Inflammatory and Immune Effects of Intra-Articular Mesenchymal Stem Cells in Horses

THESIS

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

By

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2013

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Abstract

Mesenchymal stem cells have demonstrated anti-inflammatory and immunomodulatory capabilities as well as modest efficacy in animal models of joint injury, warranting further study as a potential treatment of equine joint disease. The goal of the study is to investigate the inflammatory and immune response to intra-articular injection of autologous, genetically modified (bone morphogenetic protein 2) autologous, allogeneic, and xenogeneic bone marrow-derived mesenchymal stem cells (MSC) in horses.

The study group consisted of 6 five-year-old Thoroughbred mares, free of musculoskeletal disease in the fetlocks. The 4 fetlock joints were injected on day 0 with 15 million MSC from the described MSC groups and were assessed for 28 days for clinical and inflammatory parameters representing synovitis, swelling and pain. Arthroscopic examination and synovial biopsies were taken at day 60 post-injection for histologic and molecular analyses. On day 120, peripheral blood mononuclear cells were isolated from each horse and co-cultured with monolayers of each MSC group for analysis of CD4 positive cells via flow cytometry and cytokines via ELISA. There was no significant difference between autologous and genetically modified autologous MSC for any parameter examined and soluble gene product was detected for at least 2 days.
Allogeneic and xenogeneic MSC produced a modest increase in peak of inflammation at 24 hours than either autologous MSC group. Most inflammatory parameters returned to baseline or control values within 7-14 days for all MSC groups. Arthroscopic examination revealed normal synovium with no grossly detrimental effect to the synovium or cartilage. Synovial histology demonstrated significantly increased inflammatory cells, lymphocyte perivascular cuffing and bone morphogenetic protein 2 antibody uptake for MSC injected joints compared to controls. Adaptive immunity was demonstrated upon re-exposure of peripheral blood mononuclear cells to xenogeneic MSC in co-culture but not for allogeneic MSC.

Genetically engineered MSC can produce gene product to detectable synovial concentrations and did not result in greater inflammation warranting further investigation into the therapeutic potential of this cell therapy. Intra-articular MSC resulted in a moderate acute inflammatory joint response that was greater for allogeneic and xenogeneic MSC than autologous MSC. Clinical management of this response may minimize this effect. Intra-articular MSC demonstrated a persistent mononuclear infiltrate for at least 60 days, the significance of which is not known. An adaptive immune response was detected for xenogeneic but not for allogeneic MSC suggesting that a second intra-articular injection of xenogeneic MSC would result in a stronger inflammatory response.
Acknowledgments

The author would like to acknowledge Dr. Akikazu Ishihara for his technical assistance with data collection and analysis, Dr. Jennifer Dulin for assistance with general anesthesia, Rebekah Sanchez-Hodge and Heather Lane for their assistance with data collection, and Tim Vojt for medical illustration.
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Major Field: Veterinary Clinical Sciences
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Chapter 1: Introduction

Direct intra-articular injection of bone marrow derived mesenchymal stem cells (BMDMSC) has demonstrated modest efficacy in models of cartilage defects, osteoarthritis, cruciate ligament and meniscus damage in goats, pigs and rats.\textsuperscript{1-3} In horses with superficial digital flexor tendinopathy, recent studies have demonstrated that intra-lesional BMDMSC may reduce the reinjury rate after returning to racing.\textsuperscript{4} Direct intra-articular BMDMSC injection improved repair quality in a cartilage defect model in horses,\textsuperscript{5} warranting further study of these cells for potential treatment of equine joint disease.

Mesenchymal stem cells from equine bone marrow are considered multipotent\textsuperscript{6,7} and can be expanded and genetically manipulated in standard culture conditions making them an attractive target for gene therapy.\textsuperscript{8,9} Genetic alteration of cells allows for the release of soluble factors that may provide an enhanced therapeutic response.\textsuperscript{10} Bone morphogenetic protein 2 has demonstrated chondrogenic effects and safety both in vitro and in animal models, including horses,\textsuperscript{11,12} when placed in the articular environment.\textsuperscript{13} Bone morphogenetic protein 2 was therefore selected as a marker gene to assess cell-mediated gene expression in vivo and the inflammatory and adaptive immune response to these genetically engineered autologous BMDMSC.
Human MSC have demonstrated immune evading or modulatory capabilities in co-culture experiments with PBMC. Human and equine BMDMSC do not express MHC class II antigen, which would be expected to reduce MSC antigenicity. Although MSC immune evasion has been demonstrated in vitro in species other than the horse, an immune response to allogeneic subcutaneous, intravenous, intraperitoneal, and intracardiac stem cell injections has been reported in mice and pigs. To the authors’ knowledge, an immune response to allogeneic intra-articular MSC has not been investigated.

Few studies have characterized joint inflammation after intra-articular injection of MSC in any species, with limited reports in the horse. A recent short-term (72 hr) safety study compared the inflammatory effects of autologous or allogeneic placenta-derived MSC after intra-articular injection of 7.5 x 10^6 cells in horses. Although not statistically different, for several outcome parameters measured, the allogeneic MSC had a greater degree of inflammation for approximately the first 36 hours than the autologous MSC or saline control. We investigated further the inflammatory reaction and adaptive immune response to BMDMSC after intra-articular injection of a greater dose (15 x 10^6 cells) and of greater immunogenic diversity by using autologous, genetically engineered autologous, allogeneic or xenogeneic BMDMSC. Our hypothesis was that there would be no significant difference in the clinical and synovial inflammatory response among cell groups, but a detectable adaptive immune response to intra-articular injections of xenogeneic mesenchymal stem cells. We further hypothesized that there would be no difference in response to an injection of a genetically modified cell.
Chapter 2: Materials and Methods

