BMP-7 Protects against Progression of Cartilage Degeneration after Impact Injury

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ABSTRACT: In vivo studies were used to characterize a model of cartilage injury leading to osteoarthritis progression in the medial femorotibial joint of sheep. In three subsequent studies, bilateral impact injuries were created and one joint received intraarticular injections of 340 μg of rhBMP-7 protein in a collagen particle carrier while the contralateral knee received the vehicle alone. Sheep were allocated to three groups that received intraarticular injections on day 0 (group A), 21 (group B), or 90 (group C) after experimental knee injury. In each group the, joints were evaluated for signs of osteoarthritis progression 90 days after the last treatment using India ink stained area, OARSI histological scoring, cartilage sGAG content, immunostaining for apoptosis (TUNEL), caspase-3, collagen degradation (Col 2 3/4C short collagen epitope), and the endogenous (pro-) form of BMP-7 protein. Knee joints that received rhBMP-7 immediately after injury had small focal lesions at the injury site that did not progress into the surrounding cartilage. Joints that received BMP-7 3 weeks after injury were improved and had limited progression compared to controls, but joints that received the protein 12 weeks after injury had no statistically significant improvement. These studies suggest that BMP-7 may be chondroprotective after traumatic injury in patients if it is administered within 3 to 4 weeks of the index injury. The mechanism of protection after sublethal injury appeared to be an increased survival of chondrocytes that are able to participate in the repair process. © 2008 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 27:602–611, 2009

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Osteoarthritis (OA) constitutes a very large socioeconomic burden because this disease creates a life long, nonfatal disability.[1] The relationship of previous injury to the development of knee OA has been established by meta-analyses and case series[2–4] showing progressive cartilage loss or "chondropenia"[5] that may arise from compromised chondrocyte viability.[6–8] Strategies that prevent cartilage degeneration in the posttraumatic knee would be very valuable. Although there is experimental evidence that this goal may be attainable,[9–15] recent reviews of OA treatment strategies in clinical trials concluded that definitive evidence for disease modification in OA is scant.[9–10] Prevention of OA after injury would require stimulation of repair mechanisms and promotion of an anabolic metabolism in the face of catabolic mediators such as IL-1. Members of the bone morphogenetic protein (BMP) family are better known for their role in bone repair, but these proteins have well-established anabolic and anticatabolic effects in articular tissues including upregulation of chondrocyte metabolism[11–14] in the presence of IL-1[15,16] and fibronectin fragments.[17,18] This effect is synergistic with IGF-1,[19] and aged osteoarthritic chondrocytes still respond to BMP-7 with increased anabolic activity and viability.[12,13] Bobacz[20] and others have shown that BMP-7 improves the amount, quality, and integration of repair tissue in experimental cartilage defects.[21–23] Models of knee OA after mechanical injury include contusive impact applied through an arthrotomy,[24] arthroscopically,[25] or to the closed joint[26] in the rabbit,[27] dog,[26,28] sheep,[29] and horse.[25] In the following studies the contusive impact model was used in the sheep knee (stifle) joint because of its similarity to the human knee,[30,31] the sheep's comparable body mass. Time points for analyses were chosen with two aims in mind: to demonstrate efficacy for BMP-7 treatment in posttraumatic osteoarthritis, and to explore the window of opportunity for successful therapy. Our hypotheses were that BMP-7 would have a protective effect against the development of osteoarthritis if administered early in the disease process, and that delaying treatment after the index injury would result in declining efficacy. Intraarticular injections of BMP-7 putty formulation were administered at the time of injury, 3 weeks, and 12 weeks after injury. The aim of these studies was to investigate the protective effect and timing of administration after a contusive impact injury to the medial femoral condyle of sheep.

MATERIALS AND METHODS

All phases of this experiment were compliant with the guidelines of the Canadian Council on Animal Care and institution ethics review. There were three phases of animal experiments including: determination of BMP-7 bioavailability, animal model development, and assessment of timing of BMP-7 administration on efficacy in vivo.

Pharmacokinetic Trial

Bioavailability studies were done by using a single BMP-7 injection into one joint of eight normal adult sheep. BMP-7 was administered as commercially available putty approved for use in spinal fusion surgery (BMP-7 Putty, Stryker Biotech, Hopkinton, MA). Each dose contained 0.34 μg of rhBMP-7 protein adsorbed onto 100-mg bovine-derived type I collagen, 23-mg carboxymethylcellulose (CMC), and physiologic saline

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made up to a volume of 0.35 mL. The contralateral knee received inactive vehicle containing the same constituents with the exception of the BMP-7. Synovial fluid collection and BMP-7 concentration measurement was done at 0, 3, 24, 48, 72, and 120 h using custom luminescence ELISA. This assay had a detection limit of 2 ng/mL and used an alkaline phosphatase conjugated antibody raised in rabbits against rhBMP-7. Negative controls consisted of pooled normal sheep synovial fluid ($n = 20$ normal joints), and standard curves for optical density versus BMP-7 concentration were constructed using synovial fluid with rhBMP-7 (Stryker Biotech) added.

Model Development

Characterization of the in vivo impact model used a group of 10 adult female Cheviot cross sheep (3–6 years old) that received bilateral medial femoral condyle impact injuries but no treatments. They were sacrificed in groups of two at 0, 1, 3, 6, and 9 months for macroscopic and histological assessments as described below.

