INTRA-ARTICULAR ADMINISTRATION OF ALLOGENEIC EQUINE BONE MARROW DERIVED MESENCHYMAL STEM CELLS

by

LINDSEY HELMS BOONE

(Under the Direction of John Peroni)

ABSTRACT

The overall aim of the studies presented herein were to investigate the intra-articular use of allogeneic bone marrow derived mesenchymal stem cells (BMSCs) in horses for future use in treatment of osteoarthritis.

First, the survivability as well as the capacity of BMSCs to proliferate and differentiate along the chondrogenic lineage in the face of clinically relevant concentrations of allogeneic synovial fluid was investigated *in vitro*. This study showed that short-term exposure of equine BMSCs to 100% allogeneic synovial fluid alone was able to maintain cellular viability and proliferation as well as support chondrogenesis.

Next, platelet rich plasma (PRP) was investigated as a cellular delivery agent for equine BMSCs. Clinically relevant concentrations of autologous and allogeneic PRP were used to culture equine BMSCs and their effects on cellular viability, proliferation, and chondrogenesis were evaluated. No difference in cellular viability, proliferation, or chondrogenesis was seen with regards to the derivation (autologous or allogeneic) of the PRP.

Lastly, the systemic and local safety of intra-articular equine allogeneic BMSC administration was evaluated. Intra-articular administration of equine allogeneic BMSCs produced no adverse systemic responses in the ten horses evaluated. Moderate yet transient changes to the local synovial environment were observed.

INDEX WORDS: Equine, Osteoarthritis, Osteochondral defect, Bone marrow, Mesenchymal stem cell, Autologous, Allogeneic, Synovial fluid, Platelet rich plasma

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DEDICATION

To my parents, Doris and Carl Helms, and to my husband, S. Shaun Boone, for their unyielding love and endless support. Mother, you are and always have been an example of a highly educated woman who has never wavered in achieving her goals no matter the obstacles. You have not only been my mentor, but also my greatest friend. Father, you have always been one of my biggest supporters and one of my greatest teachers. Thank you for always waking me up and showing me the "birds". Shaun, you have been the rock upon which I have stood the last four years. You are truly my best friend and the greatest partner in life.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Osteoarthritis is one of the leading causes of decreased athletic performance in horses and one of the leading causes of disability in middle-aged to elderly human patients. A therapy that targets both inflammatory processes that are perpetuated and helps to regenerate articular cartilage is yet to be identified, but is needed for these patients. Mesenchymal stem cells (MSCs) have demonstrated both immunomodulatory and regenerative properties and for this reason have gained interest in human and veterinary articular cartilage repair research. The studies presented herein were undertaken to further understand the intra-articular use of equine allogeneic bone marrow derived mesenchymal stem cells (BMSCs).

Chapter 2 contains a review of the literature regarding intra-articular administration of MSCs in focal osteochondral defects or models of osteoarthritis. Section I reviews the pathophysiology of osteoarthritis to help the reader understand the underlying inflammatory destruction that occurs to the articular cartilage. Section II provides the reader with the current understanding on the prevalence of osteoarthritis in human and equine patients. Section III is provided to help the reader understand the importance of an appropriate animal model and why the equine is a suitable model for translational research of human patients. Section IV describes the inability of articular cartilage to adequately repair itself and why better biological therapeutics are needed for cartilage regeneration. Section V and VI give a brief overview of the current medical and

surgical strategies for osteoarthritis treatment and articular cartilage repair. Section VII describes the concept of using multiple regenerative tools to enhance regeneration in a multimodal approach. Section VIII is an overview of the different kinds of stem cells that can be used in articular cartilage repair and the rationale for the use of bone marrow derived mesenchymal stem cells in the presented studies. Section IX describes the administration of stem cells and what we know about their immunomodulatory abilities for potential allogeneic application. Section X describes platelet rich plasma and its role in cellular delivery and scaffolding in articular cartilage defects. Finally, Section XI, discusses the rationale for the studies that are presented herein.

Chapter 3 evaluates the effects that short-term culture in high concentrations of allogeneic synovial fluid has on BMSC viability, proliferation, and chondrogenic differentiation. The purpose of this study was to ensure that exposure to synovial fluid after intra-articular injection would not result in substantial cell death, a concern that is often described among equine practitioners as a reason that they avoid intra-articular MSC therapy. If significant cell death is not induced after exposure to clinically relevant concentrations of synovial fluid, then another question that is often asked by equine practitioners is whether the cells can change their phenotype to a chondrogenic phenotype after exposure to synovial fluid or do the cells that they administer need to be pre-differentiated. It is for these clinical reasons that this project was undertaken prior to proceeding to the remainder of the projects presented in this dissertation.

Chapter 4 evaluates the effects of short-term exposure of platelet rich plasma (PRP) on BMSC viability, proliferation, and chondrogenic differentiation. PRP has been used with varying success in management of human osteoarthritis patients. It is often

used as a cellular suspension and delivery agent for MSC therapy of equine tendinopathy or desmopathy, but it's effects on BMSCs has not been evaluated in clinically relevant concentrations of exposure. Additionally, if allogeneic BMSCs are used for therapy, autologous PRP is used for cellular suspension and delivery. So, this study was conducted to determine if PRP caused deleterious effects to BMSCs prior to intra-articular injection and if there was a difference in these effects when there was an autologous or allogeneic relationship between the BMSCs and PRP lines used.

Chapter 5 reports the results of intra-articular administration of equine allogeneic BMSCs in 10 horses. This study was conducted to determine the safety of the patient and the local joint environment after allogeneic intra-articular administration of BMSCs.

Chapter 6 summarizes the findings of the studies presented herein and discusses future projects for further investigation into the interaction of allogeneic BMSCs with the osteoarthritic environment.

CHAPTER 2

LITERATURE REVIEW

SECTION I. PATHOPHYSIOLOGY OF OSTEOARTHRITIS

The American Academy of Orthopedic Surgeons defines osteoarthritis as a group of overlapping distinct diseases, which may have different etiologies, but with similar biologic, morphologic, and clinical outcomes. The pinnacle outcome of this disease process is joint pain resulting in loss of performance due to articular cartilage damage with accompanying changes in the subchondral bone and surrounding soft tissues. Osteoarthritis (OA) is not just a disease of the articular cartilage rather it is a global joint disease with all elements of the joint participating in the orchestrated degradation of the cartilage's extracellular matrix.[1]

The role of the synovial membrane in osteoarthritis

The role of the synovial membrane in the symptoms and pathology of OA was previously underestimated and further research into the contribution of the synovial membrane is warranted. During early OA the synovial membrane becomes thickened altering its filtering capabilities and allowing further leakage of destructive cytokines while at the same time allowing high molecular weight lubricin and hyaluronic acid to leak from the joint into systemic circulation.[2] This thickening of the synovial villi and synovial membrane with or without an increase in synovial fluid volume (effusion) is termed synovitis. The degree of synovitis has been shown to correlate with the degree of pain experienced by patients and the progressive deterioration of articular cartilage.[3-8] In

addition to synovial hyperplasia, the synovial lining is infiltrated with a mixed immunocellular infiltrate comprised predominantly of macrophages (65%) and T cells (22%) accompanied by much smaller amounts of B cells (5%), plasma cells (<1%), mast cells, natural killer cells, and dendritic cells.[9-17] Equine synoviocytes stimulated with LPS have been shown to produce significant amounts of PGE_2 in vitro supporting the argument that the synovial membrane may be a large contributor to OA.[18]

The innate immune system is primarily responsible for mediating the destructive inflammatory events perpetuated by the synovial membrane in both acute and chronic osteoarthritis. Cellular stress and matrix breakdown result in release of endogenous damage-associated molecular patterns (DAMPs) which activate toll-like receptors (TLRs) and receptors for advanced glycation end-products (RAGE) which are responsible for triggering signaling pathways that are ultimately responsible for increasing production of pro-inflammatory cytokines.[19] The innate immune system of the synovial membrane is further activated by the release of molecular products of articular cartilage breakdown, mainly aggrecan, which activate complement and initiate formation of membrane attack complexes promoting further cartilage breakdown.[20,21] This innate immune system is fueled by pro-inflammatory cytokines that further promote synovitis while enhancing cellular production of matrix degrading enzymes promoting further deterioration of articular cartilage.

The role of the subchondral bone in osteoarthritis

For a long time the subchondral bone was thought to be a separate biological entity from the articular cartilage due to the presence of the calcified cartilage layer which was thought to act as a biochemical barrier between the articular cartilage and

subchondral bone.[22] Permeability of the calcified cartilage allowing small particle exchange via nanopores has been demonstrated in normal murine stifle joints.[23] This permeability of the calcified cartilage is markedly altered during osteoarthritis. Early in the stage of the disease, the subchondral becomes thin and more porous.[24-27] As the disease progresses the subchondral bone thickens becoming sclerotic and resulting in reduced porosity.[24-28] The zone of calcified cartilage also becomes thickened leading to advancement of the tidemark.[29,30]

Abnormal loading of chondrocytes induces chondrocyte proliferation, clustering, and a change to a more hypertrophic, terminally differentiated phenotype causing a shift in the balance of anabolic and catabolic factors towards catabolism.[31] Hypertrophic chondrocytes up regulate expression of angiogenic factors, such as hypoxia inducible factor alpha (HIF-1α) and vascular endothelial growth factor (VEGF), contributing to the advancement of the tidemark and stimulating vascular penetrance of the tidemark allowing for a more direct communication between the subchondral bone and articular cartilage for large molecule transport.[22] The degree of vascular invasion of the tidemark and subchondral bone has been correlated with disease severity and represents an important step in the pathogenesis.[32,33] Expression of VEGF stimulates osteoclastogenesis, contributing further to thinning of the subchondral bone.[34] These hypertrophic chondrocytes also increase expression of matrix degrading enzymes, matrix metalloproteinases (MMPs), MMP 1, MMP 3, and MMP 13, resulting in articular cartilage degradation that can be seen clinically as microcrack or fissure formation.[35,36] This degradation of the articular cartilage overlying the subchondral bone further

contributes to the cellular cross talk between the subchondral bone and cartilage that perpetuates osteoarthritis.[37]

Hypertrophic chondrocytes phenotypically resemble chondrocytes responsible for endochondral ossification during bone and joint development increasing their synthesis of extracellular matrix.[31] However, the extracellular matrix that is produced is abnormal with a shift from predominantly type II collagen to a biomechanically inferior, heterogenous matrix of collagen type I[31,38], collagen type X[31], fibronectin[38], and tenascin[38] with increased expression of Runx2 (hypertrophy transcription factor), and alkaline phosphatase.[31,39] Due to this reversion in phenotype, the articular cartilage becomes calcified around these chondrocytes further contributing to an upward shift of the tidemark and thinning of the articular cartilage.

The role of pro-inflammatory cytokines in osteoarthritis

Osteoarthritis is primarily orchestrated by two pro-inflammatory cytokines, IL-1β and TNFα, and the prostaglandin, PGE2.[40] The secretion of the inflammatory mediators induce further release of pro-inflammatory cytokines (IL-6, IL-17, IL-18), chemokines, and other inflammatory mediators (nitric oxide, oncostatin-M, leukemia inhibitory factor).[5,41] Both IL-1β and TNFα up regulate expression of the principle catabolic enzymes involved in extracellular matrix destruction, MMPs (MMP 1 interstitial collagenase, MMP 3 stromelysin, and MMP 13 collagenase 3)[42-44] and a disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 4 and 5 (ADAMTs 4 and 5).[45,46] This leads to down regulation of synthesis and even destruction of the major components of articular cartilage matrix, mainly collagen type II[47-49], aggrecan

(predominant proteoglycan)[50,51], and other small proteoglycans[47,49]. Articular cartilage destruction leads to pain and loss of function.

SECTION II. PREVALENCE OF OSTEOARTHRITIS IN HUMAN AND EQUINE PATIENTS

Osteoarthritis is the leading cause of disability in humans, particularly middle-aged to elderly patients.[52-54] In 2005 it was estimated that approximately 27 million US citizens were diagnosed with OA and this number is estimated to rise by 2030 to approximately 20-25% of the US population affecting an estimated 70 million people.[52-54] The economic burden of this disease was estimated to increase by $185.5 billion dollars per year in 2009.[55] Of the US citizens afflicted with OA that are women, only 24% report an ability to achieve levels of physical activity that are recommended to maintain health.[56] This limited activity level in the majority of US citizens' affected by osteoarthritis leads to subsequent health problems (i.e. obesity) resulting in further economic hardship to the U.S. Economy.

The exact incidence of OA in the equine industry is difficult to accurately estimate and is limited by many factors related to the patient, owner, and veterinarian. In 1998 the USDA's National Animal Health's Monitoring System conducted a survey of horse owners and or operators of horse facilities to identify the national incidence of lameness and laminitis. In this study, approximately 50% of horse owners or operators identified one or more horses on the property that were lame in a 12 month period with the majority of these lameness requiring consultation of a veterinarian.[57] Approximately one-third to one-half of these horses were perceived to be lame due to joint-related problems as reported by the owner.[57] These horses were not used by the owner for

performance purposes due to lameness for an average of 110 days.[57] This survey highlighted the impact that lameness has on a large proportion of the equine population resulting in significant loss of use.

More recently, studies have focused on the epidemiology of joint injury in thoroughbred racehorses and/or performance horses. These studies do not accurately reflect the true prevalence of joint disease in the total equine population because these horses only represent less than 15% of the U.S. equine population.[58] A recent study evaluating 50 thoroughbred racehorses euthanized within 60 days of racing, found partial or full-thickness cartilage erosions accompanied by other synovial-related pathological conditions consistent with osteoarthritis in 33% of the 2-3 year old racehorses.[59]

According to the American Horse Council, in 2005 the horse industry had a direct economic effect of $39 billion annually on the U.S. economy with performance horses (racing and showing) contributing to half of this economic effect ($21.4 billion). Therefore, the economic impact of lameness due to joint injury is quite substantial and new therapeutics aimed at cartilage regeneration to restore function are warranted.

SECTION III: EQUINE MODELING OF OSTEOARTHRITIS

Given the substantial physical and economic burden that OA has on human patients coupled with its complex pathophysiology, reliable *in vivo* animal models that closely resemble naturally occurring disease are vital to establishing and validating novel therapeutics prior to initiating human clinical trials.