**Horses and Study Design**

Six horses (5 year old Thoroughbred mares), free of musculoskeletal abnormalities in the metacarpo(tarso)phalangeal (fetlock) joints as determined by palpation, range of motion, and distal limb flexion, were included in the study. Limbs were assigned in a block design such that every horse had each of 4 fetlock joints injected on day 0 with 1 of 4 BMDMSC treatments: 1) autologous (auto), 2) genetically modified autologous (auto-BMP2), 3) allogeneic (allo) (from a different study horse), or 4) xenogeneic (xeno)[human]. Limbs were assigned such that BMDMSC treatments were equally divided between fore- and hind limbs. At day -30, 4 horses in the study group were randomly chosen to serve as an injection and vehicle control by a single fetlock injection of 1 mL of Gey’s balanced salt solution (GBSS) and synovial fluid sampled as per the MSC protocol through 14 days. Clinical and synovial fluid parameters were evaluated serially from day -30 to day -16 (day -30, -29, -28, -23 and -16 after GBSS injection) and for 28 days after MSC injection that occurred on day 0. Horses were confined to stall rest during the study period and limbs were not bandaged from days -30 to 60. Non-steroidal anti-inflammatory drugs were not administered so as to not interfere with the inflammatory response and provide a baseline clinical response to the injections. On day 60 post MSC injection, a subset of joints from all 6 horses (6 auto-BMP2, 3 auto,
3 allo, 3 xeno) underwent arthroscopic visualization and synovial biopsy for histologic and molecular analyses with distal limb bandages placed post-operatively for 14 days. Peripheral venous blood was obtained at 120 days after MSC injection for evaluation of adaptive immunity in co-culture assays designed to detect an adaptive cell mediated immune response specific for the autologous, allogeneic, or xenogeneic cell sources. Normal synovium was obtained from the palmar/plantar recess of the fetlock joints of 4 horses euthanized for reasons other than lameness or osteoarthritis to serve as uninjected controls for histologic and molecular analyses. All procedures were approved by the Institutional Animal Care and Use Committee at The Ohio State University.

**MSC Isolation and Characterization**

Bone marrow-derived mesenchymal stem cells from these 6 study horses were isolated and cryopreserved prior to the start of this study. Briefly, 60mL of bone marrow was obtained from the sternum under general anesthesia and was concentrated using a gravitational marrow separator with subsequent culture in monolayer. Mesenchymal stem cells were identified based on plastic adherence under standard cell culture conditions: Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum (FBS) at 37°C in a 5% CO$_2$ atmosphere. Mesenchymal stem cells were divided into aliquots of 1x10$^6$ cells and were placed in 1 mL of FBS containing 10% DMSO and frozen at -80°C for future use. Upon thawing, the cells were passaged 2-4 times, were harvested by trypsinization, counted using a hemacytometer, and viability confirmed with trypan blue exclusion stain. Bone marrow-derived xenogeneic MSC were similarly expanded and processed.
The multipotential nature of these BMDMSC (equine and human) was confirmed by differentiation into osteogenic, adipogenic, and chondrogenic lineages in vitro using the manufacturer’s recommended protocol for human MSC. For osteogenic differentiation, cells were maintained in monolayer culture for approximately 14 days and stained for production of mineral with von Kossa. For adipogenic differentiation, cells were maintained in monolayer culture for 21 days and stained for lipid production with oil red O. For chondrogenic differentiation, cells were expanded in monolayer culture and approximately $2.5 \times 10^5$ cells were formed into a pellet. Cells were maintained in culture for 21 days and then fixed in formalin, sectioned (4 µm) and stained for glycosaminoglycan production and chondrocyte morphology with toluidine blue.

Bone marrow-derived MSC (equine and human) were further characterized based on cell surface expression of MHCII and cluster of differentiation (CD) 90. Briefly, BMDMSC were trypsinized, washed twice with phosphate buffered saline, counted by hemacytometer, and 1 million cells were placed in two separate tubes. To serve as flow cytometry control cells to compare to MSC, PBMC were obtained from the same horses and isolated by Ficoll density gradient from 30 mL of heparinized venous blood per horse by following established company protocols. Briefly, 15 mL of blood mixed with 15 mL of PBS containing 2% FBS was carefully layered on top of 15 mL of Ficoll (2 tubes per horse to process a total of 30 mL blood) and centrifuged at room temperature for 30 minutes. The upper plasma layer was removed, and the PBMC layer at the plasma-Ficoll interface was removed and retained. The PBMC were washed twice with PBS, counted using a hemacytometer and placed in two separate tubes (1 million cells/tube) as
a control cell type. Mesenchymal stem cells and PBMC were mixed with either anti-equine MHCII monoclonal antibody,^k^ anti-mouse secondary antibody,^l^ and 7-Aminoactinomycin D (7AAD) fluorescent chemical^m^ or with anti-human CD90 primary monoclonal antibody,^n^ and 7AAD. The cells were then incubated for 2 hours at 4°C in a dark atmosphere, washed twice with PBS, and analyzed with a flow-cytometry analyzer.\(^o\)^ For each sample, all cells were gated and analyzed using forward and side light scatter. The numbers of viable MHCII positive or CD90 positive cells were calculated by counting the total cells and MHCII(+) 7AAD(-) cells in the first tubes, and the total cells and CD90(+) 7AAD(-) cells in the second tubes.

**Production of BMP2 by Transduced BMDMSC**

Cryopreserved BMDMSC for each horse were thawed, seeded at approximately 500,000 cells per culture flask (25 cm\(^2\)), maintained until approximately 80-90% confluence, and then transduced with an adenovirus (Ad) serotype 5 vector with E1 and E3 deletions encoding the human BMP2 gene (Ad-BMP2) at 100 multiplicity of infection (MOI).\(^{26}\) To verify gene expression, transduced MSC of 3 horses were maintained in culture with media collected at day 0 (2 days post-transduction), 1, 2, 7, 14, 21, and 28 and BMP2 protein production confirmed with ELISA.\(^p\)

**Generation of \(hbmp2\) Engineered Autologous BMDMSC**

Two days prior to MSC injection of the joints, a subset of equine MSC (80-90% confluence) were designated as the auto-BMP2 group and were transduced with 100 MOI Ad-BMP2 after estimating cell numbers per flask with an ocular lens grid square (1 cm x 1 cm). The cells were maintained in culture for 2 days until peak gene expression was
Cells were then harvested via trypsinization, counted using a hemacytometer and viability confirmed using trypan blue stain, and approximately $15 \times 10^6$ cells were transferred to another flask. This injection dose was then washed twice in GBSS. The cell pellet was resuspended in GBSS to create an injection volume of 1 mL and an injection dose of $15 \times 10^6$ cells transduced with the human BMP2 gene ($hbmp2$). The other MSC groups (auto, allo, xeno) were trypsinized, counted, and washed in a similar manner in preparation for injection.

