three layers, and rabbits allowed immediate unrestrained cage activity. One female rabbit (6.4 kg, 32 months old) had an unscheduled death immediately after surgery due to complications from anesthesia, and the femur ends were collected and analyzed as initial defects (D0). Buprenorphine was administered post-operatively twice a day for at least 3 days as needed. Safety was monitored by body weight and observations of knee pain and effusion. Knee effusion was monitored by two observers during 70 days and scored on a scale of 0–4: 0 = no effusion, 1 = slight knee swelling, 2 = clear effusion or swelling, 3 = a lump, and 4 = a very large lump (≥1.5 cm diameter) over or near the surgical sutures. Daily scores were averaged in each group of rabbits ($N = 6$) and graphed vs the day post-surgery up to day 40. The area under the curve was then calculated using a trapezoidal rule. The cumulative knee effusion score was plotted using Prism 6 (GraphPad, La Jolla, CA, USA). Rabbits were euthanized at day 70 under anesthesia by IV injection of sodium pentobarbital and the femoral ends fixed in 4% paraformaldehyde/100 mM cacodylate, pH 7.4. Femur ends and cryosections were analyzed by inverted epifluorescent microscopy (Northern Eclipse, Empix, Mississauga, ON, Canada), for fluorescent chitosan. Defects were scored for macroscopic appearance using digital images acquired with a dissection microscope [Fig. 1(C and D)], based on the following tissue color grading: 1 = depression (tissue void), 2 = red/grey, 3 = beige, 4 = red–white, and 5 = white, homogeneous tissue.

Micro-computed tomography (micro-CT)

Femur ends trimmed of their condyles were micro-CT scanned (SkyScan1172, Skyscan, Kontich, Belgium) at an image size of 2000 × 1048 pixels, pixel size resolution 9.8 μm, two-frame