One of the first considerations when utilizing an animal model for osteoarthritic research should be the species of interest. Species used are often divided into small animal (murine and lapine) and large animal (canine, caprine, ovine, porcine, bovine and

equine) research species. Small animal species are often used due to minimized genetic diversity of these animals as well as more affordable experimental costs. However, these models fall short in translational applications due to their joint size and un-relatable biomechanics. Of the large animal species used, the horse is the most suited for translational osteoarthritic research for many reasons. First of all, the horses are used for athletic performance. Since spontaneous OA occurs commonly in the athletic horse, prevention and treatment strategies related to OA have been extensively researched in the equine industry in an attempt to preserve athletic performance. For this reason, many biological assays used for joint assessment have been developed and used for research in the horse.[60] Macroscopic and microscopic articular cartilage grading systems similar to human articular cartilage grading systems have also been well established and utilized in equine articular cartilage research.[61] In addition to athletic performance, horses much like humans can undergo controlled exercise rehabilitation after musculoskeletal injury, but unlike humans horses must bear weight immediately after surgery where as humans can be gradually transition to weight bearing. Researchers often try to reduce this immediate effect of weight bearing by creating osteochondral defects in minimal to non-weight bearing regions of the articular cartilage such as the lateral femoral trochlear ridge.[60,62] However, this does represent a significant disadvantage in the use of equine research.

Most importantly, equine joint(s) have similar physical and biomechanical properties to human joints. Clinically significant osteochondral defects that necessitate articular cartilage repair in humans are greater than 10 mm in diameter ($550mm^3$)[60], a defect size that cannot be achieved in small animal species as well as the majority of large animal species used for articular cartilage repair. Articular cartilage defects created

for translational research are typically 8-15 mm in diameter.[60,62]. Additionally, the thickness of equine cartilage (1.75 mm at the medial femoral condyle) is more closely resembles the thickness of human articular cartilage (2.35 mm at the medial femoral condyle) than any other commonly used research species.[60,62] The size of the equine joint also allows serial arthroscopic evaluation providing important information on the progression of the repair *ante mortem* while having treated defects that are large enough for arthroscopic biopsy during the course of the study. The larger joints also allow for adequate tissue volume for biochemical and biomechanical analyses *ante* and *post mortem*. The main disadvantage in the use of this animal species for an osteoarthritic model is related to the cost and facility requirements.

When considering modeling for OA it is important to understand that there are two main goals for treatment of OA. The first goal should be modifying the inflammatory environment so that a balance is re-established between anabolic and catabolic mechanisms of the synovial environment. Secondly, repair the damaged tissues (articular cartilage and/or intra-synovial soft tissues) so that biomechanical function is restored. Due to the complexity of OA pathology, many researchers elect to focus on the latter goal creating osteochondral defects in animal models and repairing them with various cells, growth factors, and/or scaffolds. Much of this research has helped improve patient outcomes, but many of the treatment strategies that have been established through this type of research fall short when used to treat patients with concurrent OA. Therefore, animal models of OA have been established in an attempt to re-create both the inflammatory and tissue destructive nature of OA. Both types of research have value in developing our knowledge of treating joint-related injury.

Numerous experimental models of OA have been described in the horse and include chemical induction[63-72], articular instability[73,74], osteochondral fragmentation with exercise[75-82], trauma[83,84], and disuse[85-87]. The equine carpal osteochondral fragment model is the most commonly employed model for OA in the horse, but this model still falls short in adequate simulation of naturally occurring OA.

SECTION IV: ENDOGENOUS ARTICULAR REPAIR

Articular cartilage is incapable of adequate self-repair due to the reduced cellular population of articular cartilage in a sea of extracellular matrix as well as the low mitotic and metabolic activity of this cellular population. Chondrocytes are unable to migrate to the site of cartilage injury and initiate repair because of the surrounding extracellular matrix. Full thickness cartilage defects heal primarily by in growth of subchondral fibrous tissue and formation of fibrocartilage that is biomechanically inferior and cannot bear the compressive, tensile, or low-frictional forces of innate articular cartilage leading to mechanical failure.[88] The depth, size, location of the defect, age of the patient, and concurrent disease (OA) affect the adequacy of the endogenous repair. [88] This repair tissue is primarily composed of type I collagen compared to predominance in hyaline cartilage of type II collagen.[88] This cartilage may also express markers of chondrocyte hypertrophy such as collagen type X and Runx 2 further establishing juvenile matrix repair that is poorly integrated into the tissue and incapable of holding up.[88]

SECTION V: CURRENT MEDICAL THERAPY FOR OSTEOARTHRITIS

Non-steroidal anti-inflammatory medications (NSAIDS)

NSAIDs are the most commonly employed analgesic and anti-inflammatory medication for OA. NSAIDs block cyclooxygenase conversion of arachidonic acid to

prostaglandins and leukotrienes. The most commonly utilized NSAIDS in equine medicine are non-selective cox inhibitors, phenylbutazone and flunixin meglumine, and selective cox inhibitors, firocoxib. Topical application of diclofenac induced a significant reduction in lameness scores 36-72 hours after treatment of chemically induced OA, but no reduction was seen in assayed inflammatory mediators (PGE2). [89] Lameness improvement was not observed with application of diclofenac after mechanical induction of OA using the equine carpal fragment model, but horses in which diclofenac was applied had improved radiographic scores and improved articular cartilage matrix composistion.[90] Phenylbutazone and firocoxib were found to have similar effects on lameness and joint effusion scores in horses with naturally occurring OA. [91]

Intra-articular corticosteroids

Corticosteroids are the main therapy utilized in horses with chronic osteoarthritis and are highly effective in temporarily alleviating lameness.[92] Corticosteroids suppress arachidonic acid metabolism stabilizing cellular membrane phospholipids and limiting prostaglandin and leukotriene production. Corticosteroids also limit production of pro-inflammatory cytokines, particularly IL-1β.[93] The most commonly used intra-articular corticosteroids in the horse are triamcinolone acetonide, methylprednisolone acetate, and betamethasone. Methylprednisolone acetate has been shown to have deleterious effects on articular cartilage matrix both *in vitro*[94-96] and *in vivo*[77,97,98] and are therefore often times reserved for "low" motion joints or joints with progressive, severe OA unresponsive to other intra-articular corticosteroids. Triamcinolone acetonide has been shown to be chondroprotective in vitro[99,100] and in vivo[76] and is preferentially used in "high" motion joints.[100]

Viscosupplementation

Hyaluronic acid (HA) is an important component of both the synovial fluid and the extracellular matrix of cartilage.[101,102] During osteoarthritis, HA is broken down at a much faster rate than in normal joints resulting in fragmented, low molecular weight HA molecules.[103,104] These smaller HA molecules cause reduced viscosity of synovial fluid leading to reduced boundary lubrication and shock absorption.[105] They also stimulate TLRs responsible (TLR 4) for perpetuating the inflammatory cycle of OA.[106,107] Intra-articular administration of HA temporarily restores synovial fluid viscoelasticity[108], exerts a mild anti-inflammatory effect, and improves patient pain and function.[109-111] Despite some evidence that HA can be transiently chondro-protective after articular injury, little beneficial effects in articular cartilage repair have been reported after HA administration.[112-114]

HA is often used in combination with other intra-articular therapeutics in treatment of equine musculoskeletal disease. Clinically relevant concentrations of HA were shown to sustain similar equine BMSC viability and proliferation as control media during short-term culture indicating that mixing HA with BMSCs for injection does not have deleterious effects on BMSC viability.[115] In fact, HA is a common intra-articular delivery agent for BMSC treatment of equine experimental osteochondral defects[116] or experimentally induced osteoarthritis.[117]

While these drugs primarily do show some beneficial effects on clinical assessments of these horses they do little to treat progressive deterioration of articular cartilage.

SECTION VI: CURRENT SURGICAL THERAPIES FOR ARTICULAR CARTILAGE REPAIR AND OSTEOARTHITIS

Debridement and lavage

Currently, focal or multifocal osteochondral defects with or without concurrent osteoarthritis are minimally treated with arthroscopic debridement of the abnormal cartilage and lavage to remove debris and temporarily reduce the pro-inflammatory mediator load on the joint. Significant benefit, however, for arthroscopic debridement and lavage for treatment of articular cartilage repair has been shown to have little benefit in human knee OA[118,119] and equine knee OA. [120] This limited benefit with debridement and lavage alone is why cartilage-resurfacing techniques were pursued to stimulate the endogenous repair of articular cartilage.

Marrow stimulating techniques

Bone marrow stimulation techniques for articular cartilage repair include pridie drilling, abrasion arthroplasty, mircrofracture.[121] Microfracture is the newest of these described techniques and is considered the first line of treatment for focal cartilage defects. Microfracture involves debridement of all unstable/damaged cartilage within the focal cartilage defect down to the subchondral bone, ensuring that the calcified cartilage is removed, and then perforations are made in the subchondral bone plate with a sharp awl approximately 3-4 mm apart.[121] Penetrance of the subchondral bone plate allows leakage of several bone marrow components, but mainly bone marrow stromal cells. These cells are extruded from the subchondral bone and form a clot within the focal cartilage defect to assist with endogenous cartilage repair. This mechanism is complicated in OA joints due to the significant cross talk that takes place between the

subchondral bone and cartilage. Results of microfracture repair show that the repair tissue displays limited characteristics of hyaline cartilage and deteriorates with time. [121]

Microfracture has been studied in the horse with good to poor quality repair[120,122-125] that can be augmented with gene therapy with interleukin-1 receptor antagonist and IGF-1[126], direct implantation of bone marrow concentrate[127], intra-articular administration of equine bone marrow derived mesenchymal stem cells.[116]

Articular cartilage resurfacing techniques

Current cartilage resurfacing techniques include osteochondral transplantation and autologous chondrocyte transplantation.

Autologous chondrocyte transplantation (ACT) was originally described in an animal model by Peterson, et. al. (1984) and then described for use in human patients by Britterberg, et.al. (1994).[128,129] The original technique involved a two-step surgical procedure. The initial surgery was performed to obtain an osteochondral plug from a non-weight bearing articular surface from which chondrocytes were isolated and culture-expanded. The second surgery involves implantation of cultured autologous chondrocytes beneath a sutured periosteal flap. ACT causes increased patient morbidity due to articular cartilage harvestation for chondrocyte preparation requiring two surgical procedures[130], a relatively low cell yield[130], de-differentiation of chondrocytes during culture expansion[131], reduced chondrogenesis of chondrocytes obtained from older, osteoarthritic donors[132-134], graft hypertrophy[135], graft delamination[135], and adhesions in 18[136]-25[137]% of patients.

Other forms of ACT have been developed due to concerns associated with uneven chondrocyte distribution, leakage of chondrocytes beneath the periosteal flap,

contribution of the periosteal flap to graft hypertrophy, and the increased anesthesia time and degree of surgical exposure required for the creation of a periosteal flap during first generation ACT.[138,139] Second generation ACT involves the seeding of autologous chondrocytes on different scaffolding materials such as collagen, fibrin glue, hyaluronan, alginate, and agarose.[140-142] Patients with patellofemoral chondral lesions treated with second generation ACT showed significant improvement after 2 and 5 years in EQ VAS, but a decline in improvement in IKDC and Tegner scores from 2-5 years.[143] Slightly better improvements were observed in 50 patients treated with second generation ACT These patients demonstrated significant improvement in IKDS, EQ-VAS, and Tegner scores at 2 years with maintenance or slight improvement at 5 years. MRI evaluation at 5 years revealed complete filling and integration in 65% of grafts.[144] T

Third generation ACT or matrix associated autologous chondrocyte transplantation (MACT) involves seeding of a porcine type I/III collagen bilayer membrane with autologous chondrocytes, fitting the membrane to the defect and implanting the defect using fibrin glue as an adhesive.[138] Marlovitz, et. al. observed significant improvement in KOOS, IKDC, and Tegner up to 5 years after MACT in 18 patients.[145] MRI scores (MOCART) were significantly improved in patients as early as 3 months with 82% of defects showing complete integration and maintained up to five years with 76% of defects having normal filling.[145] Significant improvement measured by established sport-specific subjective scoring systems (KOOS, Noyes, Tegner) up to 5 years after MACT in 70 active patients with a 74.3% return to pre-injury level of sports activity.[146] Ebert (2011) evaluated 41 patients after MACT of the knee and found significant improvement in similar sports-specific subjective scores (KOOS) at 5 years

follow-up as well as significant improvement in MRI scores with 89% defects showing good to excellent filling.[147] Mehner, et. al. showed significant improvement in Meyer, Lysholm-Gilquist, and ICRS scores at 5 years, but no significant difference in Tegner score.[148]

In a block randomized prospective trial comparing ACT and MACT, both ACT and MACT had significant improvements in clinical outcome scores compared to pre-operative values at 1 year, but MACT showed greater clinical improvement than ACT using the Cincinatti knee scoring system (59.1% ACT patients with good to excellent outcomes compared to 72.3% MACT patients with good to excellent outcomes). After 1 year post-op 79.2% of ACT patients compared to 66.6% MACT patients had good to excellent repair tissue measured by the ICRS scoring system.[138] Biopsy of this repair tissue revealed hyaline or hyaline and fibrocartilage in 42.9% of ACT patients compared to 36.4% of MACT patients.[138] Therefore, the clinical and histological outcomes of ACT and MACT are similar, but reduced anesthesia times and reduced synovial exposure during implantation has led many clinicians to prefer MACT to ACT.

Disadvantages of ACT include the limited quantity of chondrocytes that can be obtained from non-weight bearing surfaces of the joint, dedifferentiation of chondrocytes in culture, and the harvestation of abnormal chondrocytes from degenerative joints.[149]

Comparison of current articular cartilage repair strategies

MF heals chondral defects with fibrocartilage[150-152] where as chondral defects are repaired with hyaline cartilage or a mixture of hyaline and fibrocartilage in ACT techniques.[145,153-158] Larger chondral defects can also be treated with ACT due to

manipulation of cellular yield by the volume of tissue that is harvested from the patient and the duration of culture expansion compared to MF.[145]

The use of these articular cartilage repair strategies are indicated for patients with focal cartilage defects, but their use in patients with concurrent osteoarthritis is controversial. One retrospective analysis looked at 44 patients treated with focal cartilage defects and concurrent knee OA treated with MACT and showed significant improvement in ICRS-IKDC scores up to two years and significant improvement in EQ-VAS scores up to 5 years.[159] Tegner scores showed steady improvement over a 5 year period, but the patient's activity never achieved the activity level prior to onset of symptoms.[159] The grafts had a much higher failure rate (27.3%) compared to grafts within focal osteochondral defects without concurrent OA.[159] Therefore, a cellular therapy that could modulate the ongoing inflammation within an osteoarthritic joint and possibly stimulate cartilage regeneration would be a promising therapy and this is why stem cell therapy has been looked at for articular cartilage repair.