**Intra-Articular MSC Injection**

Intra-articular injections were performed in the standing horse after sedation (xylazine hydrochloride 0.2-1.1 mg/kg i.v.) through a lateral collateral sesamoidean ligament approach for every joint. At day 0, all 6 horses had a dose of approximately $15 \times 10^6$ cells injected into the fetlock joint within approximately 60-90 minutes from trypsinization. All joints were injected within a 14 day period by a single investigator (JHP) blinded to the MSC group being administered.

**Synovial Fluid Analyses**

Synovial fluid (1 mL) was aspirated from each fetlock joint on days -30, -29, -28, -23, and -16 followed by a 2 week washout period, and then on days 0, 1, 2, 7, 14, 21, and 28 post injection. A single investigator (JHP), blinded to the MSC treatments, performed all arthrocenteses throughout the sampling period. Approximately 0.3 mL of synovial fluid was aliquoted into an EDTA tube for cytology. The remaining fluid was placed in a microcentrifuge tube, spun at 12,000 rpm for 5 minutes, and the supernatant frozen at -80°C for ELISA analysis of interleukin-6 (IL-6),$^p$ interleukin-10 (IL-10),$^p$ and
BMP2. Cytologic analysis of synovial fluid was performed within 30-60 minutes of collection for total nucleated cell count, and total protein concentration via refractometry. Differential nucleated cell counts were performed by a board certified veterinary clinical pathologist (MLW) blinded to the treatment groups.

**Clinical Monitoring**

Pulse, respiration, temperature and lameness at the walk were monitored daily throughout the study. Clinical signs of inflammation were monitored on the same days as synovial fluid collection and included joint swelling (circumference and edema score), and pain (range of joint motion) as previously described. Pain-free range of motion was assessed through fetlock flexion until the horse raised its head or moved its limb in resistance while measuring degree of flexion with a hand held goniometer. Fetlock circumference was measured with a tape at the middle aspect of the proximal sesamoid bone. Both measurements were taken in triplicate and averaged. Distal limb edema was assessed on a 0 to 4 scale (0-no swelling; 1-minimal swelling localized to the injection site; 2-mild swelling localized to the fetlock joint; 3-moderate swelling extending proximally towards the carpus or tarsus; 4-marked swelling extending to or above the carpus or tarsus. All observations were made by a single investigator (JHP) blinded to the MSC group administered.

**Arthroscopic Evaluation, Synovial Biopsy and Processing**

Synovial biopsies were obtained and processed from 4 normal control horses and the 6 study horses. Approximately 60 days after the BMDMSC injection, the 6 study horses underwent general anesthesia for arthroscopic visualization and synovial biopsy
of the palmar/plantar pouch of the fetlocks using a rongeur. In all 6 horses, hind fetlocks were explored arthroscopically, as well as any front fetlock that was injected with the auto-BMP2 group. This resulted in 6 auto-BMP2, 3 auto, 3 allo, and 3 xeno biopsies that were obtained and further analyzed. For all horses, a synovial biopsy was fixed in 10% formalin for sectioning and staining for routine histology and immunohistochemical analysis. A different synovial biopsy was flash frozen in liquid nitrogen and stored at -80°C for DNA and RNA extraction.

**Histology, Histomorphometry, and Immunohistochemistry**

Formalin fixed synovial biopsy specimens were sectioned (4µm) and stained using hematoxylin and eosin (H&E) and anti-CD3 antibody for morphology and identification of lymphocytes, respectively. Slides stained with H&E were evaluated semi-quantitatively for the distribution and severity of inflammation and overall cellularity including inflammatory cells and synovial cells. Scores were assigned over 5 fields at 20x by each of 3 observers (JHP, ALB, DSR) and the median score determined. For overall cellularity (including inflammatory cells, synovial cells and stromal cells), the following criteria were used: none (0), minimal (1), mild (2), moderate (3), and marked (4). For inflammatory cell severity (including neutrophils, lymphocytes, macrophages and plasma cells), the following criteria were used: none (0), minimal (1-few scattered inflammatory cells), mild (2-multifocal areas of inflammatory cells), moderate (3-multifocal to coalescing areas of inflammatory cells), marked (4-area confluent with inflammatory cells). For inflammatory cell distribution, the following criteria were used: 1-25% of field were inflammatory cells (1), 26-50% of field were inflammatory cells (2),
51-75% of field were inflammatory cells (3), 76-100% of field were inflammatory cells (4). If present, synovial microthrombi were quantified by hand counting, averaging the number of microthrombi and unaffected vessels at 20x over 5 fields per slide.

These same specimens were sectioned (4µm) and stained with CD3 antibody. Briefly, a polyclonal rabbit anti-human CD3 antibody at a dilution of 1:100 was used and counterstained with biotinylated goat anti-rabbit antibody at a dilution of 1:200. Equine positive control (lymph node) and isotype matched negative controls to assess for non-specific background staining were included. CD3 positive perivascular lymphocytes were quantified by counting and averaging the number of perivascular cuffs and unaffected vessels at 20x over 5 fields per slide. Perivascular cuffs were given a score for size: >50% of cells around circumference of vessel are lymphocytes (1), confluent single layer of lymphocytes surrounding vessel (2), confluent 2-4 layers of lymphocytes surrounding vessel (3), confluent >4 layers of lymphocytes surrounding vessel (4).

**Synovial Biolocalization of MSC and In Vivo hbmp2 Gene Expression**

Tracking of injected cells and detection of expression of *hbmp2* by the injected cells was performed by real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR) of the synovium, PCR for the cytomegalovirus (CMV) promoter in the synovium, immunohistochemical staining for BMP2 in synovium, and BMP2 concentration in synovial fluid using ELISA.

