Effect of BMP-2 and BMP-7 on Articular Cartilage Following an Impact Load

I. Specific Aims

Posttraumatic osteoarthritis (PTA) is the degeneration of articular cartilage in response to an injurious event. An *in vivo* animal model utilizing a pendulum device to deliver a single impact load to rabbit medial femoral condyles less than their fracture threshold has previously demonstrated significant chondrocyte apoptosis following impaction[6,7,9]. Recently, we described that this single high-energy impact load causes PTA in rabbits via a decrease in chondrocyte metabolism[8]. Our hypothesis is that intraarticular supplementation of BMP-2/BMP-7 heterodimers in this model will significantly decrease the amount of chondrocyte apoptosis and posttraumatic osteoarthritis. Our specific aims are as follows:

1. *To determine the effect of BMP-2/BMP-7 heterodimers on chondrocyte apoptosis following an impact load of articular cartilage. Following a single impact load of rabbit medial femoral condyles, we will perform intraarticular knee injections of heterodimers of BMP-2/BMP-7 to the injured articular cartilage. Comparing the extent of chondrocyte apoptosis between sham and experimental groups will be accomplished using light microscopy, fluorescence microscopy, and immunochemical techniques. When appropriate, paired Student's t-test will be used to compare data.*
2. *To determine the effect of BMP-2/BMP-7 heterodimers on the development of posttraumatic osteoarthritis following an impact load of articular cartilage. Following a single impact load rabbit medial femoral condyles, we will deliver heterodimers of BMP-2/BMP-7 to the injured articular cartilage via intraarticular injection, and we will analyze the development of PTA among experiment groups versus sham controls via morphologic, histologic, and biochemical techniques. When appropriate, paired Student's t-test will be used to compare data.*

II. Background and Significance of Research Question

Posttraumatic osteoarthritis is the clinical syndrome of joint degeneration that occurs after a well-defined injury to a joint such as joint loading or dislocation, articular fracture, capsule injury, meniscal injury, or ligamentous injury. It has been estimated that posttraumatic osteoarthritis accounts for 12% of the overall prevalence of osteoarthritis at estimated cost of $3 billion annually[10]. The pathogenesis of posttraumatic osteoarthritis, however, is poorly understood[11,12].

Apoptosis is a biologically conserved and highly regulated form of active cell suicide and has been linked to numerous diseases[15,19]. Prior work from our lab has demonstrated that *in vivo* chondrocyte apoptosis can be stimulated by a single impact load[9]. We have recently demonstrated that a single energy impact load on rabbit medial femoral condyles can cause posttraumatic osteoarthritis in young rabbits via a decrease in cellular metabolism. The question remains, however, does the apoptosis observed following an impact load contribute to the development of PTA? Furthermore, if apoptosis could be altered following an impact load, will this affect the eventual development of PTA?

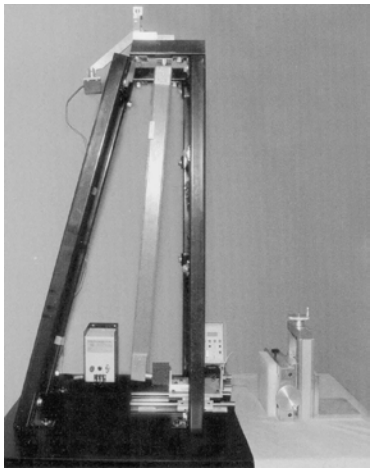
Bone morphogenetic proteins (BMPs) are a group of cytokines that regulate bone and cartilage formation[4,26,27]. A few groups helped detail the cartilage protection effects of BMP-7 in various models (chondral defects, impact load, and anterior cruciate ligament transection) and animals (dog, goat, rabbit, and sheep)[2,3,13,14,18,20,22,25]. Furthermore, an multi-center industry sponsored OP-1/BMP-7 phase II double blind, randomized, placebo-controlled trial is underway in the hopes of treating patients with osteoarthritis (www.clinicaltrials.gov, clinical trial ID: NCT01111045). Similarly, BMP-2 has demonstrated cartilage protection effects in multiple models including mouse, rabbit, and human[5,16,29,30]. In nature, BMPs function as homodimers or heterodimers. In fact, certain BMP heterodimers may possess enhanced activity over homodimers and thus enhance clinical utility[1,4,21,23,33]. In a rodent spinal fusion model, combined expression of BMP-2/BMP-7 heterodimers lead to greater bone volume and number of mechanically stable fusions than either homodimer alone[33]. The mechanism of enhanced activity of BMP heterodimers remains to be elucidated; however, explanations include enhanced receptor stimulation or decreased inhibitor interaction[23,32].