Efficacy and Timing of BMP-7 Administration

Three- to 6-year-old Cheviot cross sheep ($n = 24$) were used in three weight-matched experimental groups (A, $n = 6$; B, $n = 9$; and C, $n = 9$) that underwent experimental knee injuries and received BMP-7 by intraarticular injection. Surgical procedures were performed under general anesthesia using diazepam (0.5 mg/kg, Sabex, PQ), ketamine (2.2 mg/kg Biamedia-MTC Animal Health, ON), and halothane 1 (0.5–2.5%, Lavatrie, PQ) with aseptic technique used throughout. All sheep underwent minimally invasive bilateral knee joint arthrotomies to create contusive impact injuries to the middle weight-bearing third of the medial femoral condyle using a spring-loaded impact device equipped with a 6.0-mm diameter aluminum indenter tip. A 227.3-kg load cell (MLP-500, Transducer Techniques, Temecula, CA) was located between the impactor and indentor tip to allow data collection by a personal computer equipped with data acquisition software (LabVIEW, National Instruments, Austin, TX) using a 5000-Hz acquisition rate. Appropriate load cell calibrations were made using weight standards, and test impacts on cadaveric ovine osteochondral segments were done using paravital staining with fluoroscein diacetate and ethidium bromide to assess chondrocyte injury. Impulse duration was $6.1 + 2.8$ ms. A single operator (M.H.) applied two side-by-side impact injuries while an assistant stabilized the flexed knee joint with sand bags, with the shaft of the femur vertical and the greater trochanter and pelvic stabilized against a heavy steel restraint. This positioning resulted in stabilization of the femur while the impact load was applied resulting in a 30 MPa impact force.[29] Standardized descriptions and digital photographs were taken of the resulting injury before routine closure of the joints. Treatments were allocated to the experimental groups according to Figure 1, where the index injury was time = 0 and treatments (rhBMP-7 or vehicle alone) were administered to alternate knees. Group A ($n = 6$ sheep) was administered intraarticular BMP-7 (340 μg) in one knee and inactive vehicle in the contralateral knee intraoperatively at day 0 and again 7 days later. In group B ($n = 9$), the same treatments were administered on postoperative days 21 and 28, and in group C ($n = 9$) treatments were administered on postoperative days 90 and 97. All injections were made with the sheep anesthetized or sedated. The animals were housed in 12 m^2 pens, and no attempt was made to limit their daily activity during the study. Postoperative daily monitoring

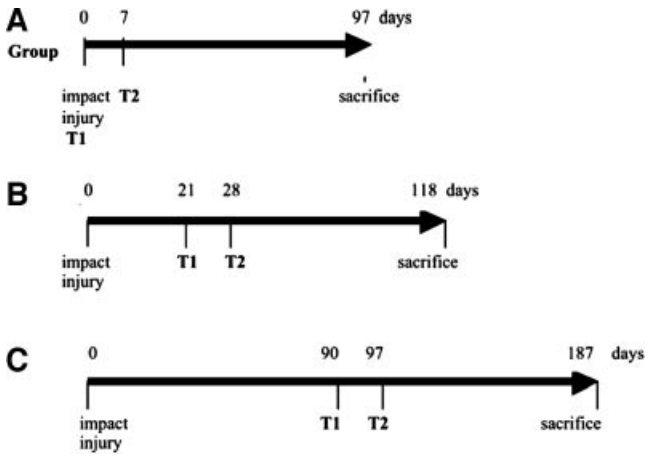

Figure 1. The flow chart shows the timing of BMP-7 and vehicle administration to experimental groups A, B, and C. T1 is the first intraarticular BMP-7 injection and T2 is the second. Postmortem assessments were made 90 days after T2 in each case.

included lameness, joint effusion, incision complications and concurrent health problems. All sheep were sacrificed 90 days after their last BMP-7 treatment. Daily lameness score was recorded as absent (0), mild lameness at the trot (1), mild lameness at the walk and trot (2), or reduced weight bearing at the walk, unable to trot (3). In all groups lateral to medial and anterior to posterior knee joint radiographs were taken to rule out preexisting disease. At the time of sacrifice, all animals had radiographic (group A) or micro-CT images (groups B and C) using 90-micron isotropic pixel resolution (GE Explore Locus Micro-CT scanner, London, ON) read by an investigator who was blind to the group allocation. Osteophytes, subchondral lucency, osteochondral bodies, or areas of soft tissue mineralization were identified in two or more planes and their volume measured using a region of interest tool. Synovial fluid (250 μL) was collected starting at time = 0 and each arthrocentesis allowed for leukocyte counting and differential cell counts, which were done manually. Synovial fluid volume, color, and viscosity were recorded.

During all postmortem macroscopic assessments the evaluators were blind to treatment allocations. All knee joints were disarticulated and digital photographs were taken using standardized lighting conditions. India ink (Nation Focus Distribution, Mississauga, ON) was applied to the articular surface for 20 s, then washed off using running tap water for 60 s to identify areas of cartilage fibrillation, thinning, erosion, and eburnation. Areas of India ink uptake were traced onto small pieces of polyethylene plastic applied to the wet joint surfaces of the medial condyle and the ink-stained area was measured using semiautomated morphometry software (Northern Eclipse, Mississauga, ON). Areas were rounded to the nearest 5%. A general linear model (SAS Institute, Cary, NC) was applied to these data to determine repeatability and interoperator variance. Areas were compared between treatment and control limbs using a student's t-test for paired data.