In vivo expression of *hbmp2* was determined by real-time RT-PCR using methods previously described. \(^{28}\) RNA was extracted using the NucleoSpin® RNA XS kit from synovial tissue of auto-BMP2 injected joints (n=6), auto injected joints (n=3), allo
injected joints (n=3), xeno injected joints (n=3) and non-injected control joints (n=6). RNA was also extracted from cultured equine BMDMSC with and without Ad-BMP2 transduction (100 MOI) as positive and negative controls. Transgene expression of \textit{hbmp2} was quantified by TaqMan® real-time RT-PCR with the ABI PRISM 7000 Sequence Detection System.\textsuperscript{v} Mean fold change was calculated using commercially available primers and probe sets for the \textit{hbmp2} genes relative to expression of the non-transduced BMDMSC and relative to expression of the endogenous 18S RNA genes using the \(2^{-\Delta\Delta C_t}\) method.\textsuperscript{29}

Biolocalization of BMP2-transduced BMDMSC was determined using quantitative PCR as previously described.\textsuperscript{30} Genomic DNA was extracted using a QIAmp DNA Mini Kit\textsuperscript{w} from synovial tissue of auto-BMP2 injected joints (n=6), auto injected joints (n=3), allo injected joints (n=3), xeno injected joints (n=3) and non-injected control joints (n=6). DNA was also extracted from cultured equine BMDMSC with and without Ad-BMP2 transduction (100 MOI) as positive and negative controls. Quantitative PCR was performed using primers that amplify CMV sequences, which were detected by the internal fluorogenic probe labeled with FAM reporter dye, and ABI Prism 7000 sequence detection instrument. Each of triplicate reactions contained 100 ng of test DNA, and a standard curve was generated using plasmid containing \(5\times10^1\) through \(5\times10^7\) copies of CMV.

Formalin fixed synovial biopsy specimens were sectioned (4\(\mu\)m) and stained with polyclonal rabbit anti-human BMP2 antibody\textsuperscript{x} at a dilution of 1:100 and counterstained with biotinylated goat anti-rabbit antibody\textsuperscript{t} at a dilution of 1:200. Antibody specificity
was assessed and positive controls were generated by fixing, sectioning and staining a pellet of BMDMSC after transduction with Ad-BMP2 (100 MOI). Briefly, BMDMSC were expanded in monolayer and transduced with Ad-BMP2 as described. Cells were trypsinized and approximately $5 \times 10^5$ MSC underwent centrifugation and were formed into a pellet that was maintained in culture for an additional 5 days. Negative controls were subject to the same protocol without gene transduction. Isotype matched negative controls were generated to assess non-specific background staining. Intensity, percent tissue positive and percent cell number positive of BMP2 immunostaining for cell injected groups compared to control synovium from normal horses was scored over 5 high power fields by 3 observers (JP, ALB, DR) and the median score was determined. Intensity of stain uptake was assessed at 20x and was graded none (0), minimal (1), mild (2), moderate (3), or marked (4). Percent of the tissue sample positive for stain was assessed at 4x as 1-25% of field (1), 26-50% of field (2), 51-75% of field (3), or 76-100% of field (4). Percent of cells positive for stain was assessed at 20x and graded using the same system.

Synovial fluid BMP2 protein concentration was analyzed by ELISA following the manufacturer’s protocol. Control samples consisted of synovial fluid from joints injected with non-transduced autologous BMDMSC and baseline synovial fluid in the autologous groups before injection. A standard curve was generated using company provided standards, and BMP2 concentrations determined using the optical density and equation for the slope of the curve.

**Adaptive Immunity In Vitro Co-culture Assays**
Adaptive immunity against auto, auto-BMP2, allo, and xeno groups was assessed in PBMC collected from all 6 horses at day 120. PBMC isolated by Ficoll gradient as described above were added to semi-confluent (70%) monolayer cultures of auto, auto-BMP2, allo, and xeno. The PBMC from each of the 6 horses were applied to each MSC well on day 0 for an in vitro co-culture experiment in 20:1 ratio (1×10^6 PBMC added onto 5×10^4 MSC monolayer). The PBMC and MSC were assigned to the following 9 experimental groups: (A) PBMC alone (negative immunoactivation control), (B) MSC alone (MSC control), (C) auto-BMP2 alone (genetically engineered MSC control), (D) PBMC plus auto (autologous immunoactivation), (E) PBMC plus auto-BMP2 (autologous genetically engineered immunoactivation), (F) Unexposed PBMC plus allo (eg, randomly selected equine MSC group that was not injected into the horse in whom PBMC were harvested) [allogeneic immunoactivation], (G) Exposed PBMC plus allo (eg, equine MSC group that was injected into the horse in whom PBMC were harvested) [allogeneic amnestic immunoactivation], (H) PBMC plus xeno (human) [xenogeneic immunoactivation], and (I) PBMC plus LPS (positive control).

The activation of a cell mediated immune response in the PBMC was evaluated on days 1, 3, and 6 by flow cytometry for the number of CD4 positive lymphocytes, trypan-blue staining for PBMC counts, PBMC viability, MSC viability, and ELISA of supernatant culture media for IL-6, IL-2, IFNγ, and IL-10 concentrations. All ELISA analyses were performed using commercially available kits. Monoclonal mouse anti-equine CD4 antibody, secondary FITC rat anti mouse antibody and 7AAD were applied.
to the PBMC samples and incubated for 2 hours at 4°C in a dark atmosphere prior to analysis by flow cytometry.