We hope to demonstrate in an *in vivo* animal impact load model that BMP-2/BMP-7 heterodimers will prevent the development of chondrocyte apoptosis, decreased cellular metabolism, and progression to posttraumatic osteoarthritis. This project could be the first to demonstrate the potential of heterodimeric BMPs in an *in vivo* model of posttraumatic osteoarthritis with the promise of improving the care of the injured patient.

III. Previous work

Borrelli J, Jr., Burns ME, Ricci WM, and Silva MJ. **A method for delivering variable impact stresses to the articular cartilage of rabbit knees.** *J Orthop Trauma,* 16(3): 182-8, 2002.

This article details the development of an open joint model using a pendulum-type device to deliver a controlled impact to the posterior aspect of New Zealand White rabbit medial femoral condyles. The device was able to deliver a reproducible, rapid, and measurable impact which allowed future investigations into the effects of primary cartilage injury. The impact apparatus is shown on the figure to the right.

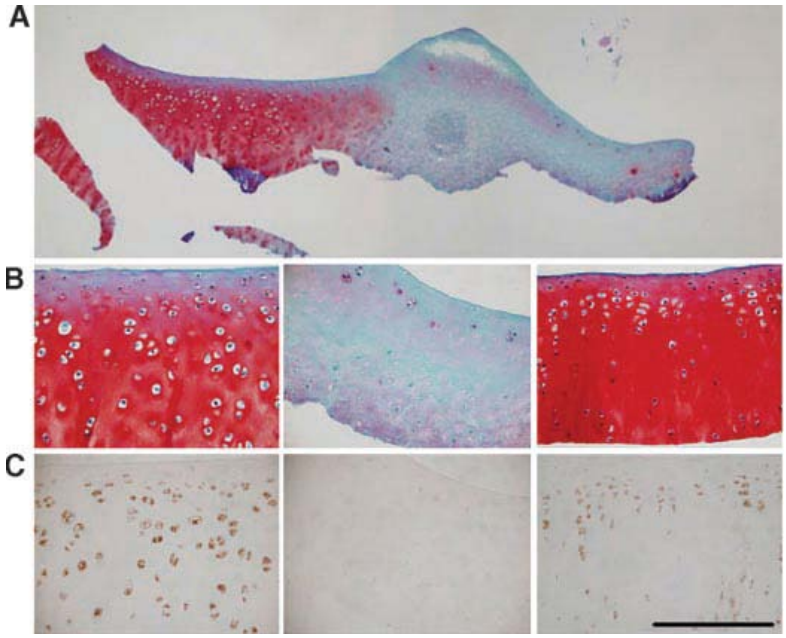


Borrelli J, Jr., Tinsley K, Ricci WM, Burns M, Karl IE, and Hotchkiss R**. Induction of chondrocyte apoptosis following impact load.** *J Orthop Trauma,* 17(9): 635-41, 2003.

Using the model system developed in the previous article, Borrelli et al investigated the incidence of chondrocyte apoptosis in response to a low and high impact load. Animals were euthanized 10 days following the impact load. A statistically significant increase in apoptotic chondrocytes was observed in the high impact load group versus either the control or low impact load group. Apoptosis was evaluated using multiple techniques including light microscopy, fluorescence microscopy, and electron microscopy.