Samples of synovial membrane, meniscus, medial femoral condyle, and medial tibial plateau were harvested according to a standardized plan that sampled the injury site as well as the surrounding femoral condyle and the opposing nonmeniscus covered portion of the tibial plateau. Osteochondral blocks were cut using a low-speed diamond saw (Buehler Industries, IL), and then decalcified in 40% formic acid:20% citric acid. Ammonium oxalate was used to check for completeness of

decalcification. Tissue blocks underwent routine paraffin embedding, and sections (5 μM) were stained with toluidine blue, hematoxylin and eosin, and safranin-O. Slides for TUNEL staining were prepared as per Bolam et al.[25] using an ApopTag™ Plus peroxidase detection kit (#S7101 Chemicon International, Temecula, CA). Negative and positive controls for TUNEL staining were included in the kit but confirmatory studies in ovine cartilage were done using nuclease (Sigma-Aldrich, St. Louis, MO) pretreated normal cartilage and liver sections. TUNEL-positive cells were systematically counted in three histological slides from each knee using a semiautomated morphometry software (Northern Eclipse, Mississauga, ON) to identify apoptotic cell nuclei based on their color, shape, and size. To assess the progression of apoptosis outward from the index injury, a region of interest comprising a 100-power microscope field was drawn starting 1.5 mm from the periphery of the impact lesion. Cells were counted in each region of interest after thresholding the image for the brown diaminobenzidine (DAB) staining TUNEL-positive nuclei, and setting bin classifier criteria to accept only the pixel groups with sizes and shape factors that were consistent with the chondrocyte nuclei. Periodic manual counting was used to confirm the semiautomated process. To investigate the point in the apoptosis cascade where BMP-7 might intervene, immunostaining for caspase-3 was done in group A animals. A polyclonal antibody (Cayman Scientific, #160745) raised in rabbits was used with a secondary antirabbit antibody (Envision, Dako Canada, Mississauga). Deparaffinized slides were pretreated with proteinase K and incubated with the primary antibody for 1 h at room temperature followed by washing and secondary antibody exposure for 30 min. The DAB color reaction was followed by counterstaining in hematoxylin. Slides were examined in pairs from BMP-7/vehicle-treated joints and immunostaining was scored on a scale of absent (0), mild (1), moderate (2), or considerable (3).

Additional slides were prepared for Col 2-3/4 C_{short} immunostaining (C1-2C antibody, Ibex Technologies, Montreal); an epitope cleaved from the type II collagen molecule by metalloproteinases and considered a marker of OA progression. Slides from group A were also stained using an antibody that recognized the autocrine form of pro-BMP-7 produced by articular cells.[32] These slides were predigested with keratanase, keratanase 2, and chondroitinase ABC (Seikagaku, Tokyo, Japan) for 90 min at 37°C and stained with a rabbit anti-pro BMP-7 antibody (Stryker Biotech, Hopkinton, MA) using a 1:100 dilution and and secondary rhodamine conjugated goat antirabbit antibody (Pierce Endogen, Rockford, IL). For negative controls, the primary antibodies were replaced with either normal serum or secondary antibody alone. Histological sections were numbered to obscure treatments and examined by two independent reviewers and scored using the OARSI scoring system.[33] The OARSI scoring system produced a score between 0 and 24 that was the mathematical product of the lesion grade and stage. Grade (0–6) indicated depth of the lesion where lesion stage corresponds to the area of abnormal cartilage as determined by India ink staining and histological examination of multiple (12–15) sections of the femoral condyle. Cartilage sulfated glycosaminoglycan (sGAG) concentration was detected by a microplate adaptation of the dimethylmethylene blue precipitation method[34] compared against standard curves prepared with chondroitin sulfate C (#C-43841, Sigma-Aldrich). Absolute amounts were normalized to cartilage wet weight and reported as mg chondroitin sulphate C per mg wet weight of cartilage.

RESULTS

Pharmacokinetic Trial

Peak synovial fluid BMP-7 concentrations occurred 24 after intraarticular injection (1.9 ± 0.17 μg/mL) and detectable levels were present at 48 and 72 h (80 ± 1.0 ng/mL and 4.7 ± 1.9 ng/mL respectively).

Model Development

Analysis of output from the force transducer showed that the mean impact force was 30.5 ± 2.8 MPa and the lowest impacts would have been well above the threshold of injury for superficial and middle zone injury for this location and species.[29] Animals that received bilateral contusive impact injuries had transient lameness and synovial effusion for 14 days postoperatively, then no lameness except in the 9-month group that developed mild grade 1 lameness and knee joint thickening between 6 and 9 months. The two animals sacrificed immediately postoperatively had two focal 6- to 7-mm diameter areas of thin, dull, and slightly darker cartilage that retained India ink at the perimeter of the lesion. This lesion occupied two-thirds of the width of the femoral condyle. Histological sections demonstrated fissured, compressed cartilage with more prominent folds at the perimeter that extended into the middle chondrocyte zone. The subchondral bone appeared normal. Animals sacrificed at 3 months had an irregularly shaped 8–10-mm diameter area of increased ink uptake in and around the experimental site. The histological features within this zone of injury included more extensive partial thickness fissures, loss of the superficial zone layer and empty chondrocyte lacunae within the remaining cartilage that had reduced safranin-O staining (Fig. 2). Surrounding cartilage had reduced safranin-O staining, smaller partial thickness fissures, and an irregular pattern of superficial zone loss extension 2 to 3 mm away from the injury site. Animals sacrificed at 6 and 9 months had progressive osteophytosis in the medial knee compartment as well as partial and full thickness erosion of the femoral condyle, and to a lesser extent, in the tibial plateau. In most cases there was little or no cartilage remaining in the injury site, and the surrounding cartilage was undermined or delaminated, revealing calcified cartilage. There was fraying of the free border of the medial meniscus but no meniscal tears. Sheep in a related study that were sacrificed at the 2-year time point had a severe end stage OA.