**Statistical Analysis**

Repeated-measure analysis of variance (ANOVA) was used to evaluate three factors; 1) MSC group, 2) time, and 3) limb. Limb was not a statistically significant factor in the analysis. For the statistically significant factors of MSC treatment and time, multiple post-test comparisons were performed using Proc Mixed statistical models for all continuous outcomes and Proc Genmod statistical models for all categorical outcomes. Variables were considered nested within horse, with the horse factor treated as a random variable, and the distribution of data assessed by use of a subset of normality. Data were assessed for normality using the Shapiro-Wilk analysis. Histologic scores were analyzed among MSC groups and control normal synovium by a non-parametric Kruskal-Wallis with adjusted individual Mann Whitney U post-tests. Significance level was set at $P < 0.05$ for all analyses.
Chapter 3: Results

**MSC Isolation and Characterization**

Mesenchymal stem cells were successfully isolated from bone marrow and expanded in culture. Characterization of BMDMSC (equine and human) via flow cytometry revealed that the majority of cells were MHC class II negative (95 ± 0.86%) and CD90 positive (98 ± 0.69%). The multipotential nature of BMDMSC from the equine and human sources was confirmed by trilineage differentiation of all samples with positive staining for mineral, glycosaminoglycan and lipid formation in vitro. (Data not shown)

**Synovial Fluid Analysis**

All six horses successfully completed the study and synovial fluid was obtained from all horses at each time point. There was no significant difference between auto and auto-BMP2 groups both within and across time points for all outcome assessments. All BMDMSC injected groups produced greater inflammation than the GBSS control as measured by total nucleated cell count, neutrophil and monocyte counts, total protein and IL-6 concentrations (Figure 1A-E). Xeno and allo injections produced a significantly greater peak of inflammation as measured by total nucleated cell count, total protein concentration, neutrophil and monocyte (allo only) counts, and significantly greater duration of inflammation as measured by total nucleated cells in the allo group (Figure
Synovial fluid lymphocytes significantly increased in all MSC injected joints, peaked at day 2 and remained elevated for the extent of the study in 66% of MSC injected joints (Data not shown). Synovial fluid lymphocyte and monocyte counts in the allo group were significantly greater than the auto and auto-BMP2 groups on day 14 ($P < 0.05$) and with a trend at day 21 ($P < 0.08$, Figure 1C inset showing monocytes only). Interleukin-6 was significantly increased on days 1 (xeno only) and 2 (xeno and allo) (Figure 1E). Interleukin-10 was not detectable except in 1 horse with increased IL-10 concentration for all 4 MSC groups that remained greater than GBSS across the study period.

**Clinical Monitoring**

Physical exam parameters of temperature, pulse, and respiration were within normal limits throughout the study. A variable lameness response was observed within 12 hours at the walk that was not different among MSC treatments and resolved by 48 hours post-injection. All MSC groups had a significantly greater clinical inflammatory response than the GBSS control (Figure 2A-C). Xeno and allo groups produced significantly greater peak inflammation, measured as increased joint circumference, increased edema score, and reduced range of motion (Figure 2A-C). Edema in the xeno group was significantly higher than the auto and auto-BMP2 groups at days 14 and 21 and higher than all groups at day 28 (Figure 2B). The pattern of distal limb edema was distinctly proximal to the pastern and was clinically significant across all MSC groups.

**Arthroscopic Evaluation and Synovial Biopsy**
All six horses successfully underwent general anesthesia and had selected fetlock joints visualized arthroscopically at low and high magnification for evaluation and synovial biopsy at 60 days post injection (auto-BMP2 n=6 joints, auto n=3 joints, allo n=3 joints, xeno n=3 joints). Grossly at high magnification, no obvious abnormalities were observed in the synovium or the cartilage.

**Histology, Histomorphometry, and Immunohistochemistry**

Subjectively, H & E stained sections from all MSC injected joints had perivascular cell cuffing, a feature that was not seen in normal control synovium (Figure 3A-D). In MSC injected joints, some small caliber vessels (approximately 20-80 µm in diameter) contained fibrillar pink material, with spindle cells and hyperchromatic nuclei attached to the intimal surface, interpreted as fibrin microthrombi (Figure 3B inset, Table 1). Inflammatory cell severity, distribution and overall cellularity were significantly greater in the MSC injected joints than control synovium ($P = 0.002$, $P = 0.002$, $P = 0.03$ respectively, Table 2). Perivascular cellular cuffs stained positive for CD3 indicating a homogeneous cell population of lymphocytes (Figure 3D). The number of perivascular cellular cuffs was significantly increased in MSC injected joints compared to control synovium but no significant difference was observed between MSC groups (Table 1). Neutrophils and plasma cells were rarely observed. Vascular microthrombi were significantly increased in number in MSC injected joints compared to normal synovium ($P = 0.036$, Table 1).

**Synovial Biolocalization of Cells and In Vivo hbmp2 Gene Expression**
Bone morphogenetic protein 2 was significantly greater in synovial fluid from joints injected with auto-BMP2 on days 1 and 2 than auto controls (Figure 1F). Variable cellular gene expression of BMP2 was confirmed in the synovium of all MSC injected joints and normal control synovium by both RTPCR and immunohistochemistry using polyclonal anti-human BMP2 antibody. In all groups, including control synovium, the surface synovial cells and the endothelium stained positively with polyclonal anti-human BMP2 (Figure 3E-F). In MSC injected joints, the intensity and amount of tissue staining with polyclonal anti-human BMP2 was significantly increased compared to control synovium ($P = 0.049$, $P = 0.018$ respectively, Figure 3E-F, Table 2). In vitro assessment of polyclonal anti-human BMP2 antibody specificity revealed a marked increase in staining of a BMP2 transduced equine BMDMSC pellet, but a mild amount of stain uptake was observed in non-transduced negative controls. In these synovial specimens obtained 60 days after MSC injection, the CMV promoter was not detected using our PCR techniques.

**Adaptive Immunity and Inflammatory Mediators of MSC in Co-Culture Assays In Vitro**

Adaptive immunity was demonstrated by a significant increase in number of CD4 positive cells at days 3 and 6 in co-culture upon re-exposure of PBMC to the xeno group (Figure 4). For trypan-blue staining, the number of PBMC at day 6 was significantly greater in the PBMC plus xeno group compared to all other groups. The viability of proliferating CD4 positive cells was not significantly different among the experimental groups and time points. The viability and cell number of MSC were not significantly
different among the experimental groups. Cytokine analysis via ELISA of culture media revealed significant increases in IL-6 for the PBMC plus xeno group compared to all other groups. Also, the IL-6 concentrations at days 3 and 6 were significantly greater in all PBMC plus MSC groups compared to the PBMC/MSC alone and PBMC plus LPS groups. Interleukin-6 was detected in the MSC only group at very low levels (0.4 pg/mL) on day 6. Interleukin-2 concentrations were not significantly different among the experimental groups at any time points. Interferon gamma concentrations at days 3 and 6 were significantly greater in the PBMC plus xeno group compared to the MSC alone group. Interleukin-10 concentrations at days 3 and 6 were significantly greater in PBMC plus auto, unexposed allo, exposed allo and xeno groups compared to the PBMC/MSC alone (day 3 only) or MSC alone groups.
### Table 1