Borrelli J, Jr., Silva MJ, Zaegel MA, Franz C, and Sandell LJ. Single high-energy impact load causes posttraumatic OA in young rabbits via a decrease in cellular metabolism. *J Orthop Res,* 27(3): 347-52, 2009.

NZW rabbits underwent a low and high impact load and then cartilage was harvested at various time points (time 0, 1 month, and 6 months) and analyzed for development of posttraumatic osteoarthritis. Results showed cartilage matrix disruption, proteoglycan content loss, cell metabolism decrease, and loss of chondrocytes leading to cartilage with the appearance of posttraumatic osteoarthritis. *Figure:* photo of a Safranin-O–stained cartilage specimen from a High impact load animal at 6-month postinjury (A). From left to right (B), higher power Safranin-O images of preserved (unimpacted area), impacted, and sham (control) cartilage. Some remaining ‘‘viable’’ chondrocytes can be identified within the impacted specimen. BMP-2 staining of preserved, impacted, and sham cartilage (left to right; C), showing absence of BMP-2 staining of the impacted specimen compared to the preserved and sham specimens. Bar represents 100 mm.



IV. Research Design and Methods

Impact Load Protocol

Fifty-four 12 week old New Zealand White (NZW) rabbits (108 medial femoral condyles) will undergo impact loading of the right posterior medial femoral condyle using a pendulum-type device, as previously described[6-9]. First, rabbits will be anesthetized, intubated, and placed in a sternal position. Using sterile surgical technique, a posterior approach will expose the right medial femoral condyle, the weight-bearing portion of the rabbit knee. The limb will then be fixed to a polyethylene block by two 1.6 mm Kirschner wires. Next the rabbit will be placed in the lateral decubitus position and the impactor will be aligned with the medial femoral condyle. When position is set, a pendulum with attached mass will be released from a predetermined height. Prior to impact, a piece of super-low pressure sensitive film (Fuji film) will be placed between the posterior medial femoral condyle and aluminum impactor to ensure uniform target area. The weight used will be sufficient to cause significant articular damage without causing subchondral bone fracture. Each impaction will have a uniform target time to peak force of 0.021 seconds (13 Hz). A piezoelectric load cell will measure the impact force while a data acquisition program will record impact force and time to peak force (Labview; National Instruments Corp., Austin, TX). For each rabbit, the left leg will undergo a sham impaction operation complete with an arthrotomy and touching of the impactor to the articular surface without application of load, and therefore serve as an internal control for the injection groups. Following impaction or sham impaction, each incision will be closed in layers in a standard fashion.

BMP Injections

Following closure of arthrotomies, injections of control buffer, BMP-2, BMP-7, or BMP-2/BMP-7 heterodimers will be performed in bilateral knees of anesthetized rabbits. The quantity of BMP-2 (5 µg), BMP-7 (20 µg), and BMP-2/BMP-7 heterodimers (10 µg) injected into rabbit joints stems from prior work in similar systems[2,3,13,14,22,29-31,33]. In all, one hundred eight medial femoral condyles will be processed from eight groups at three main time points (7-10 days, 30 days, and 180 days). Six animals will be euthanized at time 0 as to serve as a baseline for control and impacted groups. The additional groups include 1) sham impaction with buffer injection, 2) sham impaction with BMP-2 injection, 3) sham impaction with BMP-7 injection, 4) sham impaction with BMP-2/BMP-7 injection, 5) impaction with buffer injection, 6) impaction with BMP-2 injection, 7) impaction with BMP-7 injection, and 8) impaction with BMP-2/BMP-7 injection. Fifty-four animals will allow for four medial femoral condyles in each treatment arm (group and time point) which will provide sufficient sham or impacted cartilage for statistical analysis in our proposed methods. All animals will have free cage activities with water and food ad libitum. Pain medication will be provided and antibiotics will be administered for 48 hours postoperatively. Animals will be euthanized in a standard fashion at time 0, 7-10 days, 30 days, and 180 days. The Institutional Animal Care and Use Committee (IUCUC) of the University of Texas Southwestern Medical Center is reviewing this investigation for approval.