Efficacy and Timing of BMP-7 Administration

All sheep had a lameness of grade 2 severity immediately postoperatively that declined to grade 1 within 7 days, and was no longer evident 14 days after surgery. Joint effusion was detectable during the first postoperative week but was not discernable thereafter. There was no difference in lameness scores, joint effusion or incisional complications between BMP-7- or vehicle-treated knees. Preoperative radiographs were

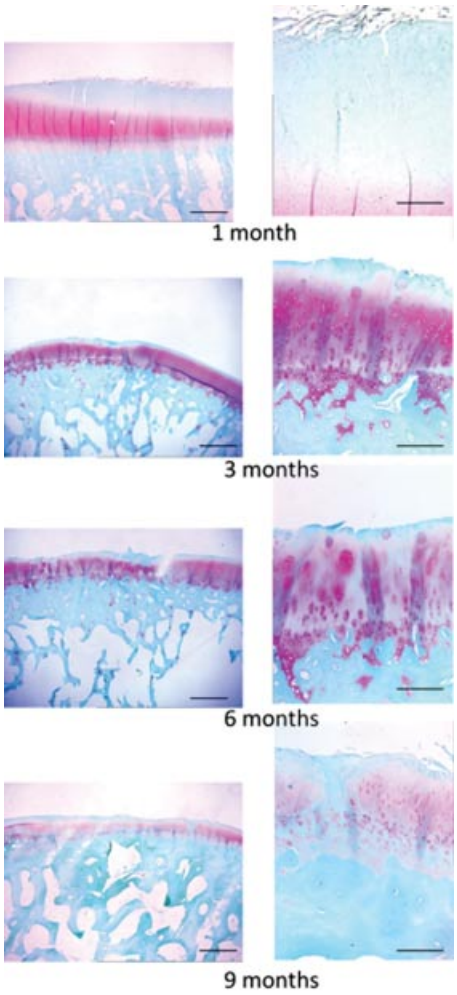

Figure 2. The histology of the impact model development studies is shown with safranin-O/fast green stained slides demonstrating progression of after impact injury 1, 3, 6, and 9 months postinjury. Left column original magnification 20 × bar = 1000 μ, right column × 100 magnification, bar = 250 μ.

normal in all sheep. The most frequent radiographic (group A) or micro-CT (groups B and C) abnormality at the end of the study was an osteophyte on the distal pole of the patella. Other findings were subchondral plate lucency in the medial femoral condyle, small osteophytes in the intercondylar notch, and a mild increase in radiodensity in the fat pad or anterior joint capsule. The frequency of radiographic or micro-CT abnormalities was not different between the three groups or between treated and control knees. Micro-CT identified very small areas of mineralization in three BMP-7 treated joints in group B and none in group C. These were composed of 1×4–6 mm-long areas of mineralized tissue within the incision or capsule adjacent to the posterior–medial joint injection site comprising a mineralized tissue volume of 6.7 to 42 mm^3. One vehicle-treated animal had similar findings.

Preoperative synovial fluid was within normal limits (clear to amber colored and viscous) and contained less than 0.33×10^9 leukocytes per liter and 0.5 g/L total protein. The predominant cell type detected was the small lymphocyte. In group A, leukocyte number and protein concentration were significantly increased from baseline 1 week postoperatively (both $p < 0.01$); but there were fewer leukocytes in synovial fluid of BMP-7-treated joints ($2.2 + 1.3 \times 10^9$/L) compared to those administered the vehicle alone ($5.2 + 3.4 \times 10^9$/L, $p < 0.05$, paired t-test). Mature neutrophils, small and large lymphocytes, and the occasional synovial cell were found on stained smears. In groups B and C there was a more modest threefold increase in leukocyte concentration 1 week after the first intraarticular injection that was not statistically different between BMP-7- and vehicle-treated knees.

Repeated analysis of India ink-stained cartilage area by three different operators on two occasions showed that intraoperator variance was 2% and interoperator variance was 2%. Ink staining allowed clear demarcation of subtle articular defects as well as the zone of injury around the impact site. The ink stained surface area of the MFC was significantly lower in BMP-7-treated knees from groups A ($p < 0.01$) (Fig. 3) and B ($p < 0.03$) (Table 1), but there was no difference in group C ($p < 0.14$) compared to vehicle-treated control joints. Tibial plateau (TP) surfaces mirrored the loss of reflectivity in the MFC surface but developed less severe wear lines and cartilage erosion in the areas not covered by the meniscus. India ink-stained areas in the tibia were not significantly different between BMP-7- and vehicle-treated knees in any group. No meniscal tears were observed, although the free border of the meniscus was irregular and frayed in joints with large cartilage defects.