Resurfacing techniques employed in horses

Ortved, et. al. (2012) retrospectively evaluated 49 horses with 65 subchondral cystic lesions of the medial or lateral femoral condyle treated by arthroscopic debridement, filling of the cystic cavity with autologous cancellous bone or tricalcium phosphate granules, and resurfacing of the articular defect with 20-30 million allogeneic chondrocytes in a fibrin glue with IGF-1.[160] 74% of horses returned to or achieved their intended level of performance and 14% showed improvement, but not resolution of lameness causing reduced performance (88% overall improvement).[160] This method of repair shows improvement in functional outcome compared to horses treated with

traditional cyst debridement (64% for horses <3 yrs. And 35% for horses >3yrs.)[161] or corticosteroid injection of the cyst lining (67%)[162] particularly in older horses and horses with pre-existing osteoarthritis.

Traditional ACT has been evaluated experimentally in the horse, but it clinical use has not been reported. One study evaluated full and partial cartilage defects of the lateral trochlear ridge treated with ACT to control defects treated with a periosteal flap alone.[163] Foci of chondrocytes were found to persist in both full and partial defects after 8 weeks with significant improvement in histological scoring compared to control defects indicating that ACT improved cartilage repair. It is important to highlight this improvement in cartilage repair in partial thickness defects because ACT is not commonly employed for partial thickness defects in human articular cartilage repair. Also, this study unlike many other experimental studies evaluating ACT accounted for the contribution of the periosteum to defect repair in the control defects. Periosteal flaps alone have been described and shown to contribute to the repair process and are therefore an important factor to control.[164,165] A modified approach to ACT has been evaluated in horses in an attempt to eliminate the need for a second surgery. A one-step surgical procedure where cartilage tissue was harvested, fragmented, attached to either a PDS scaffold or a cartilage autograft implant system, and placed into the cartilage defect was found to improve the quality of tissue repair compared to empty defects.[166]

Articular cartilage resurfacing with autologous chondrocytes requires specialized training and equipment as well as specialized laboratory equipment that is not readily or commercially available to the equine practitioner or surgeon in a private practice setting.

It is for this reason as well as reasons related to chondrocyte culture that other biological strategies for articular cartilage repair are sought.

SECTION VII: MULTIMODAL REGENERATIVE APPROACH FOR JOINT DISEASE (OSTEOCHONDRAL DEFECTS AND/OR OSTEOARTHRITIS)

The use of biologics for musculoskeletal disease modification of injured synovial tissue is a vastly growing field. These biologic therapies are mainly targeted toward enhancing the body's own biomechanically inferior repair by establishing a therapeutic plan that integrates one or more of the three pillars of regenerative medicine: cells, growth factors, and scaffolds. Cellular therapeutics are used to increase the reparative cell number at the site of injury. These cellular therapeutics include defined cell phenotypes (chondrocytes, synoviocytes, fibrochondrocytes, etc.) or stem cells (embryonic stem cells, induced pluripotent stem cells, or adult/fetal derived mesenchymal stem cells). Growth factor supplementation or stimulated production is used to enhance exogenous or endogenous cellular proliferation and/or migration to the site of injury. Growth factor supplementation can be performed using either one or more growth factors thought to enhance the repair of the targeted tissue. The tissue's environmental growth factor profile can be enhanced either through direct supplementation or gene therapy of delivered cells. Scaffolds are used to facilitate the spatial migration of exogenous or endogenous cellular therapy to enhance tissue uniformity and biomechanical strength of the injured tissue. Currently all of these treatment modalities have been evaluated alone or in combination for treatment of osteochondral defects and/or osteoarthritis. However the plethora of literature evaluating articular cartilage repair strategies in the face of OA fail to utilize a uniform cell type, cell dose, or cell treatment regime. In addition, many studies try to

enhance the anabolic effects of the cells utilizing direct or indirect supplementation of various growth factors believed to enhance cartilage repair and/or utilize various biologic and non-biologic scaffolds to enhance cellular migration and engraftment. These various factors of treatment coupled with varying assessments utilized to prove adequate cartilage repair make interpretation difficult. The remainder of the discussion will focus on what is known about the intra-articular administration of stem cells in experimentally induced and naturally occurring OA. In addition, an autologous biologic scaffold rich in growth factors, platelet rich plasma, will be discussed due to it's interest in OA therapy in combination with stem cells.

SECTION VIII: STEM CELLS

Stem cells are immature progenitor cells that are capable of self-renewal and multi-lineage differentiation.[167] They are primarily classified as embryonic or non-embryonic stem cells. Embryonic stem cells are considered totiopotent if derived from the morula (1-3 days after oocyte fertilization) or pluripotent if derived from the blastocyst (4-14 days after oocyte fertilization) during embryonic development.[167] One type of non-embryonic stem cell, the induced pluripotent stem cell (iPSCs), can exhibit embryonic-like characteristics including pluripotency. These cells are derived from terminally differentiated tissues and reprogrammed to a pluripotent state by overexpression of certain transcription factors. Totipotent cells can give rise to all tissues of an organism including extra-embryonic tissues where as pluripotent cell can give rise to tissues of the three germ layers; endoderm, ectoderm, and mesoderm.[167] Non-embryonic stem cells include hematopoietic and non-hematopoietic stem cells of fetal or adult tissues. They are multi-potent meaning that they can differentiate into one of the

three germ layers. MSCs are non-hematopoietic stem cells capable of multi-potent differentiation along the mesodermal lineage.[167]

Embryonic stem cells

ESCs have grown some interest in articular cartilage repair, because MSCs can exhibit senescence after approximately 20 or more population doublings limiting their use to a certain degree as an available commercial source for allogeneic administration. However, ESCs have been reported to have poor efficiency of exclusive chondrogenic differentiation, but chondrogenic differentiation of human ESCs has been demonstrated.[168,169] *In vivo* studies evaluating the use of ESCs in articular cartilage repair are limited. One of the first reports of ESCs, evaluated the safety of intra-articular injection of ESCs in normal stifle joints of 25 SCID mice.[170] Eight of these mice developed teratomas within the joint and it was concluded that ESCs were not safe for intra-articular use until further optimization was performed.[170] A subsequent study by the same research group, showed the formation of hyaline-like repair tissue in osteochondral defects of immunosuppressed rats without teratoma formation.[171] Cartilage matrix formation was demonstrated after direct implantation of 2×10^6 chondrogenically differentiated human ESCs in a HA hydrogel in an experimentally created osteochondral defect of the femoral trochlear groove in 40 rats.[168] Histological scores improved significantly over the 12 weeks study period, but the repair tissue was poorly integrated and hypertrophic chondrocytes persisted in the tissue.[168] These results do not adequately support enhanced chondrogenesis of ESCs due to persistence of chondrocyte hypertrophy. Poor repair with ESCs was also demonstrated one year after $5\text{-}7 \times 10^5$ ovine ESCs in fibrin glue were directly implanted in surgically created osteochondral defects of

the medial femoral condyle of sheep. The repair tissue formed after treatment with ovine ESCs was not different macroscopically, microscopically, or biomechanically compared to untreated defects.[172]

Equine ESC-like cells displaying similar features as human and murine ESCs have been isolated and expanded in culture, but these cell lines have failed to form teratomas after injection into SCID mice, which could represent a unique characteristic of equine ES-lice cells.[173-176] ES-like cells showed marked structural improvement of a chemically induced superficial digital flexor tendinopathy in two horses.[173] No structural improvement was seen, however, after intra-lesional injection of 1×10^6 ES-like cells in chemically induced superficial digital flexor tendinopathy, but cellular retention at the site of injury was significantly greater for ES-like cells compared to autologous or allogeneic MSCs.[174] Similar leukocyte infiltration of the lesion was present after treatment with ES-like cells compared to autologous or allogeneic MSCs.[174]

Induced pluripotent stem cells (iPS)

iPS cells are derived from terminally differentiated cells that are reprogrammed to a pluripotent state by overexpression of certain transcription factors such as Oct 4, Sox 2, c-Myc and Klf-4.[167] iPS cells may provide an alternative cellular source for ESCs because unlike ESCs they can be harvested autologously from a readily available, differentiated adult tissue source. Like ESCs they can maintain their ability to proliferate indefinitely while maintaining an undifferentiated state. Murine iPSCs subjected to chondrogenic differentiation showed increased production of glycosaminoglycan and collagen II with little collagen type X staining indicating formation of hyaline-like cartilage.[177] These chondrogenically differentiated iPS cells were mixed with agarose and

placed into 3 mm core cartilage defect that was created in a porcine femoral cartilage explant.[177] After 21 days of *in vitro* culture cells showed matrix production and matrix integration. This repaired cartilage core was then forced from the cartilage explant to obtain the integrative repair strength. The integrative repair strength was significantly higher than cartilage explant cores that were filled with the cartilage that was biopsied to produce the core or agarose gel alone.[177] To the author's knowledge, no *in vivo* studies have been published evaluating the use of iPSCs in articular cartilage repair. iPS cells have been successfully isolated by three research groups from the equine fibroblast, but no studies have been conducted evaluating their *in vivo* use in musculoskeletal disease.[178-180]

Mesenchymal stem cells (MSCs)

The first multi-potent stromal precursor cell was described by Friedenstein in 1974 and was obtained from the bone marrow.[181] This described cell due to its capacity for self-renewal and differentiation was named a mesnchymal stem cell by Caplan in 1991.[182] Today, MSCs are defined by the International Society of Cellular Therapy as plastic adherent cells capable of tri-lineage differentiation into adipose, cartilage, and bone as well as express CD73, CD90, and CD105, but does not express CD11b, CD14, CD34, CD31, CD45, and MHC II on their surface.[183]

One of the first reports of mesenchymal stem cells isolated from the horse was published in 1998 by Fortier, et. al. evaluating chondrogenic differentiation of BMSCs. [184] Since then MSCs have been derived from both adult and fetal equine tissues. Adult tissues from which equine MSCs have been isolated include: peripheral blood,[185] adipose[186-188], bone marrow[187,189,190], gingiva,[191] periodontal ligament,[191], tendon[189,192],

articular cartilage[193]. Fetal tissues from which equine MSCs have been isolated include: umbilical cord blood and tissue[188,194-196], amniotic tissue and fluid[197,195,198], and wharton's jelly[195].

A few studies have compared the difference in chondrogenic capacity of chondrocytes and MSCs. [199-201] MSCs are gaining popularity for articular cartilage repair because they are able to overcome some of the inherent problems with chondrocyte culture, most notably chondrocytes inability to maintain their capacity for chondrogenesis with age.[18,133,202] MSCs are able to produce a neo-tissue with characteristics of cartilage, but chondrocytes produce a neo-tissue with superior characteristics of cartilage compared to age and donor matched MSCs. However, these studies only used neonatal to juvenile chondrocytes and MSCs and do not consider the chondrogenic capacity of the cells with age. .[18,133,202] Kopesky, et. al. compared chondrocytes and BMSCs seeded in self assembling peptide hydrogels obtained from foal and adult sources. Adult BMSCs supplemented with TGFβ-1 produced superior sulfated glycosaminoglycan and proteoglycan content compared to adult chondrocytes and similar sGAG and PG content compared to foal chondrocytes.[203] Foal and adult BMSCs with TGFβ-1 produced a neo-tissue with higher dynamic stiffness than foal and adult chondrocytes (~2x higher dynamic stiffness).[203] These studies highlight that BMSCs are an important cellular therapy to be considered for articular cartilage repair due to their capacity for chondrogenesis and their ability to maintain this capacity for chondrogenesis with age.

MSCs from different tissue origins can have distinguishing properties and some researchers feel that MSCs show greater regenerative capacity if they are obtained from the similar tissues as the tissue that is attempting to be regenerated. For enhancing

articular cartilage repair these tissues could be derived from the articular cartilage, synovium, synovial fluid, intra-articular soft tissues (meniscus and cruciate ligaments), subchondral bone, or infrapatellar fat pad of the stifle. MSCs obtained from intra-articular mesenchymal tissues have been shown to have genetic expression profiles that more closely resemble the genetic expression profiles of healthy articular chondrocytes compared to extra-articularly derived MSCs.[204] In fact, human meniscal-derived, anterior cruciate ligament-derived, and synovium-derived MSCs as well as chondrocytes have expression levels 2.5 times that of extra-articular mesenchymal tissues of the proteoglycan genes PRELP, OGN, and ECR4.[204] Human[204,205], murine[206] synovium derived MSCs have shown enhanced chondrogenesis compared to other adult derived MSCs (BMSCs, AMSCs, PMSCs, MMSCs) *in vitro*. Intra-articular administration of synovium derived MSCs enhanced collagen type II and sulfated glycosaminoglycan content of surgically created osteochondral defects of minipigs.[207] Hyaline-like matrix was also demonstrated in femoral osteochondral defects of rabbits after treatment with synovium derived MSCs encapsulated in a type I collagen/HA/fibrinogen gel.[208] Other experimental models have shown that intra-articular injection of intra-articularly derived MSCs promote hyaline cartilage repair[209-211], but to date no direct comparisons of osteochondral defect repairs with intra-articular derived MSCs and extra-articular derived MSCs has been reported.

Equine articular cartilage-derived MSCs were shown to have decreased proliferative capacity, but improved cartilage like-matrix formation compared to BMSCs.[193] Articular cartilage-derived MSCs did not express markers of chondrocyte hypertrophy and endochondral ossification indicating inadequate cartilage matrix

formation, but BMSCs did express these markers indicating possible superiority of articular-cartilage derived MSCs.[193] Despite this suggested superiority, the difficulty associated with harvesting this tissue coupled with the patient morbidity sustained during harvest, has limited their use for equine articular cartilage repair.

Bone marrow and adipose are the most commonly used tissues for clinical MSC therapy in veterinary medicine due to tissue availability and ease of tissue harvest as well as a their increased cellular proliferation rates and high cellular yield following culture expansion. BMSCs are more commonly used for articular cartilage repair due to numerous studies that have demonstrated greater matrix production after chondrogenic differentiation of BMSCs compared to ADMSCs.[205,206,212-218] However some studies have demonstrated similar[194,219] or even reduced chondrogenesis of BMSCs compared to ADMSCs *in vitro*[187]. The differences seen in their chondrogenic capacity in these studies may be affected by the growth factors used to induce chondrogenesis. It has been shown that BMSCs and ADMSCs require differential growth supplementation for chondrogenesis. BMSCs have increased chondrogenesis when supplemented with dexamethasone and TGFβ-3 with or without BMP-6 compared to ADMSCs that have increased chondrogenesis when supplemented with dexamethasone, TGFβ-3 and BMP-6.[218,220,221] BMP-6 supplementation induces AMSC expression of the TGFβ receptor, ALK-5, which has reduced or absent expression in undifferentiated AMSCs.[218,220,221] Even still, BMSCs are predominantly the MSC of choice in articular cartilage repair studies.