Mean ± SEM immunohistochemical and histomorphometric analysis of CD3 positive perivascular cuffs and vascular microthrombi in MSC injected joints and control synovium. *Values are significantly different ($P < 0.05$) between each MSC group compared to control. Control synovium was obtained from normal fetlock joints of horses not in the study.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Auto</th>
<th>Auto-BMP2</th>
<th>Allo</th>
<th>Xeno</th>
<th>Control</th>
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<tbody>
<tr>
<td>CD3+ perivascular cuffs % present *</td>
<td>33.8 ± 4.3</td>
<td>28.5 ± 4.5</td>
<td>28.6 ± 3.5</td>
<td>34.6 ± 8.3</td>
<td>2.1 ± 2.1</td>
</tr>
<tr>
<td>Size score (1-4) *</td>
<td>1.9 ± 0.4</td>
<td>2.4 ± 0.2</td>
<td>2.1 ± 0.2</td>
<td>1.9 ± 0.3</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>H&amp;E microthrombi % present *</td>
<td>6.27 ± 5.2</td>
<td>16.4 ± 6.6</td>
<td>10.1 ± 0.8</td>
<td>5.4 ± 1.1</td>
<td>1.5 ± 1.5</td>
</tr>
</tbody>
</table>

### Table 2

Median (range) of histologic (inflammatory) and immunohistochemical (hBMP2 positive stain uptake) of synovium from MSC injected joints compared to control. *Values are significantly different ($P < 0.05$) in MSC injected joints compared to controls. See Table 1 for control definition.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Auto</th>
<th>Auto-BMP2</th>
<th>Allo</th>
<th>Xeno</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>H&amp;E inflammation scores</td>
<td>1 (0.5-3)</td>
<td>2 (1-3)</td>
<td>1.5 (1.5-3.5)</td>
<td>2 (1.5-2)</td>
<td>0.5 (0-2)</td>
</tr>
<tr>
<td>Severity (0-4) *</td>
<td>1 (0.5-2)</td>
<td>2 (1-3)</td>
<td>1.5 (1-3)</td>
<td>1 (1-2)</td>
<td>0.5 (0-1)</td>
</tr>
<tr>
<td>Distribution (0-4) *</td>
<td>1.5 (1-3)</td>
<td>3 (1-4)</td>
<td>2.5 (2-4)</td>
<td>1 (1-2)</td>
<td>1 (0-2)</td>
</tr>
<tr>
<td>H&amp;E cellularity score Cellularity (0-4) *</td>
<td>2 (2-2)</td>
<td>3 (1-3)</td>
<td>3 (2-3)</td>
<td>1 (1-2)</td>
<td>1 (1-2)</td>
</tr>
<tr>
<td>Polyclonal hBMP2 scores</td>
<td>2 (2-2)</td>
<td>3 (1-4)</td>
<td>2 (2-3)</td>
<td>2 (1-2)</td>
<td>2 (1-3)</td>
</tr>
<tr>
<td>% tissue positive *</td>
<td>2 (2-3)</td>
<td>3 (1-4)</td>
<td>2 (2-3)</td>
<td>2 (1-2)</td>
<td>2 (1-3)</td>
</tr>
<tr>
<td>Intensity *</td>
<td>2 (2-3)</td>
<td>3 (1-4)</td>
<td>2 (2-3)</td>
<td>2 (1-2)</td>
<td>2 (1-3)</td>
</tr>
<tr>
<td>% cells positive</td>
<td>2 (2-3)</td>
<td>3 (1-4)</td>
<td>2 (2-3)</td>
<td>2 (1-2)</td>
<td>2 (1-3)</td>
</tr>
</tbody>
</table>
Figure 1 (A – F) – Mean ± SEM synovial fluid analysis for mesenchymal stem cell injected joints compared to controls. A – Total nucleated cell count (cells/µL) with inset highlighting days 7-28. B – Neutrophil cell count (cells/µL). C – Monocyte cell count (cells/µL) with inset highlighting days 7-28. D – Total Protein concentration (g/dL). E – Interleukin-6 concentration (pg/mL). F – Bone morphogenetic protein 2 concentration (pg/mL). Data from auto and auto-BMP2 groups were not significantly different and were combined for graphics only. Different symbols (*) indicate significantly different ($P < 0.05$) groups (A-E) or time points (F). All MSC groups were significantly different than Gey’s Buffered Salt Solution (GBSS) injected joints up to or including day 7.
Figure 2 (A – C) – Mean ± SEM clinical parameters of synovitis and pain. A – Fetlock circumference (cm). B – Edema score (0-4). C – Pain-free range of motion (degrees). Data from auto and auto-BMP2 groups were not significantly different and were combined for graphics only. Different symbols (*, †) indicate significantly different ($P < 0.05$) groups. All MSC groups were significantly different than Gey’s Buffered Salt Solution (GBSS) injected joints up to or including day 7.
Figure 3 (A – F) – Histologic and immunohistochemical analysis of synovium from MSC injected joints harvested 60 days post injection compared to non-injected normal fetlock synovium (control). A – H&E control synovium. B – H&E synovium from MSC injected joint with allo group indicating increased mononuclear inflammation. Inset with black arrow indicates vascular microthrombi and white arrow indicates an unaffected vessel. C – Polyclonal rabbit anti-human CD3 antibody applied to control synovium. D – Polyclonal rabbit anti-human CD3 antibody applied to synovium from MSC injected joint with auto-BMP2 group indicating CD3 positive perivascular cuffing. E – Polyclonal rabbit anti-human BMP2 antibody applied to control synovium. F – Polyclonal rabbit anti-human BMP2 antibody applied to synovium from MSC injected joint with auto-BMP2 group indicating diffuse staining. Scale bar (A-F) indicates 100 µm. Scale bar (B inset) indicates 20 µm.
Figure 4 – Mean ± SEM of CD4 positive cells from co-culture of peripheral blood mononuclear cells with each MSC group assessed via flow cytometry. Within time points, different letters (a or b) indicate significantly different ($P < 0.05$) groups while groups labeled with both (a,b) were not significantly different.
Chapter 4: Discussion