Histology, Histological Scoring, and Immunostaining

The impact site (MFCi) was always identifiable at the histological level, but its appearance varied according to the treatment group. In group A, BMP-7-treated joints had focal partial thickness cartilage fissures and loss of safranin-O staining at the MFCi site, but the rest of the MFC had only superficial zone cell loss while retaining safranin-O staining. There was no evidence of extrinsic repair but cartilage flow had reduced the size of injury sites leaving a healed fissure or fold (Fig. 4). The TP had

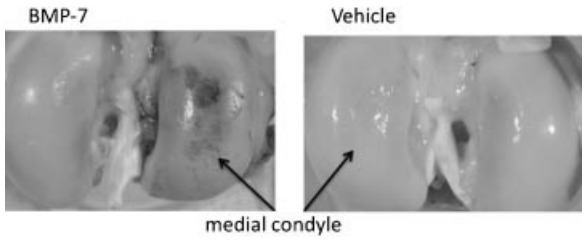

Figure 3. Macrophotographs of India ink-stained femoral condyles from sheep #53 in group A showing vehicle (left) and BMP-7 (right) treated knees. In the vehicle-treated knee the original impact injury can be identified as a circular area of ink uptake as well as the degenerate cartilage extending posteriorly covering 40% of the femoral condyle. Loss of surface sheen and reflectivity is evident compared to the BMP-7-treated knee, in which 5% of the condyle is affected.

Table 1. India Ink-Stained Area for the Medial Femoral Condyle—Groups A to C

Group A			Group B			Group C		
Animal #	BMP-7	Vehicle	Animal #	BMP-7	Vehicle	Animal #	BMP-7	Vehicle
28	5	20.6	52	6.3	8.9	50	7.4	13.3
29	20.3	40.5	53	17.9	71.1	51	46.6	38.4
30	0	60.2	54	7.1	12.2	61	33.3	24.9
31	20.8	50.4	55	10.7	35.5	62	29.3	24.0
32	10.5	70.9	56	27.8	64.7	63	30.8	31.5
33	10.2	25.0	57	18.5	6.5	64	45.7	34.0
			58	23.8	34.4	65	21.3	25.5
			59	18.9	35.8	66	29.5	32.7
			60	10.6	20.3	67	26.2	27.5
Mean	11.1	44.8	Mean	15.7	30.0	Mean	30.0	28.0
t-Test	$p < 0.01$		*t*-Test	$p < 0.03$		*t*-Test	$p < 0.14$	

A paired *t*-test was used to compare scores between BMP-7 and treated joint surfaces.

small areas of superficial zone fibrillation and loss of staining but no consistent lesion pattern. In vehicle-treated control joints, the MCFi was easily identified as an area of erosion or delamination of cartilage, with little or no matrix staining and extensive loss of chondrocytes. The surrounding area was disrupted by areas of delamination, clefts, and cartilage erosion accompanied by moderate to severe loss of safranin-O staining. When the superficial cartilage zone remained, chondrocyte nuclei were small and condensed or absent. The TP had diffuse superficial and middle zone fibrillation and fissures as well as loss of safranin-O staining. In groups A and B, BMP-7-treated knees appeared to have less centrifugal progression from the impact site, resulting in a smaller more superficial lesion (Table 2). Differences in the depth of the lesion reflected by the grades of the OARSI score were greater in group A than group B, and total scores were significantly different in BMP-7- versus vehicle-treated knees in both groups ($p < 0.0004$ and $p < 0.015$, respectively, paired *t*-test). No difference in histological scores was evident between the BMP-7- and vehicle-treated knees in group C ($p < 0.36$).

The abnormalities observed in the calcified cartilage and subchondral bone of both BMP-7- and vehicle-treated femoral condyles consisted of tidemark duplication and a small number of sites where blood vessels were encroaching on the calcified cartilage. The subchondral bone of the TP was normal in all cases. There were no degenerative changes or mineralization in the body of the menisci or synovial membrane. Collagen particles, a