The effect of MSCs co-cultured with chondrocytes on chondrogenesis has been evaluated.[132] MSCs have been shown to enhance chondrocyte proliferation while

preventing chondrocyte de-differentiation when co-cultured in micromass pellets. The chondrocytes enhance MSC production of type II collagen and repress production of type I and type X collagen leading to a more hyaline-like extracellular matrix. These co-culture benefits were only seen when cells were in direct contact (cell-cell or cell-extracellular matrix contact) versus contact through proximity in a transwell culture system. This suggests that the articular chondrocytes and MSCs communicate through direct contact and support the MSC recruitment and synergism of an articular cartilage defect's niche.[132]

Mechanical load has also been shown to enhance the chondrogenic capacity of MSCs. Studies have shown that multiaxial loading with a combination of shear and compressive forces results in enhanced and sustained GAG production and up-regulation of genetic markers of chondrogenesis.[221] Kisiday, et. al. (2009) showed that equine BMSCs subjected to dynamic compression loading and growth factor supplementation with TGFβ-1 showed that the duration of applied dynamic compression was critical in stimulating chondrogenesis.[222] When dynamic compression was applied to equine BMSCs for 12 hours/day at 45 minute cycles GAG accumulation and 3H-proline incorporation were 64% and 74% of unloaded TGFβ-1 supplemented BMSCs suggesting an effect of dynamic compression on induction of chondrogenesis.[222]

SECTION IX: ADMINISTRATION OF STEM CELLS

Delivery of stem cells to the site of injury can be performed by local regional, or systemic administration. Local or intra-lesional injection of MSCs is commonly performed by equine practitioners for treatment of tendinopathy or desmopathy under ultrasound guidance. However, intra-lesional injection into damaged articular cartilage

lesions of an OA joint requires specialized training and equipment. Therefore, equine practitioners must rely on regional or intra-articular injection of MSCs to deliver MSC therapy. For this reason, it is important to understand the ability of the MSCs to survive in the synovial environment, to home and integrate into sites of articular injury, and/or to modulate the inflammatory environment.

Administration of bone marrow concentrate vs. culture expanded MSCs

One of the first forms of "stem cell" therapies utilized was the use of direct injection of bone marrow aspirates in musculoskeletal injury. Only 2-4% of the mononuclear cell population of bone marrow is considered to be a mesenchymal stem cell.[223] This therapy later evolved such that the nucleated cellular portion of tissue aspirates obtained from either fat or bone marrow were concentrated and then applied to the injured tissue. This therapy is appealing to some individuals for several reasons. This technique can be processed very quickly for faster therapeutic application to the patient. It only takes 1-2 hours if the processing can be performed in-house using a commercially available kit or 24-48 hours if the tissue sample needs to be shipped and processed by a commercially available laboratory. This allows the practitioner the ability to initiate therapy much earlier (3-4 weeks or greater) than culture-expanded cells. Additionally, these cells are not manipulated in culture to the extent that culture-expanded cells are meaning that they do not undergo adherence, expansion, and trypsinization through multiple passages, which can alter cellular phenotype. This cellular therapeutic also delivers portions of the bone marrow cell pool that could potentially participate in tissue regeneration.

Table 2.1 reports the studies that have evaluated the use of bone marrow concentrate for articular cartilage repair. Bone marrow concentrate applied to an extracellular matrix (hyaluronic acid membrane or collagen membrane) arthroscopically implanted into osteochondral lesions of one or both femoral condyles significantly improved clinical assessment scores of 30 human patients with up to 3 years follow up.[223] MRI assessment of osteochondral defects showed nearly complete filling of the osteochondral defects and good graft integration in the majority of these patients. Two patients consented to second-look arthroscopic evaluation and biopsy. Histopathologic evaluation of the obtained biopsies revealed the presence of collagen type II and proteoglycans, confirming repair of the osteochondral defects with hyaline-like cartilage. Similar clinical improvements after one-year follow-up have been observed in 48 human patients with osteochondral lesions of the talus treated with arthroscopically implanted bone marrow concentrate loaded matrices (porcine collagen powder or hyaluronic acid membrane).[224] In this study, 77% of patients were able to participate in high-impact sporting activities within an average of 11.3 months after surgery.[224] These findings are comparable to clinical results obtained after treatment of similar osteochondral lesions with ACT without the need of a second surgery and/or the morbidity associated with cartilage harvest from a non-weight bearing articular surface.

Bone marrow concentrate was used to repair large osteochondral defects of 12 horses after microfracture.[127] Bone marrow concentrate was shown to enhance the microscopic and macroscopic scores of the repair tissue as well as improve the composition of the repair tissue compared to defects treated with microfracture alone.

To date there are no studies that have directly compared the efficacy of a cellular concentrate to culture expanded MSC therapy from the same tissue source for articular cartilage repair.

Effect of normal and osteoarthritic synovial fluid on MSCs

Few studies in vitro have evaluated the effect of clinically relevant volumes of normal or osteoarthritic synovial fluid on MSC viability and proliferation. Synoviocytes cultured in 100% autologous OA synovial fluid had reduced cellular proliferation compared to synoviocytes cultured in control media supplemented with FBS.[225] This was one of the first *in vitro* studies to indirectly demonstrate that 100% synovial fluid could support non-MSC cellular viability and proliferation without additional nutrient supplementation.[225] Other studies, have used varying concentrations of synovial fluid for short-term monolayer culture of MSCs, but have not evaluated cellular viability or proliferation. [226,227] Few studies *in vivo* directly assess cellular viability after intra-articular injection. Viable caprine BMSCs have been recovered from synovial fluid after intra-articular injection in a model of OA up to 7 days.[228] These studies demonstrate that *in vitro* and *in vivo* exposure to synovial fluid maintains cellular viability, but the degree of cellular viability maintained is not assessed. Therefore, the direct effect of synovial fluid exposure cannot be determined and evaluation of the degree of cellular death or loss after synovial exposure is warranted.

The majority of studies that evaluate the effect of synovial fluid on MSCs, measure the ability of MSCs to chondrogenically differentiate after exposure to varying concentrations of synovial fluid, but the concentrations of synovial fluid that are supplemented are small in volume (<50% v/v) and not clinically relevant. [226,229,230] Ovine

BMSCs in monolayer culture exposed to 20% autologous non-osteoarthritic synovial fluid caused significant changes to cellular morphology within 24 hours of exposure. The cells shortened, becoming round, oval, or polygonal and the cells migrated toward one another forming grid-like aggregations. [226] This stimulated migration after exposure to synovial fluid is supported by enhanced *in vitro* migration of synovial progenitor cells from synovial membrane explants after exposure to 100% autologous osteoarthritic synovial fluid.[225] This migration and aggregation of cells resemble clustering of chondrocytes prior to endochondral ossification and imply that these cells are differentiating toward a chondrogenic phenotype in the presence of synovial fluid.[231] Two weeks after culture, the previously mentioned ovine BMSCs demonstrated positive type II collagen, safranin-O, and toluidine blue staining demonstrating chondrogenesis.[226] Chondrogenesis of equine BMSCs in micromass pellets cultured in n up to 50% autologous non-osteoarthritic synovial fluid has been demonstrated, but only in one BMSC line.[229] This is the only study to demonstrate chondrogenesis of equine BMSCs in response to synovial fluid.

MSC chondrogenesis in the presence of osteoarthritic synovial fluid has been demonstrated. Human corticospongious progenitor cells subjected to chondrogenic media supplemented with 5% synovial fluid obtained from healthy donors or osteoarthritic donors showed increased proteoglycan and type II collagen production compared to human corticospongious progenitor cells chondrogenically differentiated with media supplemented with 5% synovial fluid obtained from rheumatoid arthritis patients.[232] This demonstrates that BMSCs can differentiate in the face of an inflammatory environment to a degree and that there is a threshold of inflammation above, which detrimental effects on

MSC chondrogenesis occur. This inflammatory threshold is ill-defined because these studies fail to evaluate the synovial fluid or the OA stage of the joint from which the synovial fluid was obtained (i.e. acute OA vs. chronic OA). Conditioned medium obtained from culture of OA synovial membrane was shown to inhibit chondrogenesis of human MSCs in pellet culture, but the degree of inhibition varied between the different synovial membranes indicating variability in the inflammatory inhibition among patients.[233] Additionally the cause of this inflammatory threshold has not been identified. It is likely that the major inflammatory cytokines, IL-1β and TNFα, contribute to this reduction in chondrogenesis. Supplementation of chondrogenic medium with IL-1α and TNFα inhibited human MSC chondrogenesis in pellet cultures. More studies are warranted to understand the effects of osteoarthritic synovial fluid on MSC chondrogenesis.

If cells are to be administered autologous, potentially the disease state of the patient (osteoarthritic vs. non-osteoarthritic) could affect the capacity of MSC differentiation. Studies evaluating the capacity for chondrogenic differentiation of MSCs obtained from osteoarthritic donors have produced controversial results, with some studies demonstrating hindered chondrogenesis[228,233,234] and others demonstrating similar chondrogenesis of MSCs from osteoarthritic and non-ostearthritic donors.[234,235] These studies are primarily performed with the use of human MSCs and to the author's knowledge, no studies have evaluated the chondrogenic capacity of equine MSCs obtained from osteoarthric donors to non-osteoarthritic donors.

These studies suggest that synovial fluid contains stimulatory signals for chondrogenic differentiation of MSCs, but after cellular tissue integration these MSCs

will also receive signals from the local environmental niche, which will include cells, such as chondrocytes and synoviocytes. Autologous ovine synoviocytes co-cultured with BMSCs have also been shown to induce similar morphological changes as synovial fluid exposure and induce chondrogenesis.[226] Culture of MSCs in micromass with chondrocytes has also been shown to induce MSC chondrogenesis.[236,237]

Homing capacity of stem cells after intra-articular injection

Intra-articularly administered BMSCs have been shown to preferentially migrate to the synovial membrane and intra-articular soft tissues (cruciate ligaments and meniscus).[238] However, reports of labeled MSCs in repaired cartilage after OA induction have demonstrated that MSCs do have the potential to home to the articular cartilage, but they do not preferentially do so. For example, intra-articular injection of lagamorph BMSCs labeled with supermagnetic iron oxide nanoparticles (SPIO) seven days after creation of a 4 mm articular cartilage defect in the femoral trochlear groove showed homing and retention of the SPIO labeled BMSCs 4 weeks after injection, but cellular retention in the defect was abolished by 12 weeks post-injection.[239] GFP labeled BMSCs have also been reported in the repaired articular cartilage of donkeys after intra-articular injection into radiocarpal joints after chemical induction of OA.[240] These studies as well as other studies presented in Table 2 demonstrate that MSCs can integrate into the articular cartilage. However, these cells prefer to integrate into the synovial membrane and it is this interaction that warrants further investigation particularly in regards to the interaction of the innate immune cells of the synovial membrane and the injected MSCs.

Intra-articular administration of MSCs may prevent OA progression

Table 2.2 displays experimental and clinical studies, which have utilized intra-articular MSC therapy in osteoarthritis. As demonstrated in this table, there is wide variation in the OA model used or degree of natural injury, the type of cell source injected, the number of cells injected, the cellular delivery agent used, and the timing of injection making comparison difficult.

The first paper to describe promising therapeutic results after intra-articular administration of MSCs was reported by Murphy, et. al. In this study, 10 $x10^6$ autologous GFP-transduced BMSCs suspended in HA were intra-articularly administered 6 weeks after mechanical induction of OA in 24 goats. Improvements in macroscopic and microscopic pathology of the articular surface of the medial femoral condyle and neomeniscus of joints treated with BMSCs compared to joints treated with HA alone suggested that intra-articular administration of BMSCs could dampen the progression of OA.[241]

Several other studies have shown that intra-articular administration of MSCs can reduce the progression of OA (Table 2.2), but the one experimental study that has evaluated the intra-articular administration of equine BMSCs did not show significant reduction in OA progression. In this study, intra-articular administration of autologous equine BMSCs or adipose derived stromal vascular fraction failed to prevent experimental OA progression. BMSC administration significantly decreased PGE2 concentration in the synovial fluid of treated and contralateral joints compared to ADVSF administration. No other treatment effects were observed and neither treatment had a significant effect on macroscopic or microscopic cartilage and synovial pathology.[242]

This is in contrast to an experimental study in which $5.4 \pm 6.9 \times 10^6$ autologous GFP transduced BMSCs were intra-articularly injected into the radiocarpal joints of 27 donkeys after chemical induction of OA.[240] This study showed reduced progression of OA, as measured by radiographic and histological examination, 6 months after injection. However, the scientific methods used call to question the results.

Enhanced repair tissue has been demonstrated in osteochondral defects treated with equine BMSCs. One of the first studies to evaluate the use of equine BMSCs evaluated the repair of a large (15 mm) femoral trochlear ridge osteochondral with 12×10^6 autologous pre-chondrogenically differentiated BMSCs suspended in fibrinogen directly injected into the defect under arthroscopic guidance[243] Early improvement in the repair tissue within the defect was observed, but this improvement compared to controls was not sustained at 8 months demonstrating that BMSC treatment resulted in early improvement of defect healing.[243] More recently, intra-articular injection of 20×10^6 autologous equine BMSCs one month after surgical creation and microfracture of an osteochondral defect of the medial femoral condyle resulted in significant improvements in the quality of the reparative tissue.[116]

Potentially, the mechanism by which intra-articular administration of MSCs inhibits articular cartilage degradation and/or matrix regeneration is an indirect rather than direct effect. Given that studies have demonstrated preferential homing of MSCs to the synovial membrane and/or intra-articular soft tissues with minimal homing and integration into articular cartilage, it is likely that MSCs directly modulate the synovium's orchestration and perpetuation of the inflammatory events central to the pathogenesis of OA, which indirectly reduces cartilage destruction.

To date, only one prospective multicenter clinical trial evaluating the intra-articular use of autologous BMSCs has been published. In this study, 39 horses with surgically confirmed moderate to severe OA unresponsive to other intra-articular medications were treated with autologous BMSCs with a minimum follow-up time of 21 months.[244] Seventy-seven percent of these horses were able to return to work. Of these 38% returned to their prior level of performance and the remainder returned to work, but at a lower level of performance.[244] Of the horses that did not return to work, the majority of the cases (29/39) had severe stifle OA.[244] This study demonstrates great promise for functional return of horses with chronic, severe OA and prompts further evaluation of BMSC therapy for horses with natural OA.