Our study showed that direct intra-articular injection of BMDMSC produced significant, acute, clinical inflammation that was greater with allogeneic and xenogeneic MSC (non-self MSC) than autologous MSC. Xenogeneic cells induced an adaptive immune response as measured by the presence of circulating lymphocytes that became activated when re-exposed to the original xenogeneic cells in co-culture. This anamnestic cellular immune response was not detected to allogeneic or autologous cells. MSC injection using our protocol had a sustained (> 60 day) subclinical biologic response in the joint seen as a mild lymphocytic synovitis. Intra-articular injection of autologous MSC genetically engineered with the hBMP2 gene was similar to autologous MSC.

To the authors’ knowledge, this is the first study reporting a significantly greater acute inflammatory stress response (< 48 hrs) after intra-articular injection of MSC from an allogeneic or xenogeneic source. The causes of this greater immediate stress response (reaction) of the joint to non-self cells are unknown, but could be a response to cell size and function variability, non-self protein secretion, or any other innumerable changes that occur when propagated non-self biologic material is placed in vivo. Our results showing this early response will likely spawn future work to investigate this further. Intravenous injection of stem cells in human clinical trials often contains immunosuppression as a component of the protocol to minimize this known possible risk. [www.clinicaltrials.gov,
Since all BMDMSC were cultured and processed similarly including multiple wash steps prior to injection, cell processing was not considered the reason for the difference among MSC groups. The acute inflammatory stress difference observed is likely a direct function of allogeneic and xenogeneic MSC recognition when combined with the highly sensitive joint environment.

Based on the data from Carrade et al., a greater number of total nucleated cells and greater joint circumference was observed in horses injected intra-articularly with allogeneic cells than with autologous cells, although the difference was not significant. Synovial fluid total nucleated cell counts averaged over 50,000 cells/µL using one-half the dose than in our study. Inter-animal variability was significant and may have limited the power of the study such that no statistical differences were detected between MSC groups. The authors justified and used each animal as their own control when comparing MSC groups in an attempt to limit this variability. Based on the published data, we used each animal as its own comparison for our groups, used adult mares, and to further minimize variability, used age-matched mares of registered Thoroughbred descent. We believe our selection of tight inclusion criteria for our experimental animals and experimental block design for inter-treatment comparisons were effective in permitting us to find the effects of cell group in our study while staying within financial limits.

Our study demonstrated for the first time an anamnestic adaptive immune response after intra-articular injection of xenogeneic (human) MSC. An adaptive immune response has been demonstrated for allogeneic injections of subcutaneous, intravenous, intraperitoneal and intracardiac MSC in mice and pigs and xenogeneic (human)
intracardiac MSC injections in rats. This suggests that an allogeneic or xenogeneic immune response is possible despite MSC possessing a relative lack of MHCII expression (as in our study) prior to injection. Further studies have demonstrated a lack of an immune response in vitro after inducing MHCII expression on MSC via IFNγ suggesting that if present, the response may occur through another mechanism. An adaptive immune response was not demonstrated for allogeneic MSC in vitro in our study, based on no significant differences in number of CD4 positive cells in previously exposed PBMC, suggesting that lymphocyte activation did not occur or was not detected using our methods. Additional work from our laboratory [Bertone unpublished data] has demonstrated some level (~ 50%) of MHCI expression by flow cytometry on juvenile equine MSC and therefore some level of MHCI expression may be present in these adult BMDMSC. MHCI may be responsible for inciting the anamnestic lymphocytic cellular immune response upon re-exposure to xenogeneic cells. Allogeneic cells did not incite a detectable lymphocytic immune reaction suggesting that equine MHCI antigen may be sufficiently similar between animals to be immunotolerated. After injection into the intraarticular environment, it is currently unknown if MSC change phenotype or cell surface protein expression that may alter immunogenicity.

In co-culture with PBMC, all MSC groups demonstrated significantly increased levels of IL-6 of which the xeno group was significantly greater than the other MSC groups. Increased levels of IL-6 have been demonstrated previously in co-cultures of human MSC with PBMC from unrelated donors and may function to augment specific T helper cells. Further, increased concentrations of IL-10 were observed for all
MSC/PBMC groups by day 6. Increased IL-10 has been demonstrated in MSC human co-culture experiments, the role of which has been debated but may help suppress the immune cell response.\textsuperscript{32}

Our findings confirmed the previous published results of a marked, acute synovial inflammatory stress response after intra-articular MSC injection in horses\textsuperscript{24}, characterized by a marked yet transient influx of neutrophils (approximately 80%). Our study also demonstrated moderate to marked limb swelling radiating proximally from the injected joint in all cases except saline treated controls. This limb swelling improved significantly in all MSC groups throughout the study period. Horses were not bandaged for 60 days post MSC injection, were not administered anti-inflammatory drugs and were not exercised in order to assess the full clinical response to the MSC injections uninfluenced by physical effects and medications. The clinical inflammatory response may be ameliorated by more typical clinical practice of post-injection bandaging and non-steroidal anti-inflammatory drugs. We propose that this swelling may be related to microthrombi in the synovial vasculature, MSC migration in veins or lymphatics causing cellulitis or lymphangitis, or both. This mode of potential migration or clearance of cells from the joint would also explain why MSC biolocalization was not achieved in the synovium at 60 days and BMP2 protein detection was limited to less than 7 days in synovial fluid. In the authors’ previous work, autologous cells injected intramedullary into rabbit distal femurs were detected in regional lymph nodes, but not in abdominal or thoracic organs, suggesting that locally administered cells may travel through the lymphatics.\textsuperscript{bb} Additional contributing factors to the inflammatory response may include
the dose of injection or the overall sensitivity of the fetlock joint to these cells. In our study, an injection dose of 15x10^6 MSC was used in fetlock joints compared to 3x10^6 MSC in fetlocks,^a 7.5x10^6 MSC in equine radiocarpal joints,^24 approximately 10.5x10^6 MSC in equine middle carpal joints,^25 and 20x10^6 MSC in equine medial femorotibial joints. However, a study directly evaluating the safety of various cell doses is lacking. The high dose used in our study combined with the presence of non-self living cells could have contributed to a greater stress response in our model. In rats after a relative high dose MSC injection (10x10^6) in a model of intra-articular tissue injury, free bodies of scar tissue containing fibroblastic-like cells were observed in both the sham and tissue injured knee joints whereas joints injected with 1x10^6 MSC did not demonstrate this finding,^1 suggesting an MSC dose effect. However, despite evidence of a marked acute inflammatory stress response after intra-articular injection with MSC in our study (< 48 hours), this reaction revealed no gross abnormalities in the joints upon arthroscopic evaluation at 60 days post-injection.