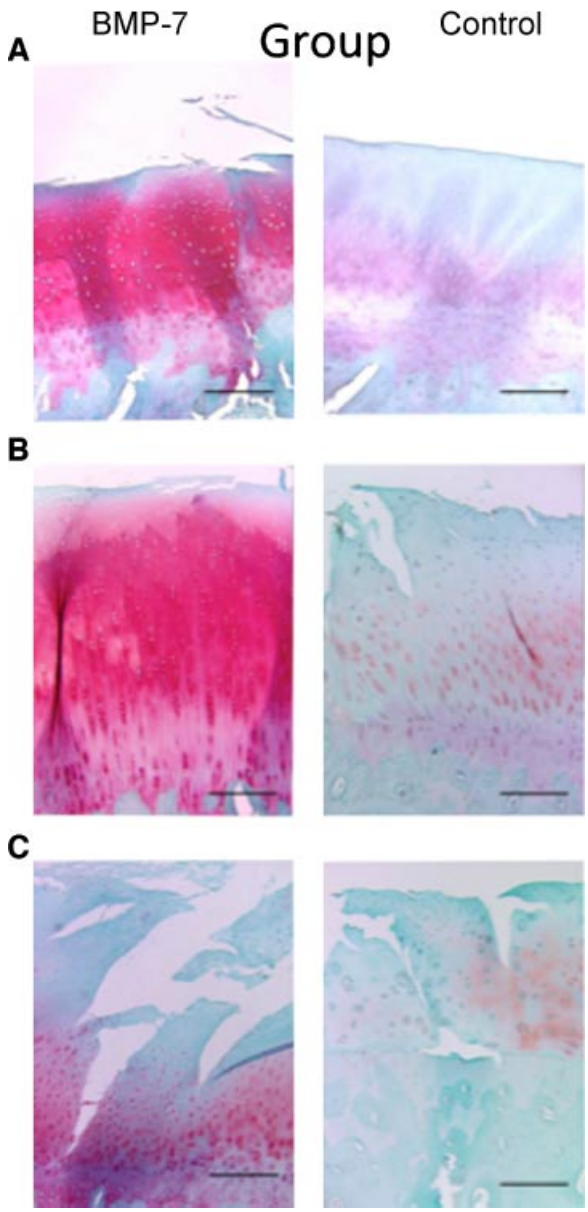

Figure 4. The histology of the efficacy and timing experiments is shown with safranin-O/fast green stained slides from sheep with the median histological scores for each treatment group. Treated (left) and control (right) images are shown for each sheep. BMP-7-treated joints in the left column have retained more histological architecture and safranin-O staining compared to control joints in the right column, particularly in groups A and B. Animals in group C show improvement over controls but fissures and injury at and around the index injury site persisted, resulting in no statistical differences between treated and control joints. ×20 original magnification bar = 1000 μ.

Table 2. OARSI Histological Scores for the Medial Femoral Condyle of Sheep in Groups A, B, and C

Group A	BMP-7		Vehicle		BMP-7	Vehicle	
Animal	Stage	Grade	Stage	Grade	Total Score		
28	1	1	2	3	1	6	
29	2	1	3	4	2	12	
30	1	2	3	4	2	12	
31	2	2.5	3	4	5	12	
32	1	4	4	3.5	4	14	
33	1	4	2	5	4	10	*t*-Test
				Mean	3.0	11.0	0.0004
Group B	BMP-7		Vehicle		BMP-7	Vehicle	
Animal	Stage	Grade	Stage	Grade	Total Score		
52	1	3	1	5	3	5	
53	2	1.5	4	3	3	12	
54	1	3	2	3.5	3	7	
55	1	3.5	3	3.5	3.5	10.5	
56	3	1.5	4	4	4.5	16	
57	2	3	1	3.5	6	3.5	
58	2	2.5	3	3	5	9	
59	2	2	3	2	4	6	
60	2	2	2	3	4	6	*t*-Test
				Mean	4.0	8.3	0.015
Group C	BMP-7		Vehicle		BMP-7	Vehicle	
Animal	Stage	Grade	Stage	Grade	Total Score		
50	4	2	2	2	8	4	
51	2	2	2	2	4	4	
61	2	4	3	2	8	6	
62	1	4.5	3	4.5	4.5	13.5	
63	2	4.5	1	4	9	4	
64	1	3.5	2	4	3.5	8	
65	1	1	3	3	1	11	
66	2	2	2	2	4	4	
67	3	2	3	3	6	9	*t*-Test
				Mean	5.00	7.06	0.362

component of the putty formulation, could be seen in all knees as irregular, acellular, amorphous-staining bodies within the synovial membrane surrounded by macrophages; however, there was no evidence of synovial membrane activation or hypertrophy in any group.

In all cases TUNEL staining localized apoptotic chondrocytes in the impact injury site, but the pattern of immunostaining in the surrounding femoral condyle varied according to treatment group (Fig. 5). In all of the vehicle-treated joints the cells were TUNEL positive throughout all cartilage layers in the impact zone and in the adjacent cartilage wherever fissures or erosions were present. By contrast, BMP-7-treated joints had significantly fewer apoptotic chondrocytes adjacent to the impact area, and when present, these cells were limited to the perimeter of fissures or damaged cartilage in the MFCi zone (Table 3). Differences between BMP-7- and vehicle-treated knees were not evident in group C. Caspase-3 immunostaining (group A only) showed a similar trend where BMP-7-treated knee joints had mild positive staining in superficial zone cells adjacent to injury sites, whereas in vehicle-treated joints, mild to

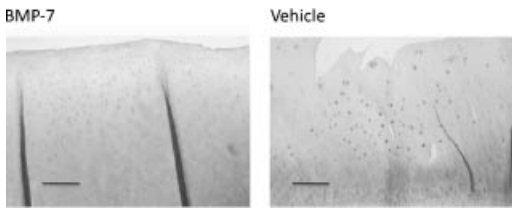

Figure 5. TUNEL stained histological slides from BMP-7- and vehicle-treated knee joints. The left image shows scattered faintly positive cells in a BMP-7-treated knee, the right image demonstrates many TUNEL positive cells adjacent to an area of injury in a vehicle-treated knee. Semiautomated counting of TUNEL positive cells from similar generated the data in Table 3. Immunostaining, ×100 original magnification, bar = 250 μ.

Table 3. Mean and Standard Deviation of TUNEL-Positive Cells from Semiautomated Counting in Three 200× Microscope Fields Adjacent to the Experimental Injury Sites

Group	OP-1	Vehicle	Paired t-test $p <$
A	11.3 ± 8.9	44.8 ± 12.3	0.00002
B	9.1 ± 10.3	48.0 ± 20.0	0.027
C	27.7 ± 9.9	33.7 ± 19.7	0.09

Knees from group A and B had significantly fewer TUNEL-positive cells, whereas group C treated at 12 and 13 weeks postinjury was not significantly improved.

considerable positive staining was present in all zones of chondrocytes distant from the original injury (Fig. 6). A striking aspect of this was that even in the most severely damaged cartilage in BMP-7-treated joints there was little caspase-3 staining.