Immunogenicity of MSCs and their allogeneic administration

MSC therapy in our veterinary species is primarily conducted in an 'autologous' manner such that tissue is harvested, cells are isolated and expanded, and then returned to the patient from which the tissue was originally harvested for treatment of a musculoskeletal injury. However, allogeneic administration could provide several patient, therapeutic, and research advantages. Some studies have demonstrated that certain patient-related factors such as gender, age, and disease state can affect proliferation and chondrogenesis of MSCs prompting some clinicians to seek young, non-osteoarthritic donors for allogeneic MSC banking. [228,245] MSCs derived from tissues that are not readily available for autologous transplantation due to patient morbidity concerns and/or difficulty in tissue harvest, but may have superior capacity for articular cartilage repair, such as articular cartilage progenitor cells, could be harvested, expanded, and banked for allogeneic use.[246] These allogeneic MSCs could be extensively characterized by tri-

lineage differentiation, immunophenotyping, and immunogenicity to provide a more defined product than autologous MSCs, which are rarely characterized prior to therapeutic application due to the expense and time, related to characterization. This would decrease the variability of MSC lines and allow a more uniform therapeutic research.

MSCs have been shown to modulate and suppress certain aspects of both the innate and adaptive immune systems making allogeneic administration of the cells therapeutically possible theoretically. MSCs express low levels of MHC I, but lack MHC II and co-stimulatory molecule expression (CD 80 and CD 86) allowing them the ability to go unrecognized to a certain extent by the recipients immune system after allogeneic transfer.[247-250] However, some studies have demonstrated that activation of MSCs by exposure to pro-inflammatory cytokines can up regulate surface expression of MHC II.[251] When activated *in vitro,* MSCs exhibit several immunosuppressive effects. They can inhibit allo-antigen or mitogen activated B-cell[252,253],T-cell[247-250], and NK-cell proliferation.[254] Activated MSCs can also stimulate the release of certain anti-inflammatory mediators from immune cells. For instance, MSCs have been shown to stimulate production of IL-10 from plasmacytoid dendritic cells which in turn stimulates regulatory T cell production.[255,256]

In vivo experimental studies that have directly compared the safety and efficacy of autologous MSCs to allogeneic MSCs are limited.[257-260] Persistence of donor allogeneic MSCs within the tissue of interest for 4 or more weeks has been demonstrated[257,261,262] indicating that an innate or adaptive immune response is not present to a degree that would result in complete removal of the transplanted cells. The majority of these

experimental studies have used un-activated allogeneic MSCs, but due to the need for activation *in vitro* for MSCs to exert many of their immunosuppressive effects, interest in use of activated MSCs is growing.

MSCs have been shown to be activated by synovial fluid from osteoarthritic donors. BMSCs obtained from patients undergoing total hip arthroplasty were cultured in 20% (v/v) synovial fluid and showed increased IL-6 expression and IDO activity with a trend for decreased production of TNFα.[227] Conditioned medium obtained from culture of BMSCs in 20% OA synovial fluid significantly inhibited lymphocyte proliferation.[227] Conditioned medium obtained from short-term culture of human BMSCs exposed to TNFα and IFN γ was shown to downregulate expression of inflammatory cytokines (IL-1β) and catabolic enzymes (MMP 1, MMP 3, ADAMTS 5) in synovial and cartilage expants as well as up regulate expression of IL-1 receptor antagonist in cartilage explants. Conditioned medium also caused down regulation of collagen II expression explants, suggesting altered matrix production. Interestingly, conditioned medium did increase production of the inhibitor of nuclear factor kappa B alpha (IκBa) in chondrocytes and synoviocytes obtained from explants. Nuclear factor kappa B alpha is an important transcription factor for enhancing pro-inflammatory cytokine gene expression in OA and its inhibition after exposure to conditioned medium in synoviocytes and chondrocytes represents along with alterations of gene expression in synovium and cartilage further highlight a possible immunoregulatory role of MSCs in treatment of OA.[263] These studies would suggest that pre-activation of MSCs is not necessary prior to intra-articular injection, but further work is warranted.

MSCs from different species exert different immunomodulatory effects. For instance, human MSCs primarily use IDO expression to reduce T cell proliferation, but murine MSCs primarily secrete NO to inhibit T cell proliferation.[264] Therefore, it is important that the specific *in vitro* and *in vivo* immunomodulatory effects of equine MSCs be more thoroughly evaluated before clinical application of allogeneic administration of MSCs in the horse is pursued. Equine MSCs have been shown to express MHC I, but have not been shown to express MHC II or co-stimulatory molecules.[265-268] A recent *in vitro* study compared immunogenic properties of equine bone marrow, adipose, umbilical cord, and umbilical tissue-derived MSCs.[265] This study demonstrated that equine BMSCs and ADMSCs could reduce T cell proliferation without activation, but when activated equine BMSCs, ADMSCs, UC-MSCs, and UCT-MSCs significantly inhibited allo-antigen and mitogen activated T-cell proliferation.[265] Equine BMSCs, ADMSCs, UC-MSCs and UCT-MSCs constitutively expressed TGFβ, but its expression was increase after MSC activation.[265] PGE2 and IL-6 were not constitutively expressed, but secretion significantly increased after MSC activation.[265] These MSCs also significantly decreased production of TNFα and IFNγ by mitogen activated T cells.[265] IDO was not produced by any of these MSCs before and after activation, but NO was produced by activated MSCs.[265] These findings demonstrated that equine MSCs can have significant effects on T cell proliferation after exposure to an inflammatory stimulus. This is important when considering that one of the most important cell-cell interaction of MSCs after intra-articular injection takes place at the synovium, which is predominantly infiltrated with macrophages and T cells in OA. Further work is necessary to understand how equine MSCs influence the innate and adaptive immune systems and how these cells

exert their effects *in vivo*, meaning do we need to pre-activate them toward an anti-inflammatory phenotype prior to injection or do we allow the microenvironment influence the MSCs.

In vivo allogeneic MSC interactions have been studies sparsely in the equine literature and primarily focus on safety data. One of the first studies to evaluate the safety of allogeneic MSC administration in the horse, evaluated the safety of intra-lesional injection of surgically induced superficial digital flexor tendinopathy in two horses. Three separate core lesions were created and subsequently injected with 1×10^6 autologous or allogeneic BMSCs in autologous bone marrow supernatant and autologous bone marrow supernatant as a control.[269] Horses were euthanized 10 and 34 days after BMSC administration. Cellular retention within the lesions was relatively low, but the number of cells retained within the lesion was similar.[269] A cellular immune response was detected after both autologous and allogeneic BMSC administration, but no difference in the quantity of peri-lesional immune cells was detected between the treatments as measured by LFA-1 antibody staining of leukocytes.[269] This was followed by a small descriptive case series of 16 horses with naturally occurring tendinopathies treated with allogeneic ADMSCs suspended in autologous PRP.[270] Fourteen of these 16 horses were reported to 'recover', but there degree of recovery meaning if they returned to work and if so there level of performance upon return was not documented in the study. Additionally, the severity of the lesions was not reported and these horses were not compared to a population of horses that received autologous ADMSCs in PRP or recovered with traditional therapeutic approaches (rest and controlled exercise) making it difficult to understand the efficacy of allogeneic use.[270] Fifty percent of these horses experienced

transient limb edema and pain after injection, but it is difficult to know if the response was due to the ADMSCs, PRP, or both. The previous study in which two horses received allogeneic BMSCs in autologous bone marrow concentrate did not report adverse events such as edema and pain suggesting that the possible response seen was due to injection with PRP. The author has observed focal edema and pain after intra-lesional injection of naturally occurring tendinopathies by PRP alone, but reports in the equine literature of PRP reaction after intra-lesional injection are sparse. More work is warranted to validate the safety and efficacy of allogeneic MSC administration in tendon and ligament disorders of the horse.

The safety of intra-articular MSCs has been studied. $7.5x10^6$ autologous, syngeneic, or allogeneic umbilical cord tissue or umbilical cord blood-derived MSCs suspended in normal saline were injected into the radio-carpal joint of 16 non-OA horses (foals, half-siblings, and their dams).[266] Differences among treatment groups were not detected in regards to vital parameters, lameness, and joint swelling were noted. Injection with UCB or T-MSCs caused a significant, but transient increase in the nucleated cell count and total protein of synovial fluid after injection. This study showed that allogeneic administration of UCB or T-MSCs into normal joints did not elicit a greater local inflammatory response than autologous administration. There are currently no other reports evaluating the use of equine allogeneic MSCs in either normal or abnormal joints.

Allogeneic BMSCs administered via intra-arterial or intravenous regional limb perfusion did not cause significant lameness in 5 of 6 horses. The one horse in which severe lameness was induced after allogeneic BMSC administration horse had received an initial injection of allogeneic BMSCs that was abandoned early in the procedure due to

spillage of the cells. Regional limb perfusion was performed 6 weeks later, and after the second injection of allogeneic BMSCs, the horse developed severe lameness and dermal necrosis due to arterial thrombosis of the medial palmar artery. This complication was attributed to the procedure not the allogeneic nature of the cells or the repeated administration of allogeneic BMSCs.[271] Severe venous thrombosis after intravenous allogeneic regional limb perfusion has been observed by the author, but a high dose of cells was used to perform this injection (50 million equine allogeneic BMSCs) and patient related factors were attributed to this adverse event. Two other horses administered the same dose of allogeneic BMSCs did not develop complications related to the regional limb perfusion (Mumaw, et. al. Unpublished data). It is likely that these complications observed after regional limb perfusion of equine allogeneic MSCs is related to the pooling of blood within the arterial and venous system in conjunction with high cell volumes results in thrombosis of the vessel and is not related to the allogeneic nature of the MSCs.

Another concern that has been raised in the human literature is the possible existence of alloantibodies after MSC administration and whether repeat MSC administration would result in a severe immunologic response. Depending on the degree of musculoskeletal injury, repeated administration may be pursued by a practitioner. Repeated intra-dermal injection of allogeneic UCT-MSCs failed to elicit an immediate or delayed hypersensitivity reaction in 16 horses.[272] Allogeneic UCT-MSCs did not suppress or enhance T cell proliferation obtained from horses *in vitro* prior to and after repeat intra-dermal injection.[272] This is the only study that has evaluated repeat

administration of allogeneic MSCs in an equine model and further work is warranted particularly if considering the use of allogeneic MSCs in OA therapy.

Allogeneic administration of MSCs presents a unique opportunity for therapeutic and research development, but our level of knowledge in regard to the safety and efficacy of allogeneic MSC administration is far behind the commercial drive of this therapy.

Effect of other intra-articular medications on MSCs.

Due to the concern of possible synovial sepsis with intra-articular administration of biological therapeutics, many equine practitioners administer antibiotics with these biological therapeutics. One study evaluated equine BMSC viability after 48 hours in culture with 50-500μg/mL of gentamicin, amikacin, penicillin, enrofloxacin, or ceftiofur and showed reductions in cellular viability after culture with enrofloxacin (200μg/ml) and amikacin (500μg/ml).[273] Conclusions on the effects of gentamicin and ceftiofur on BMSC viability could not be made due to an interaction of the antibiotic with components of the viability assay (Alamar Blue).[273] A more recent study did show a significant reduction in BMSC viability (>95% cell death) after 45 minutes of incubation with 150 mg of gentamicin and after 2 hours of incubation with 250 mg amikacin[115]. Incubation with hyaluronic acid (22 mg) was shown to maintain cellular viability (80% viability) after 6 hours of culture[115]. Theses studies indicate that the concurrent administration of intra-articular antibiotics with equine BMSCs is not recommended.

Many practitioners have shown interest in concurrent administration of BMSCs with intra-articular corticosteroids. Preliminary data from the UGA VTH regenerative medicine laboratory suggest that co-administration of equine BMSCs with clinically relevant doses of corticosteroids (methylprednisolone acetate and triamcinolone) do not

produce deleterious effects on BMSC viability (Mumaw, et. al. 2012 Unpublished data). However, it would be interesting to evaluate the effects of culture with these corticosteroids affects the immunomodulation of BMSCs, i.e. does it prevent there activation to become anti-inflammatory. Concurrent systemic NSAID administration has also been evaluated for its effects on BMSC chondrogenesis. Chronic NSAID administration with acetaminophen and naproxen has been shown to affect gene expression of human BMSCs causing up regulation of collagen X, a marker of chondrocyte hypertrophy.[274] This primarily has to do with the collection and expansion of the cells, but direct effects of NSAIDs administered at the time of BMSC injection have not been evaluated.

SECTION X: PLATELET RICH PLASMA

PRP is obtained from autologous plasma of the patient and is processed via centrifugation, filtration, or apheresis to achieve a platelet concentration above baseline concentration. The precise mechanism of PRP is unknown, but the conceptual mechanism of action of PRP is related to the activated release of growth factors stored in the platelets alpha granules enhancing the healing process. The growth factors contained in the platelets alpha granules include transforming growth factor β (TGF-β), platelet derived growth factor (PDGF- AB and BB), insulin-like growth factor (IGF-1), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), fibroblast growth factor.[275] These factors primarily promote angiogenesis into the lesion (VEGF and FGF), enhance cellular proliferation (PDGF, FGF, and TGF-β), and promote extracellular matrix formation (TGF-β and IGF-1).[275]

The ideal platelet concentration for treatment of musculoskeletal injuries is poorly defined, though, a platelet 'dose' of greater than 1,000 x 10^3 platelets/µl PRP is needed to enhance wound healing this equates to a 2-6 fold increase in the platelet concentration from human baseline platelet concentration (150 – 450 x 10^3 platelets/µl).[276] Other studies suggest that a minimum of a five-fold increase of baseline platelet concentration is necessary to promote wound healing.[277] Current recommendations in human orthopedics are to concentrate the platelets 4-6 times that of the baseline concentration.[275] Greater platelet concentration that what has been recommended does not enhance wound healing and may in fact be deleterious to wound healing.[278] Platelet concentration also does not correlate with growth factor concentration and can vary greatly among individuals.[279] PRP in humans is typically administered for 3 or more treatments, 2-4 weeks apart.[275] The equipment and techniques employed to produce PRP have great variability in platelet concentration achieved as well as the degree of leukocyte and red blood cell depletion.[280] Additionally, the treatment protocols utilized and the presence or absence of activation prior to administration varies greatly among studies. This coupled with the lack of placebo controlled, randomized clinical trials, makes interpretation of study results challenging for the clinician.