Detection of chondrocyte apoptosis (Aim 1)

Apoptosis will be detected in animals euthanized at seven days post impaction using several well-established methods to quantify chondrocyte apoptosis including conventional light microscopy with H&E staining, fluorescence microscopy with Hoechst 33342 staining, and immunocytochemical techniques[24]. Furthermore, every effort will be made to quantify specimens in a blinded fashion or by computerized quantification software. First, conventional light microscopy with hematoxylin and eosin staining will help identify the typical histologic changes of apoptosis including: cytoplasmic changes, condensed nuclei, and cell shrinkage as well as gross morphologic changes in specimens. Next, fluorescence microscopy with Hoechst 33342

nuclear staining will be used to identify nuclear condensation. Terminal 2'-deoxyuridine 5'-triphosphate (dUTP) nick end labeling (TUNEL) will be used to detect strand breaks in nuclear DNA and is a sensitive and efficient method to detect apoptosis. Finally, immunocytochemistry will be performed with an activated caspase-3 antibody to detect catalytically active caspase, a specific indicator of apoptosis. Student's t test will be used to compare stained/labeled cells in different treatment groups.

Detection of Posttraumatic Osteoarthritis (Aim 2)

As in Borrelli et al 2009, PTA will be characterized at 30 days and 180 days post impaction[8].

Histological Assessment

To assess the initial changes within the injured cartilage as a result of the single impact load, we will perform histological assessment. This will include scoring initial damage with the damage scoring scale of Lewis. This scoring system ranges from grade 0 which is no disruption of cartilage surface to grade 3 which is complete disruption of cartilage surface. Furthermore, cartilage specimens will undergo routine histology to assess cartilage morphology using hematoxylin and eosin (H&E) and Safranin-O staining to assess the proteoglycan content of the extracellular matrix.

Immunohistochemistry

Immunohistochemistry techniques as described by Fukui et al. will be used to assess changes in BMP-2 production[8,17]. Anti-human BMP-2 goat polyclonal antisera (Santa Cruz Biotechnology, Santa Cruz, CA) will be used to detect BMP-2 in paraffin-embedded specimens, which can be visualized with the streptavidin (Sigma, St. Louis, MO) and DAB from BrDu staining kit (Zymed Laboratories, Inc., South San Francisco, CA). Donkey anti-goat (Santa Cruz Biotechnology) secondary antibody will be used localize BMP-2. For control staining, nonimmune serum will be used in place of the primary antibody. Student's t test will be used to compare numbers of stained cells in differing groups.

Biochemical: In Situ Hybridization to mRNA

In situ hybridization will be used to assess procollagen type II mRNA synthesis and 18S ribosomal RNA content, and both are an indication of chondrocyte metabolism[8]. Briefly, cRNA (riboprobes) will be generated from cDNA plasmids encoding for a fragment of type II procollagen (bovine IIB N-propeptide). This riboprobe has previously been shown to be specific for rabbit mRNA and does not cross react with other similar collagens[8,28]. Radiolabeled riboprobe will be added to hybridization buffer and hybridized at 53–58°C. Unbound probe will be hydrolyzed with RNase A, and final washes will be carried out at high stringency (53–58°C, 2xSSC/50% formamide). Slides will be exposed to Iso-MaxAutoradiography/Xray film (SciMart, St. Louis, MO) for 3 days to approximate signal strength and prepared for autoradiography by dipping in photographic emulsion. The slides will be dried, sealed, and kept in a dark box for 72 h at 48C. Sections will be counterstained by routine methods with Mayer's Hematoxylin. Bright field and dark field microscopy will then be performed. Student's t test will be used to compare numbers of stained cells in differing groups.

Timeline for completion

January 2011 – acquisition of rabbits and impact loading of animals.
Late January – February 2011 – we will be able to obtain data from specimens in the apoptosis group as well as PTA group at 30 days post injury.
June 2011 – we will start acquiring data on the PTA group at 180 days post injury.
July 2011 – October 2011 – we will complete data analysis.

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