Immunostaining for newly formed pro-BMP-7 was clearly positive in the articular cartilage and synovial membrane of BMP-7-treated joints. With one exception (sheep #32), vehicle-treated joints had little positive staining. This pattern was also observable in meniscus and to a lesser extent, subchondral bone. Col 2-3/4 C_{short} immunostaining was strong within the MCFi and surrounding superficial cartilage layer and in scattered areas of the TP in all vehicle-treated sheep. In BMP-7 treated joints of group A there was minimal staining around the experimental defect and scattered foci in the surrounding cartilage but none in the TP. Group B was similar except for foci of staining within the surrounding tissue and tibia. In group C all cartilaginous tissues were positive for this epitope.

Sulfated GAG content was measured in two sites (MFCi and TP). There was a statistical trend toward preservation of sGAG in the impact zone of BMP-7-treated joints in experimental group A ($p < 0.06$, paired t-test) but not groups B ($p < 0.47$) or C ($p < 0.58$). This trend was not repeated in the TP, where sGAG concentrations remained normal. In groups B and C, there were no differences in cartilage sGAG concentration between BMP-7- and vehicle-treated joints at any site.

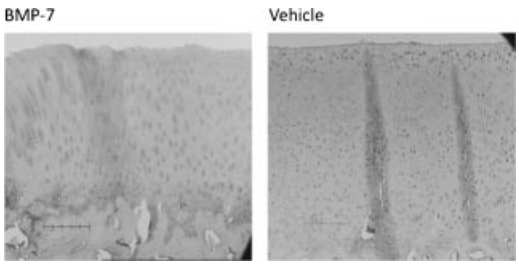

Figure 6. Caspase-3 immunostaining in BMP-7-treated joints was limited to a few cells in the superficial zone around injury sites in BMP-7-treated joints even when frank cartilage injury was present (left). Vehicle-treated joints had caspase-3-positive chondrocytes through the depth of the articular cartilage and distant from the site of injury in areas where there was no apparent damage to the articular surface (right). ×100 original magnification, bar = 250 μ.

DISCUSSION

BMP-7 was administered bound to a type I collagen vehicle in these experiments because the half-life of BMP-7 protein alone is in the order of hours. Intra-articular injections of BMP-7 putty resulted in longer BMP-7 residency at concentrations known to be efficacious in vitro. The dose (340 μg) was calculated from successful reports of cartilage repair in sheep where an implantable osmotic pump was used to deliver the protein without a carrier over a 12-week period.[23] Because slow release formulations of BMP-7 have not been developed, we used collagen particles as a prototypical delivery vehicle knowing that some inflammation could result. Small foci of inflammation were found in the synovial membrane associated with these particles; however, there was no histological evidence of a diffuse inflammatory reaction. The synovial fluid sampling intervals in this study were not designed to assess inflammation arising from the injections, but these data indicated a mild, short-lived inflammation that elevated leukocyte concentrations to 6×10^9/L but fell to within normal limits ($<1.0 \times 10^9$/L) 2–4 weeks after injections. Type I collagen is a component of periosteum, a tissue used in cell-based cartilage repair, and type I collagen scaffolds can be used in tissue engineering of cartilage[35] so it may not be deleterious in the synovial environment. Aside from the mild synovial fluid leukocytosis we have no evidence that the collagen carrier created a significant inflammation; however, this would have been difficult to discern in the face of postsurgical injury in group A but readily identifiable in groups B and C, which received the putty formulations sometime later. Nevertheless, sustained release formulations would be preferable if they created no additional inflammation and eliminated the need for multiple injections.

The 30-MPa impact force used in this study exceeds our own[29] and other's assessment of threshold of injury for adult articular cartilage in this animals.[24–28] To ensure a defect was created that would not heal spontaneously in this species,[36] we created a challenging environment for articular repair by making two side-by-side 6-mm diameter 30 MPa injuries. This impact force was within or above the range of deleterious impact forces reported for intact joints or osteochondral segments in other animal species such as the rabbit[37,38] and bovine,[24] and the resulting injury was larger than a similar model that used partial thickness defects in this species.[39] Rabbits, or a laboratory animal model, may have been more convenient and allowed larger group sizes for these studies, but the mechanism of action for BMP-7 includes some aspects of improved cartilage repair; therefore, we were reluctant to use a species such as the rabbit that retains intrinsic repair capacity in adulthood.[40] The large body weight, flock behaviors, and housing conditions of these sheep ensured that traumatic injuries would receive regular loading to make partial thickness cartilage injuries progress. The use of the impact model avoided some well-acknowledged

problems associated with rapidly progressive models of joint instability (such as anterior cruciate transection) where outcome measures must be done within a narrow time period before the prevailing instability overcomes any chondroprotective effect.[41] Subsequent cycles of reinjury and repair as well as the marked effects of body weight and activity level make interpretation of the results more difficult. The impact model used a single injury to create a predictable though slower progression of degenerative changes that allowed more opportunities for therapeutic intervention and conversion of the catabolic metabolism associated with osteoarthritis to an anabolic state.