PRP has gained interest in human OA therapy due to the enhancement of articular cartilage repair by some of the growth factors present in the alpha granules and its proposed anti-inflammatory effects. In addition, to these growth factors PRP becomes a biological scaffold upon activation making it a useful autologous cellular suspension product for cell delivery into focal osteochondral defects. A clinical case series of 115 human knees with chronic OA receiving 3 treatments of autologous PRP showed clinical

improvement at 6 months as measured by the IKDC and EQ-VAS, but this therapeutic effect was not maintained and clinical improvement worsened 12 months after treatment though the clinical assessment scores were still improved from baseline assessments.[281] Another clinical case series evaluating two different techniques of manual preparation (single or double centrifugation producing low and high platelet concentration respectively) for the treatment of 144 human knees with chronic OA also showed improvements in clinical assessment scores (IKDC, EQ-VAS, and Tegner) for a minimum of 2-6 months and varying improvement or maintenance of 6 month scores depending on the clinical outcome score used.[282] No differences were observed between the two different methods of preparation as far as clinical outcome, but when platelets were processed using a double centrifugation technique resulting in higher platelet concentration, patients experienced significantly greater pain and swelling.[282] Not surprisingly, younger patients with less OA had significantly better outcomes. Evidence for the efficacy of PRP in OA therapy is lacking and placebo controlled randomized clinical trials are warranted before *in vivo* use is recommended.[282] When the therapeutic benefits of PRP was compared to low molecular weight HA and high molecular weight HA in 150 patients with chronic knee OA, PRP and low molecular weight HA showed similar improvements in IKDC and EQ-VAS scores compared to high molecular weight HA at 2 months.[283] By 6 months improvement in clinical scores was greater in knees treated with PRP, but longer follow-up was not reported.[283] These and the majority of other studies evaluating the intra-articular use of PRP in OA demonstrate improvements in subjective pain scores and clinical outcome scores, but fail to demonstrate improvement in cartilage repair. One smaller study (14 patients) that used

ultrasound assessment of cartilage thickness as a means of evaluating cartilage repair after intra-articular PRP administration of OA knees failed to show improvement in cartilage thickness.[284] Therefore, the benefits of PRP for articular cartilage repair are still largely unknown and further studies are warranted to address it's therapeutic benefit in OA.

To date, only one report has been published evaluating the intra-articular use of PRP in equine patients with OA. This study evaluated four horses with a total of 5 OA joints. Horses were injected with 3 treatments two weeks apart. No adverse systemic or local responses were reported and lameness score improved within the first two months of treatment, but the degree of improvement steadily declined eight months after treatment. [285] This study was not controlled or blinded and further studies are necessary to provide sufficient evidence that this treatment is safe for use of OA in horses.

PRP has been shown to enhance fibroblast[286], tenocyte[287], endothelial cell[288], osteoblast[289,290] and MSC proliferation. PRP has been shown to enhance equine BMSC proliferation[291], but not at clinical relevant concentrations to what would be used as a cellular suspension for injection. Studies evaluating the use of PRP as a cellular suspension and source of growth factors for stimulation of MSCs used in OA treatment are needed.

SECTION XI: RATIONALE FOR PRESENTED STUDIES

As discussed in the previous sections of this literature review, there is much that is unknown about the intra-articular use of autologous or allogeneic MSCs in normal let alone osteoarthritic joints. Allogeneic administration of MSCs would provide practitioners with a more uniform, "off the shelf" type product to treat musculoskeletal

injuries in the more acute phases of musculoskeletal injury. MSCs provide a unique opportunity for treatment of OA in that they possess certain immunomodulatory characteristics when activated by an inflammatory environment and have the ability to form hyaline-like cartilage repair tissue. Therefore, the studies described in chapters 3-5 were conducted to understand the biological effects exposure to allogeneic synovial fluid and a biological cellular suspension agent (PRP) have on equine BMSCs as well as the local and systemic effects of intra-articular administration of allogeneic BMSCs.

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Table 2.1. Studies evaluating the use of cellular concentrates for articular cartilage repair

Cell Concentrate	Publication	Study species and study type	Additional factors	Follow Up	Findings
Bone Marrow-Direct Implantation	Fortier, et. al.[127] J. Bone Joint Surg. Am.	N=12 Equine Experimental comparative study 15 mm defects of the lateral femoral trochlear ridge	Treated with microfracture alone or with bone marrow concentrate	Second look arthroscopy at 3 months Euthanized at 6 months	Bone marrow concentrate had greater macro and micro scores for repair tissue. Matrix within the defect showed improved composition and organization when treated with bone marrow concentrate.
Bone Marrow-Direct Implantation	Buda, et. al.[223]	N=30 Human Case Series Osteochondral defects (ICRS grade III and IV) of knee Patients with diffuse OA excluded	BM loaded onto onto collagen membrane or hyaluronic acid membrane. Platelet rich fibrin gel sealed the construct	Clinical Assessments at 6, 12, 18, 24, and 36 mos. MRI 6 and 12 mos. Second look arthroscopy 12 mos. (n=4) with biopsy (n=2)	Improved clinical assessments from baseline (IKDC and KOOS) MRI showed defect filling Collagen II and PG without Collagen I was present in defect
Bone marrow-Direct Implantation	Giannini, et. al. [224]	N= Human Case Series Osteochondral defects (ICRS grade II) of ankle	BM mixed with collagen powder or loaded on HA membrane Platelet rich fibrin gel sealed construct	Clinical assessments at 6,12,18, and 24 mos. Second look arthroscopy (n=5)	Improved clinical assessments from baseline (AOFAS) 2 patients at second look arthroscopy had hypertrophic regenerated cartilage. Some staining of collagen I, II, and PG (poor hyaline cartilage repair)
Adipose-Intra-articular administration	Black, et. al.[307] Vet. Ther.	N=18 Canine Randomized clinical trial Bilateral coxofemoral OA	4.2-5 million nucleated cells/joint in PBS; Placebo animals receive PBS only	Clinical assessments at 30, 60, and 90 days Owner assessment (Cincinnati orthopedic disability)	Veterinarian assessment identified improvement in function, pan, and range of motion. Owner survey showed

					perceived improvement
Adipose- Intra-articular adiministration	Koh, et. al.[308]	N=18 Human Case Series Knee OA	Mean 1.8 x10^6 concentrated infrapatellar fat pad cells in 3 mls PRP (mean 1.28x10^6 platelets/ul)	Clinical Assessments (Western Ontario and McMaster Universities OA Index scores) 2 yrs. Pain Assessment 2 yrs. (Visual analog scale) Imaging: Radiograph and MRI 2 yrs.	Clinical improvement Pain improvement Improved MRI scores *Clinical and MRI score improvements positively correlated with cell number
Adipose- Intra-articular injection	Koh, et. al. The Knee	N=50 (25 cells + PRP, 25 PRP alone) Human Comparative clinical study Knee OA	Mean 1.9x10^6 concentrated infrapatellar fat pad cells in 3 mls PRP (mean 1.28x10^6 platelets/ul)	Clinical Assessment (Lysholm, Tegner) Pain assessment (VAS)	Some patients experienced pain for 2-3 days after injection Similar results between groups in outcome between groups, but trend for improvement with concentrated ADMSCs thereapy Younger patients with lower ICRS scores showed the greatest improvements
Adipose- Intra-articular injection	Pak, et. al.[309] J. Med Case reports	N=2 Human Case Series Knee OA	ADSC concentrate + HA + PRP + Dexamethasone 4 Additional weekly PRP + Dex treatments	Descriptive assessment outcomes at 3 mos	Improved function and range of motion. Diminished pain Improved cartilage thickness of MRI

Table 2.2 Studies evaluating the use of culture expanded MSCs after intra-articular injection for articular cartilage repair in osteoarthritis.

Tissue source	Publication	Study species and study type	Treatment	Added factors	Follow Up	Findings
BMSCs	Mokbel, et. al.[117] BMC Musculoskeletal Disorders	N=27 Donkey Experimental Chemical induction (amphotericin B) of OA in radiocarpal joints	5.4 ± 6.9 x 10^6 Autologous BMSCs/joint GFP labeled Cells injected 3 (n=9), 6 (n=9), and 9 wks. (n=9) after OA induction	HA	1, 2, and 6 mos. Lameness (AAEP grades) Radiographs (Crawford scale)	GFP+ cells were seen on the surface of the articular cartilage Reduced radiographic and histological progression of OA
BMSCs	Horie, et. al.[292] Osteoarthritis and Cartilage	Murine Experimental Mechanical induction (ACL transection and menisectomy)	2x10^6 Human BMSCs (xenogenic) or Rat BMSCs (allogeneic)/joint Cells labeled with CM-Dil Cells injected at time of surgery	PBS	Euthanized 2, 4, and 8 wks.	MSCs (xenogeneic or allogenic) enhanced regeneration of meniscus and inhibited OA progression CM-Dil cells visualized in neomeniscus, but cellular retention declined with time.
BMSCs	Emadedin, et. al.[293] Arch Iran Med	N=6 Human Case Series Naturally occurring severe knee OA	20 – 40 x 10^6 cells/joint	-	Clinical assessment (WOMAC) Pain assessment (VAS) 12 months	Improved joint function and walking distance Decreased pain 50% of patients had improved cartilage thickness and decreased subchondral bone on MRI
BMSCs	Sato, et. al.[294] Arthritis Research and Therapy	N=60 Hartley Guinea Pig Experimental Natural progression of OA that occurs in Hartley guinea pigs	7 x 10^6 human cells/joint (xenogenic treatment) Cells labeled with carboxyfluorescein diacetate succinimidyl ester Cells introduced when guinea pigs were 7 mos.	HA	Euthanized 1, 3, and 5 wks. post treatment	No adverse reactions following injection Cells seen in synovial membrane, meniscus, and cartilage after injection. HA + MSCs lower macroscopic scores than PBS, HA, and PBS + MSCs. HA + MSCs improved histo scores compared to PBS and PBS + MSCs, but not to HA alone. HA+ MSCs greater col II and less

						MMP 13 immunostaining
BMSCs	Davatachi, et. al.[295] Int. J. Rheum. Dis.	N=4 Human Natural OA Moderate to severe knee OA	8-9 x 10^6 cells/joint in normal saline	-	12 months	Improved clinical and pain scores No radiographic improvement
BMSCs and ADSVF (cell concentrate)	Fisbie, et. al,[242] J Orthop Res	N= 24 Equine Experimental Comparative Mechanical induction Carpal fragment model	16.3 x 10^6 nucleated cells/joint of adipose derived stromal vascular fraction (Concentrate not culture expanded cells)(n=12) mean of 10.5 x 10^6 BMSCs/joint (culture expanded) Horses treated 14 days after OA induction	None	Euthanized at d. 56	No improvement in lameness ADSVF treated joints had increased flexion scores compared to BMSC treated and control joints No differences in joint effusion and radiographic assessments were seen between treatments No significant treatment effect was seen in synovial total protein or WBC counts. BMSC treated joint had significant reduction in synovial fluid PGE2 compared to ADVSF treated. No significant treatment effects were seen macro and microscopically in synovial membrane and cartilage
BMSCs	Murphy, et. al.[241] Arthritis & Rheumatism	N=24 Ovine Experimental Mechanical Induction (ACL transection and menisectomy)	10 x 10^6 autologous BMSCs/joint BMSCs transduced to express GFP BMSCs injected 6 weeks after OA induction	HA	Euthanized at 6 and 20 weeks	BMSC injection reduced OA progression (Less cartilage destruction, osteophyte formation, and subchondral sclerosis); BMSC injection improved histo scores. BMSCs were found in neomeniscus and stained for col II and proteoglycan. Two sheep treated with cells were poor responders meaning that they had little evidence of OA protection and neomeniscus formation GFP + cells were present in SF 7 days after injection GFP+ cells primarily found in

						meniscus, but also seen in synovial lining and femoral condyles.
BMSCs	Chen, et. al.[296] Oral and maxillofacial surgery	N=60 Rabbit Experimental Mechanical induction (menisectomy)	$0.2x10^6$ autologous BMSCs/jt. Undifferentiated BMSCs (n=12) Pre-differentiated chondrogenic BMSCs (n=12)	HA	Euthanized at 4, 12, and 24 weeks	Improved histological scores At 4, 12, and 24 weeks, BMSC increased Sox 9, col II, aggrecan and decreased MMP13 (Differentiated > Undifferentiated) MicroCT showed greater subchondral bone volume and trabecular thickness (Differentiated > Undifferentiated)
BMSCs	Wakitini, et. al.[297] Osteoarthritis and cartilage	N=24 Human Clinical comparative study Natural knee OA	$5x10^6$ BMSCs/joint	Collagen	16 months	Clinical improvement was not significantly different between treated and control, but better arthroscopic and histological scores were present in the cell-transplanted group.
ADMSCs	Wood, et. al.[298] Journal of ocular pharmacology and therapeutics	N=6 Canine OA was not induced-imaging study of stifle	5×10^6 allogeneic ADMSCs Labeled with iron oxide or fluorescently labeled with DiD Cells injected 3x (wk 1, 3, and 5 of study)	PBS	Euthanized at 10 weeks	Cells were localized to the synovium and persisted for 4 weeks as imaged by MRI Cells produced a mild inflammatory response in the joint.
ADMSCs	Desando et. al.[246] Arthritis and Research	Rabbit Experimental Mechanical induction (ACL transection)	2×10^6 autologous cells/joint or $6x10^6$ cells/joint Cells administered eight weeks after OA induction Some cells were labeled with CM-Dil	4% rabbit serum albumin	Euthanized at 3 days, 20 days, 16 weeks, and 24 weeks.	ADMSCs seen in synovial membrane and meniscuse at 3 and 20 days. ADMSCs not observed in articular cartilage in any subject. ADMSCs significant reduction in fibrillation index at both doses at 16 and 24 wks. Low dose ADMSCs significantly increased cartilage thickness and cartilage Laverty's score. ADMSCs prevented synovial hyperplasia and inflammatory cell infiltrate at both doses. ADMSCs increased col II expression and decreased col I, TNFα and MMP1 expression in cartilage