Our study was the first to report a sustained biologic effect to intra-articular injection of MSC. Histologically, MSC injected joints had lymphocytic synovitis characterized by lymphocytic perivascular cuffs with scattered vascular microthrombi in the synovium evident 60 days after injection. There was no difference among the cell treatment groups. Concurrent and historical control samples of normal equine synovium do not have these findings. These effects may have been influenced by the large intra-articular dose of MSC including a large dose of non-self MSC, but the finding that MSC can have a sustained biologic effect shows the potential for a long-term effect from a
single injection. Mononuclear infiltrates have been demonstrated to be a characteristic of allogeneic and xenogeneic stem cell injections in rat, pig and dog models of myocardial infarction. However to the authors knowledge, this response has not been demonstrated in synovium after intra-articular injection of MSC.

The biolocalization of MSC was attempted through hbmp2 gene localization, gene expression, and protein production identified via polyclonal anti-human BMP2 antibody. The genetic modification of MSC using the hbmp2 gene significantly increased BMP2 protein in synovial fluid of auto-BMP2 injected joints for at least the first 2 days and less than 7 days post injection. The lack of detection after this time period was likely due to cell migration, cell death, or a decrease in protein production. The inflammatory response to genetically modified autologous MSC was not different than autologous MSC, with no clinical or histologic side effects observed. At 60 days, there was no detectable hbmp2 gene in synovium and no significant difference in gene expression in MSC injected joints compared to control synovium, suggesting that MSC were either not present or not detected using our techniques. Histologically, there was an unexpected, but substantial increase in intensity and synovial/endothelial expression of cellular protein detected by polyclonal anti-human BMP2 antibody in control and MSC injected synovium. The intensity and diffuse cell expression was significantly increased by MSC injection. This likely represents endogenous BMP2 production by these cell types, but cross reactivity with other BMPs or other related proteins cannot be ruled out. There was no significant difference of protein detection between MSC groups, suggesting that it was not related to a xenogeneic immune reaction.
There were several limitations of the study. Studies using living cells in animals will inherently have interactions with potential for effects that are complex, unknown, and dependent on genetic factors, immune status, and many other biologic responses that will be animal dependent. Controlling for inter-animal variability was critical to detecting the rather low level of difference expected among cell treatments in our study. A multiple MSC dose study design was utilized such that each animal received 4 doses of stem cells. This assumes that if present, a systemic response could contribute to the measured results. This was minimized by using each animal as their own control and by using genetically similar subjects (5 year old Thoroughbred mares). In the co-culture experiment, an adaptive immune response was examined only through CD4 positive cells, leaving the possibility that an immune response was generated through the CD8 pathway and not detected.

This study demonstrated an adaptive immune response to a single xenogeneic MSC intra-articular injection in horses. The genetic engineering of cells using an adenovirus vector and the human BMP2 gene did not produce a greater inflammatory response than autologous cells and cells remained functional in vivo for greater than 2 days. Our study is the first to suggest a greater inflammatory stress response to intra-articular allogeneic and xenogeneic MSC when compared to autologous MSC and demonstrated evidence of a sustained biologic response to intra-articular MSC.

In conclusion, intra-articular injection of MSC, using this protocol, produced a clinical inflammatory response that was acute and marked. Allogeneic MSC injections may have produced a significantly greater inflammatory response than autologous MSC
in our study but the difference was small and appeared clinically insignificant. The genetic engineering of MSC warrants further investigation in models of joint disease as it has potential to provide an enhanced therapeutic response.
Footnotes


b. Catalog No. 2M-302, Lonza Inc., Walkersville, MD.

c. Sigma Aldrich, St. Louis, MO.

d. MarrowStim™ Concentration Kit, Biomet Biologics LLC., Warsaw, IN.


f. Gibco Life Technologies, Grand Island, NY.

g. Hausser Scientific, Horsham, PA.

h. Lonza Inc., Walkersville, MD.

i. Ficoll-Paque™ Premium, GE Healthcare, Piscataway, NJ.

j. Stem Cell Technologies, Vancouver, BC.

k. Clone CVS20, AbD Serotec, Raleigh, NC.

l. FITC rat anti-mouse IgG1, clone A85-1, BD Biosciences, San Jose, CA.

m. BD Biosciences, San Jose, CA.
n. Clone 5E10 directly conjugated to phycoerythrin, BD Biosciences, San Diego, CA.
o. Accuri Cytometer, Ann Arbor, MI.
p. R&D Systems, Minneapolis, MN.
q. Cell Dyn 3500 Hematology Analyzer, Abbot Labs, Santa Clara, CA.
r. Oblique Hopkins telescope, 4mm 30°, magnification 1x/80xM 4mmx, Karl Storz, El Segundo, CA.
s. Dako Inc., Carpinteria, CA.
t. Vector Laboratories, Burlingame, CA.
u. Macherey-Nagel Inc., Bethlehem, PA.
v. Applied Biosystems, Foster City, CA.
w. QIAGEN Sciences, Germantown, MD.
x. Catalog No. ABIN498495, Antibodies-online Inc., Atlanta, GA.
y. Clone CVS4, Thermo Fischer Scientific Inc., Rockford, IL.
aa. SPSS, version 19, SPSS Inc, Chicago, IL.