To explore the timing of therapy we used three different regimes in groups A to C. From results presented here, BMP-7 given immediately after the injury and again 1 week later is sufficient to prevent progression of cartilage degeneration after injury. The diminished synovial fluid leukocyte concentration at 2 weeks postinjury, and preservation of structural and biochemical parameters in cartilage are evidence of chondroprotection. In related experiments we have administered additional doses of BMP-7 between 0 and 4 weeks, with no additional improvement (data not shown). More importantly, delayed administration of BMP-7 in group B still conferred protection against progression, although preservation of sGAG concentration in the medial femoral cartilage was not demonstrated. Despite this, BMP-7 administered 3 and 4 weeks after injury still mitigated cartilage degeneration because the resulting lesions were smaller and less severe than lesions in vehicle-treated knees. The authors acknowledge that a larger, longer experiment with more time points for analyses would be preferable, but careful interpretation of the results is possible. The model development phase of this experiment and others we have conducted[25,29] showed no animals will improve spontaneously, but the findings of the present study do not rule the possibly of relapse after treatment. The evidence of suppressed catabolic metabolism and lack of OA progression at early time points such as 90 and 147 days after injury should be considered preliminary, because osteoarthritis often presents an unrelenting challenge. Additional studies at longer time points are needed with a view to administering a series of carefully planned injections to maintain the therapeutic effect. The rationale for assessments 90 days after the last treatment in group A was based on the need to demonstrate a proof of principle with treatment at time = 0 after injury but probably not mimic a clinically relevant situation in health care systems where access to specialists is limited. Groups B and C were a more realistic paradigm where patients might receive treatment after diagnostic imaging and referral to a specialist weeks or months after the index injury. We feel that the declining statistical strength of the outcome measures from groups A to B to C is indicative of the difficulty of treating well-established lesions where cartilage morphology is so abnormal it is unlikely to repair. Larger or deeper defects create an incongruity requiring repair from a numerically depleted and metabolically exhausted chondrocyte population. This presents a much greater challenge compared to early OA where mild superficial lesions and biochemical derangement are present.

A limitation of these experiments could be that observations were made at only one time point, and limited investigation was done regarding the mechanism of action for BMP-7 in vivo, but some conclusions can be drawn from the data. Although BMP-7 may restore intrinsic cartilage repair in adult sheep,[23] there was no histological evidence that chondrocyte proliferation contributed to the repair. Our data show that programmed cell death is ongoing for months after a single injury, so depletion of the chondrocyte population may be taking place continuously. BMP-7 treatment in groups A and B reduced the number of TUNEL positive cells around the impact area, and there was more normal zonal chondrocyte organization compared to vehicle-treated joints that frequently had large areas of cell-depleted matrix. Because TUNEL staining is nonspecific, and represents apoptosis or late-stage necrosis, we chose to investigate whether caspase-3, and upstream mediator of cell death driven by tumor necrosis factor (TNF), would confirm our TUNEL immunostaining. Gene expression studies in BMP-7-treated cartilage have shown that production of this enzyme was suppressed.[42] In animals from group A caspase-3 staining was confined to the superficial zone in BMP-7-treated joints, and was frequently absent despite abnormal cartilage morphology present at the injury site. By contrast, vehicle-treated joints had caspase-3-positive cells throughout the femoral condyle articular surface in sites distant from the original injury. This indicates that BMP-7 interfered with caspase-mediated cell death after mechanical injury, and could play a role in inhibition of cell–cell signaling leading to apoptosis. Because there was no clear evidence of additional cells[43] participating in the repair process, our data suggest that BMP-7 allowed survival and retention of native chondrocytes that replenished and remodeled the damaged matrix. Measuring cell viability and metabolic activity per unit volume of cartilage would be a more reliable evidence of this hypothesis, but this would be technically demanding and require a larger experiment.

The chondroprotective effect we observed in our experiments may not be wholly dependent on the pharmacodynamics of the injected protein. This is consistent with our observation that rhBMP-7 induced more endogenous (autocrine) pro-BMP-7 and is in agreement with the work of Fahlgren,[44] who demonstrated an increase in BMP-7 after a capsular incision. Hyaluronic acid therapy is a comparable example where a therapeutic intraarticular injection of polysaccharide has a very short half-life but the therapeutic effect may arise by stimulating more endogenous production of hyaluronate.[45]

In addition, other downstream events contributed to cartilage preservation. The reduction in Col 2-3/4 short immunostaining shows that the catabolic metabolism driven by IL-1 and TNF that typifies osteoarthritis did not develop. Col 2-3/4C_{short} is a collagen fragment resulting from metalloproteinase degradation of hyaline cartilage collagen, and suppression of this marker indicates that catabolic metabolism was blocked to some degree. These data are consistent with other experiments that showed BMP-7 increased the survival and anabolic capacity of normal and OA chondrocytes in the face of signaling molecules that promote catabolic chondrocyte metabolism and OA degeneration[15,17,18,42,46,47] This protective effect is partly mediated by an increase in endogenous BMP-7 production in cartilage, synovial membrane, and other tissues. Taken together with previous in vitro data recently reviewed,[42] this is more evidence that BMP-7 can initiate an effective protective response that may also induce some degree of intrinsic repair.

In summary, BMP-7 afforded protection against the development of posttraumatic cartilage degeneration when this protein was administered immediately after injury and again, 1 week later. Delayed treatment 1 month after injury still prevented progression of degeneration but the original injury remained, indicating reduced but still relevant chondroprotection. Delayed treatment 12 weeks after injury was not protective, and degeneration progressed beyond the experimental injury site. It remains to be determined whether BMP-7 would prevent OA from developing in long term studies 1–2 years in duration, but it is likely that additional BMP-7 treatments would be necessary to sustain this protective effect. The basis for this effect appears to be suppression of chondrocyte loss and catabolic metabolism after sublethal injury and promotion of cartilage repair in the remaining chondrocytes.

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