						ADMSCs decreased MMP1 and TNFα expression in the synovial membrane
ADMSCs	Huurne, et. al. [299] Arthritis and Rheumatism	N= N/R Murine Experimental Chemical induced OA (collagenase) of stifle	20,000 autologous ADMSCs/joint Cells transduced with GFP (lentivirus) Cells injected 1 wk. after OA induction	Autologous serum with 4% albumin		GFP+ cells in synovium 24 hrs. after injection, but not seen 5 days later. Reduced synovial hyperplasia Reduced IL-1β mRNA (not IL-6 or 10) of synovium 24 hrs. after injection GFP Reduced cruciate ligament rupture and entheseophyte formation Reduced matrix degredation products at d.14 (not d. 42)
ADMSCs	Fatemehsadat, et. al. [300]	N= 16 Rabbit Experimental Mechanical induction (ACL transection)	1 x 10^6 Allogeneic ADMSC/joint (only one cell line used in this study) injected in media Cells injected 12 wks. after OA induction 10 rabbits were treated, 10 rabbits control injected with media	None	Euthanized at 16 wks. and 20 wks.	Treatment minimally reduced radiological scores. Histological (Mankin) scores significantly improved compared to control joints.
ADMSCs	Al Faqeh, et. al. [301] Experimental gerontology	N=20 (16 experimental/4 control) Ovine Experimental Mechanical transduction (ACL transection and medial menisectomy) Injection 6 wks. After OA induction	10 x 10^6 undifferentiated cells/ joint (n=6) or 10 x 10^6 chondrogeneically differentiated cells/joint injected in media	None	Euthanized 3 and 6 weeks	ICRS scores different between treated and untreated, but not between differentiated and undifferentiated. Cartilage regeneration in both treatment groups with differentiated cells producing cartilage thickness that was similar to normal knees
ADMSCs	Guercio, et. al.[302] Cell Biol Int	N=4 Canine Case Series	3-5 x10^6 autologous cells/joint	HA (n=2) or PRP	1 month	Functional improvement was observed by either veterinarian or owner after 1 month of treatment

		Naturally occurring OA of the elbow OA was 6 mos in duration and non-responsive to NSAIDs		(n=2)		
ADMSCs	Toghraie, et. al.[300,303] The Knee and Arch Iran Med	N=20 Rabbit Experimental Mechanical induction (ACL transection)	1 x 10^6 allogeneic infrapatellar fat pad ADMSCs/jt in media	-	Euthanized at 16 (n=10) and 20 wks. (n=10)	Improved radiology scores Improved histo scores at 20 wks.
Synovial and Bone Marrow MSCs	Horie, et. al.[304] Stem Cells	N=27 Rat Experimental Comparative Mechanical induction (menisectomy)	5 x 10^6 allogeneic synovium derived MSCs/joint (N=9) 5 x 10^6 allogeneic bone marrow derived (N=14) 5x10^6 synovium derived MSCs in control (no OA) rats (N=4) Cells obtained from dual colored Tg rats that express luciferase/LacZ Cells injected at time of sx	PBS	Euthanized at 2, 4, 8, and 12 wks. Histology Immunostaining Bioluminescence TEM RT-PCR	Synovium MSCs homed to the meniscal defect, but were not observed in the synovium, cartilage surface of cruciate ligaments. Neomeniscus formed and was positive for collagen II No differences in synovium MSCs and BMSCs in neomeniscus formation Luminescence of cells after intra-articular injection were observed in the knee up to 28 days.
Muscle MSCs	Mifune, et. al.[305] Osteoarthritis and cartilage	N=36 Murine Experimental Chemical induced OA (MIA) of stifle	5 x10^5 MDSCs/joint 12 joints received retroviral transduced MDSCs with GFP/BMP4 and sFlt1/LacZ Cells injected 2 wks. after OA induction	PRP alone or in combination with MDSCs (5.5 fold increase from baseline)	Euthanized at 4 and 12 weeks	MDSCs with PRP showed improved cartilage histo scores compared to PBS and PRP alone. No differences in histo scores were observed between MDSCs alone and MDSCs + PRP. IHC for GFP or β-gal expressing cells showed greater col II expression in MDSCs + PRP compared to MDSCs alone. Transduced MDSCs + PRP had better histo scores and greater col II expression than transduced MDSCs alone

						PRP alone did not contribute significantly to AC repair *in vivo*
Meniscal derived MSCs	Shen, et. al. [306]	N=9 Rabbit Bilateral menisectomy	6 x 10^7 MeSCs 1 or 2 weeks post-op. Cells fluorescently labeled with carboxyfluorescein diacetate	PBS	Euthanized at 4, 8, and 12 weeks	Formation of neomeniscus in treatment group with significantly greater biomechanical strength (compressive modulus). Protected OA progression: improved histology scores of articular surfaces and improved radiographic images of the knee.

CHAPTER 3

VIABILITY, PROLIFERATION, AND CHONDROGENESIS OF EQUINE BONE MARROW DERIVED MESENCHYMAL STEM CELLS AFTER EXPOSURE TO VARIOUS CONCENTRATIONS OF NORMAL ALLOGENEIC SYNOVIAL FLUID *IN VITRO*[1]

[1] Boone, L., Mumaw, J., Thoresen, M., Gogal, R., Peroni, J
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ABSTRACT

Objective: Evaluate effects of equine allogeneic synovial fluid on viability, proliferation and chondrogenesis of bone marrow derived mesenchymal stem cells (BMSCs).

Animals: Bone marrow was collected from 8 horses aged 7 -13 years old. Synovial fluid was collected from 25 horses aged 4 -20 years old.

Procedure: BMSCs were cultured in medium supplemented with 25, 50, or 100% (v/v) synovial fluid for 72 hours. Viability and proliferation were measured after 24 and 72 hours BMSCs were cultured in micromass for 28 days after which cell viability and glycosaminoglycan (GAG) content were quantified to assess chondrogenesis.

Results: A significant increase in viability was observed initially after 24 hours in culture with greater than 25% synovial fluid supplementation, but no difference in viability was observed after 72 hours of culture. A significant increase in cell number was observed in BMSCs exposed to 100% synovial fluid after 24 and 72 hours. However, no significant difference in cellular proliferation was detected between BMSCs cultured in 100% synovial fluid and medium after 24 and 72 hours. BMSCs became elongated and aggregated into three-dimensional colonies during culture. Additionally, GAG production per viable BMSC in 100% synovial fluid was significantly increased compared to medium.

Conclusions and clinical relevance: *In vitro* exposure to high concentrations of normal allogeneic synovial fluid produces little to no deleterious effects on BMSC viability, proliferation, and chondrogenesis. After intra-articular injection, BMSCs should maintain their viability, proliferation, and chondrogenic capacity during homing and implantation into normal synovial or articular tissues.

INTRODUCTION

Lameness due to osteoarthritis is one of the leading causes of reduced or lost performance in horses and causes significant economic hardship to the equine industry.[1-4] Osteoarthritis is a debilitating degenerative disease process characterized by progressive deterioration of articular cartilage with or without accompanying changes in the subchondral bone, ligaments, joint capsule, synovial membrane and peri-articular muscles.[5-8] The pivotal events responsible for this degenerative process involve biomechanical, biochemical, and genetic factors that lead to an inflammatory response within the joint.[7] Currently, the mainstay of therapy for equine patients with osteoarthritis focuses on temporary reduction of inflammation with non-steroidal or steroidal anti-inflammatory agents and/or viscosupplementation with non-sulfated glycosaminoglycans such as hyaluronic acid or polysulfated proteoglycans in an attempt to restore articular homeostasis.[6] The detrimental effects associated with repeated administration of high doses of corticosteroids have prompted clinicians to seek biological therapies with anti-inflammatory or reparative functions to assist in cartilage homeostasis restoration. Bone marrow (BMSCs) or adipose derived MSCs (ADMSCs) are more commonly used for equine musculoskeletal repair due to the ease of tissue harvest and limited morbidity associated with the tissue harvest. BMSCs are often preferred for cartilage repair strategies due to their enhanced chondrogenic capacity compared to ADMSCs.[1-9] However, this field is still in its infancy, with limited experimental knowledge involving the behavior and action of MSC in a joint environment.

Experiments looking at the chondrogenic capacity have demonstrated that supplementation with synovial fluid can have beneficial effects on chondrogenic

differentiation. Ovine synovial fluid supplementation (20%) of growth medium and ovine BMSCs co-cultured with synovial cells have been shown to change ovine BMSC cellular morphology forming aggregates in culture similar to cells undergoing chondrogenic differentiation and expressed proteins indicative of chondrgenic differentiation in the absence of exogenous chondrogenic signaling factors.[10] Autologous synovial fluid supplemented with concentrations up to 50% of growth medium induced chondrogenesis in equine BMSCs.[11] More recently, abnormal synovial fluid obtained from human osteoarthritic and rheumatoid arthritic patients supplemented in 20% growth medium was shown to enhance human BMSC immunomodulation by causing a significant increase in indoleamine 2,3-dioxygenase (IDO) and IL-6 as well as a slight reduction in TNFα expression.[12] These papers show that *in vitro* exposure to synovial fluid can induce chondrogenesis in BMSCs and potentially enhance their immunomodulatory properties indicating that BMSCs can provide a bimodal therapeutic in degenerating joints.

Both normal and osteoarthritic synovial fluid has been shown to have controversial effects on proliferation of cells obtained from various tissue sources. Human chondrocytes exposed to media supplemented with 10% (v/v) synovial fluid obtained from patients after traumatic knee injury showed a significant increase in DNA quantity after 14 days of *in vitro* culture.[13] In contrast, human synoviocytes cultured for 7 days in osteoarthritic synovial fluid showed decreased cellular proliferation.[14] To the author's knowledge, there are currently no known reports of the effects of normal autologous or allogeneic synovial fluid on cellular proliferation of equine BMSCs in the literature. The maintenance of cellular proliferation of equine BMSCs after exposure to

high concentrations of normal allogeneic synovial fluid is a novel finding that could be used to support the therapeutic use of BMSCs within the synovial environment.

Several studies have shown that BMSCs can home to a site of articular cartilage injury after intra-articular injection and begin production of an extracellular matrix consistent with cartilage (type II collagen and/or proteoglycans).[15,16] This homing capacity of BMSCs and the ability of the synovial environment to push the cells toward a chondrogenic phenotype tentatively supports intra-articular therapy of BMSCs making stem cell therapy a more readily used modality for the equine practitioner.

Additionally, allogeneic administration of BMSCs is appealing because this approach eliminates the delay associated with the culturing of autologous cells, however the potential for inflammatory reactions or incompatibility is a concern in using allogeneic cells. It is beneficial to use allogeneic cell lines because the lines can be evaluated in *vitro* for desirable characteristic of enhanced immunomodulation and chondrogenic capacity to optimize the potential therapeutic. This also provides the option of obtaining youthful allogeneic lines from younger non-osteoarthritic donors, which have been shown to enhance capacity for chondrogenesis.[17] Allogeneic administration of equine BMSCs has been shown to be tolerated well with minimal immune response. In a study using allogeneic BMSCs in experimental models of superficial digital flexor tendonitis, allogeneic BMSCs had shown to have no difference in leukocyte migration into lesions when compared to autologous BMSCs.[18] Allogeneic intra-articular MSC administration using placentally derived equine MSCs demonstrated no difference in the degree of synovial inflammation caused by allogeneic or autologously administered

MSCs.[19] This handful of studies shows that allogeneic MSCs are well tolerated and are a viable source for potential therapeutic cell lines.

Despite the wide use of BMSCs in equine practice for joint related musculoskeletal disease, little is known regarding the interactions between BMSCs and the synovial environment into which they are injected, let alone the effects of an allogeneic synovial environment on BMSCs. Therefore, the objective of this study was to evaluate the effects of allogeneic synovial fluid on the viability, proliferation, and chondrogenesis on BMSCs. While we hypothesized that exposure to normal allogeneic synovial fluid at concentrations <50% would have no significant effect on cellular viability and proliferation, but that allogeneic synovial fluid concentrations of 100% would have a significant deleterious effect on cellular viability and proliferation, we found the opposite to be true. We also validated our hypothesis that exposure to allogeneic synovial fluid at concentrations <50% would show significant enhancement of chondrogenesis compared to medium controls, but that 100% allogeneic synovial fluid supplementation would significantly inhibit chondrogenesis compared to medium controls.

MATERIALS AND METHODS

Animals

Bone marrow was aseptically harvested from 8 healthy adult horses aged 8-14 years old. Synovial fluid was aseptically harvested from 25 healthy adult horses donated to the University of Georgia aged 4 – 20 years old. The University of Georgia Institutional Animal Care and Use Committee approved all procedures involving collection of samples for this study.

Isolation of BMSCs

Bone marrow was aseptically harvested from the sternum of six healthy adult horses using an 8 gauge x 4 in. Jamshidi bone marrow biopsy needle[a] Approximately 20 cc of bone marrow was drawn from either the 4th, 5th, or 6th sternebrae into 2-35cc syringes containing 2500 units of heparin sulfate[b] in each syringe. BMSCs were obtained by plating the cells according to the plate adherency method[20] Cells were cultured in defined culture medium consisting of Dulbecco's Modified Eagles Medium with 4.5 g/L glucose and sodium pyruvate without L-Glutamine[c] supplemented with 10% fetal bovine serum[d] 0.05% L-glutamine[e] and 50 U/ml penicillin/ 50 µg/ml streptomycin[f] under standard cell culture conditions (37°C and 5% CO_2). Initial BMSC colonies were allowed to reach confluency and then manually re-distributed prior to trypsinization. Once cells had reached 70-80% confluency, cells were harvested from the plate with 0.05% trypsin-EDTA[g], reseeded at 5000 cells/cm^2, allowed to reach 70-80% confluency and cryopreserved.

Synovial fluid harvest

Synovial fluid was obtained from adult horses deemed healthy by physical examination. Between 2 -8 milliliters of synovial fluid were obtained aseptically from the radiocarpal joint, middle carpal joint, and/or tarsocrural joint. Approximately 500µl of synovial fluid was placed in tubes containing EDTA and analyzed for total protein, nucleated cell count, and cytology. Only synovial fluid deemed "normal" (yellow and clear with a nucleated cell count <2500 cells/µL and total protein < 2.5 g/dL) was processed for storage. Normal synovial fluid was centrifuged at 10,000 g for 10 minutes to remove cellular content and the cell-free supernatant was obtained then frozen at -80°C

until experimental use. Synovial fluid was pooled and aliquoted for medium supplementation.

Experimental cell culture

All experiments used cryopreserved BMSCs between passages 3-5 Cells were seeded into 96 well plates at a seeding density of 3500 cells/well for viability and proliferation studies. BMSCs were seeded into one 96 well "V" bottom plate a seeding density of 200,000 cells/well for the chondrogenesis study. The chondrogenic plate was then centrifuged at 450 g for 10 minutes for cellular aggregation and formation of micromass pellet culture. BMSCs were allowed to acclimate and attach to the culture plate for 24 hours under standard culture conditions prior to applying treatment conditions.

Cellular viability

Medium and experimental conditions were added to BMSC lines in triplicate. Control medium consisted of Dulbecco's Modified Eagles Medium with 4.5 g/L glucose and sodium pyruvate without L-Glutamine supplemented with 10% fetal bovine serum, 0.05% L-glutamine and 1% penicillin/streptomycin. 25% or 50% (v/v) synovial fluid was added to control medium and then added to the appropriate wells. 100% synovial fluid was added to the appropriate wells. After 24 or 72 hours in culture, culture medium was removed. BMSCs were trypsinized, centrifuged, and re-suspended in PBS. Cells were incubated with calcein AM[h] for 20 minutes protected from light. Cellular suspensions were analyzed with flow cytometry using FlowJo version 9.6.2[i].

Cellular proliferation

Culture conditions were performed as stated above for cellular viability. After 24 and 72 hours in culture, Hoescht 33342[j] was added to culture medium and incubated for 20 minutes then Hoescht absorbance was measured at 346/460 nm using a microplate reader. Separate culture plates were set up as described for cellular viability for cellular proliferation utilizing the Clik-iT cell proliferation assay[k] Clik-iT EDU was added to the culture plates 24 hours prior to running the assay. The assay was performed according to the manufacturer's instructions and absorbance was measured at 495/519 and 568/585 on a microplate reader.

Cellular morphology

The 96 well plates used for both the viability and proliferation assays were observed daily with an inverted microscope to evaluate BMSC confluency, morphology, adherence, and aggregation. Pictures were taken of all BMSC lines after 24 and 72 hours of culture for all culture conditions.

Chondrogenesis

After the 24 hour acclimation period, the experimental conditions were applied in triplicate to all BMSC lines. HyClone Advance STEM[l] chondrogenic differentiation medium was directly applied to control pellets or supplemented with either 25% or 50% synovial fluid. 100% synovial fluid was applied directly to the micromass pellets. Medium and/or synovial fluid was changed every 3 days for a 28-day culture period. After 28 days in culture all micromass pellets rinsed with phosphate buffered saline three times, and the quantity of viable cells within the pellets was measured by staining the pellets in parallel with Neutral Red as previously described.[21] The cells were then fixed

with 100% methanol for 10 minutes at -20°C. A 0.2% Alcian Blue 8GX in 0.1 M HCl solution was applied to the micromass pellets for 2 hours at room temperature. The pellets were then washed three times with PBS and alcian blue stain that had been taken up by the pellets was extracted by exposing the pellets to 6 M guanidine/HCl overnight at room temperature. The optical density of the extracted alcian blue was measured at 650 nm. The detection of Neutral Red content was measured at an optical density of 550 nm. Using this method we were able to detect cell viability and simultaneously quantify chondrogenesis.[22]

Statistical analysis

All analyses were performed using SAS V 9.2 (Cary, NC). A repeated measures model that recognized multiple observations as belonging to the same horse was used to test for differences in viability, proliferation and chondrogenesis of BMSCs between treatment groups and time points. The full model included fixed factors for group, time and an interaction effect of group and time and a random factor of horse. If multiple time points were not measured then the model included just the fixed factor for group and the random factor for horse. An unstructured covariance structure was used in all repeated measures models. All hypothesis tests were 2-sided and the significance level was $\alpha = 0.05$. Tukey's test was used to adjust for multiple paired comparisons.

RESULTS

Cellular viability

Cellular viability of BMSCs cultured in medium, 25% synovial fluid, 50% synovial fluid, or 100% synovial fluid after 24 and 72 hours in culture were assessed by flow cytometric analysis of calcein fluorescence (figure 3.1). There was a significant

increase in cellular viability of BMSCs cultured for 24 hours in all synovial fluid concentrations when compared to control medium (25% p=0.01; 50% p=0.04, 100% p=0.02), but no significant differences in BMSC viability were observed between synovial fluid groups. No significant differences in cellular viability were observed between BMSCs cultured in synovial fluid and BMSCs cultured in control medium for 72 hours (25% p=0.9, 50% p=0.5, 100% p=1). There were no significant differences observed within experimental groups in cellular viability with longer culture times (24 vs.72 hrs) (Medium p=0.76, 25% p=0.90, 50% p=1.0, 100% p=0.16).

Cellular proliferation

There was a significant dose-dependent increase in DNA quantity measured by Hoescht 33342 absorbance for BMSCs cultured for 24 hours with increasing synovial fluid exposure compared to control medium (25% p=0.01, 50% p=$1x10^{-12}$, 100% p=$1x10^{-12}$). There was a significant increase in DNA quantity of BMSCs cultured in 50% synovial fluid (p=$0.9x10^{-3}$) and 100% synovial fluid (p=$1x10^{-12}$) compared to culture in 25% synovial fluid for 24 hours. There was also a significant increase in DNA quantity of BMSCs cultured in 100% synovial fluid compared to BMSCs cultured in 50% synovial fluid (p=$0.65x10^{-2}$) for 24 hours. After 72 hours in culture, only BMSCs cultured in 100% synovial fluid had significantly greater DNA quantity than BMSCs cultured in control medium (p=0.01), but no significant differences in DNA quantity were observed after 72 hours among BMSCs cultured in synovial fluid (25 vs. 50% p=0.85, 25 vs. 100% p=0.35, and 50 vs. 100% p=0.99). There was a significant decrease in DNA quantity after 72 hours in BMSCs cultured in 50% synovial fluid (p=$1.9x10^{-50}$) and 100% synovial fluid (p=$1x10^{-12}$) compared to DNA quantity at 24 hours (Figure 3.2a).

There was a significant decrease in cellular proliferation, measured by the Clik-it Edu assay, of BMSCs cultured for 24 hours in 25% synovial fluid compared to BMSCs cultured in control medium ($p=0.2x10^{-3}$), BMSCs cultured in 50% synovial fluid ($p=0.2x10^{-2}$), and BMSCs cultured in 100% synovial fluid ($p=7.9x10^{-10}$). No significant differences were seen in cellular proliferation after 24 hours in culture for BMSCs cultured in 50% synovial fluid ($p=1.0$) or 100% synovial fluid ($p=0.1$) compared to culture in control medium. No significant differences in cellular proliferation were observed for BMSCs cultured in control 50% and 100% synovial fluid ($p=0.05$) for 24 hours. No significant differences were detected in cellular proliferation after 72 hours in culture for BMSCs cultured in 25% synovial fluid ($p=1.0$), 50% synovial fluid ($p=1.0$), or 100% synovial fluid compared to BMSCs cultured in control medium ($p=1.0$). There was a significant decrease in cellular proliferation with prolonged culture (24 hrs vs. 72 hrs) for BMSCs cultured in medium ($p=1x10^{-12}$), 50% synovial fluid ($p=3.1x10^{-12}$), and 100% synovial fluid ($p=1x10^{-12}$) (Figure 3.2b).

Cellular morphology

BMSCs after 72 hours of culture in medium, 25%, 50%, and 100% synovial fluid, took on a finer, elongated morphology and began to aggregate into colonies forming a web-like network (Figure 3.3). With increasing synovial fluid exposure these cellular aggregates formed three-dimensional structures. Cellular concentration per high power field was noted to increase with increasing synovial fluid concentration. These findings were observed in all cell lines during the culture period.

Chondrogenesis

There was a significant reduction in cellular viability of the micromass pellets with increasing synovial fluid exposure compared to micromass pellet cultures maintained in control chondrogenic medium as measured by neutral red absorbance (25% $p= 1.3x10^{-8}$, 50% $p=8.5x10^{-8}$, 100% $p=1x10^{-12}$) (Figure 3.4a). There was a significant increase in total GAG content measured by alcian blue absorbance of BMSC lines cultured in 25% ($p=1.8x10^{-8}$) and 50% ($p=8.5x10^{-8}$) synovial fluid, but a significant decrease in total GAG content of BMSC lines cultured in 100% ($p=1x10^{-12}$) synovial fluid compared to BMSC lines cultured in chondrogenic medium (Figure 3.4b). There was a significant increase in total GAG content measured by alcian blue absorbance of BMSC lines cultured in 25% and 50% synovial fluid, but a significant decrease in total GAG content of BMSC lines cultured in 100% synovial fluid compared to BMSC lines cultured in chondrogenic medium ($p<0.001$; Figure 3.4b). When total GAG content was divided by cellular quantification to evaluate the GAG content per viable BMSC, a significant increase was observed with 50% or greater synovial fluid supplementation (50% $p=0.02$, 100% $p=7.6x10^{-7}$). There was a significant difference in GAG content per BMSC between micromass pellet cultures exposed to 25% synovial fluid and 100% synovial fluid ($p=0.1x10^{-3}$) and between micromass pellet cultures exposed to 50% synovial fluid and 100% synovial fluid ($p=0.02$) with cells exposed to 100% synovial fluid having the highest GAG content per BMSC (Figure 3.4c). Gross observation of the chondrogenic pellets after 28 days in culture revealed a slight increase in size of micromass pellets cultured in up to 50% synovial fluid (Figure 3.4d). Micromass pellet cultures exposed to 100% synovial fluid were smaller than all other micromass pellets

observed, and pellets cultured in 100% synovial fluid had a gelatin-like matrix surrounding the pellet (Figure 3.4d arrow).

DISCUSSION

The use of BMSCs for treatment of intra-articular soft tissue injuries, synovitis and/or osteoarthritis is gaining popularity due to BMSCs regenerative and immunomodulatory effects. Intra-articular administration of BMSCs rather than direct implantation at the site of injury requires that the cells maintain their viability during homing and implantation of tissues. Once to the site of injury the synovial environment would ideally support cellular proliferation and/or differentiation toward a chondrogenic phenotype to maintain viability, proliferation, and chondrogenic capacity after *in vitro* exposure to increasing concentrations of normal allogeneic synovial fluid provides evidence that the cells are able to survive once injected into joints.

Synovial fluid supplementation up to concentrations of 100% was able to enhance cellular viability as compared to control media at 24 hours. This indicates that the cells were not detrimentally affected by short-term culture, even in 100% synovial fluid. The ability of synovial fluid to enhance cellular viability compared to optimized culture medium *in vitro* provides hope that the cells will survive well following joint injection. While this was not maintained at 72 hours, it is possible that the increased cellular concentration in the synovial fluid metabolized the nutrients at a faster rate resulting in an inability to maintain the greater levels of viability as control.

Exposure of BMSCs to 100% synovial fluid resulted in maintenance of cellular proliferation compared to media after 24 and 72 hours of culture. Cellular proliferation as measured by quantification of DNA was enhanced in a dose dependent manner after 24

and 72 hours of culture in synovial fluid. This enhanced cellular proliferation was not seen after 24 and 72 hours of culture in synovial fluid when cellular proliferation was measured by DNA incorporation of EDU (5-ethylnyl-2-deoxyuridine). However, using this assay, no significant differences in cellular proliferation was observed between cells cultured in media and cells cultured in 50 and 100% synovial fluid after 24 and 72 hours. The discrepancies in observed cellular proliferation between these two assays could be due to variations in the measured absorbance of the compounds after incorporation into the BMSCs DNA. An additional possibility is that the morphological changes that increased the interactions between cells created a contact inhibition preventing further proliferation, and the early increase in cell number resulted in culture conditions that prevented further proliferation. Despite these differences, the results of both assays demonstrate that BMSCs cultured in high concentrations (>50%) of allogeneic synovial fluid are at the very least able to proliferate to the same degree as BMSCs cultured in control medium. Increased BMSC proliferation in the face of high concentrations of synovial fluid is further supported by observations of BMSCs in culture. Figure 3.3 demonstrates the dramatic increase in BMSC number per high power field that was observed after 72 hours of culture in 100% synovial fluid.

Exposure to high concentrations of synovial fluid caused morphological changes to equine BMSCs. Cellular observation revealed elongation and thinning of BMSCs cultured in high concentrations of synovial fluid (≥ 50%). Cell numbers increased and the cells began to cluster and migrate towards one another, eventually then stacking on top of one another to form three-dimensional cellular aggregates (Figure 3.3 c and d). These findings indicate alterations in cellular adherence, signaling, and networking with the

addition of synovial fluid to the culture media. Cartilage-producing cells undergo cellular proliferation and aggregation during endochondral ossification *in vivo*, suggesting that exposure of BMSCs to high concentrations of synovial fluid *in vitro* can cause changes of the BMSC phenotype such that they are pushed toward chondrogenesis. Changes to BMSCs morphology, adherence, and cellular contact were only observed when BMSCs were cultured in 50 and 100% synovial fluid. Other studies that have evaluated cellular morphological changes in the face of synovial fluid supplementation described widening of ovine BMSCs such that they took on a polygonal shape after 2 weeks of culture in 20% autologous synovial fluid.[10] Much like our equine BMSCs, the ovine BMSCs were observed to aggregate into grid-like cellular clumps that stained positive for collagen type II and proteoglycans.[10] In addition to differences in cellular morphology, equine BMSCs also formed cellular aggregates in culture. Though, staining of these cellular aggregates were not performed in monolayer culture after 72 hours, chondrogenesis of BMSCs exposed to high concentrations of synovial fluid were demonstrated (Figure 3.4). These findings support the "priming" of BMSCs after exposure of allogeneic synovial fluid toward a chondrogenic phenotype.

Chondrogenesis (GAG/BMSC) was enhanced in BMSCs after exposure to high concentrations of synovial fluid. Long-term pellet culture of equine BMSCs in synovial fluid did cause significant decreases in cellular number as assessed with the neutral red assay. Cellular quantification was decreased compared to medium in BMSC micromass pellets cultured in 25% and 50% synovial fluid, but no significant difference in cellular quantification of the pellets between these two synovial fluid concentrations was observed. There was, however, a significant reduction in cellular viability with

supplementation of 100% synovial fluid suggesting that there is a threshold of synovial fluid supplementation between 50 and 100% that results in a significant reduction the ability of the cells to either proliferate or support higher numbers of cells over the extended culture period. This reduction could be due to decreased nutrient diffusion to and within the pellets due to increasing viscosity with higher concentrations of synovial fluid supplementation, or more rapid differentiation of the cells in the higher concentrations of synovial fluid. If the cells initiated differentiation toward chondrogensis earlier than the cells in the medium control they would have left the cell cycle earlier, resulting in fewer cells proliferating before differentiation. Additionally, given the proliferation of the cells after short term culture in monolayer, there may have also been an increase in cellular proliferation that could not be sustained leading to decreased cellular quantification due to a reduction in nutrient availability with prolonged culture times before medium changes. In the synovial environment, cyclic, repetitive movement of the joint allows the synovial fluid to disperse and adequately bathe the articular cartilage for diffusion of nutrients to the chondrocytes. Nutrients within the synovial fluid are also constantly being replenished due to the circulatory supply of the synovial membrane that filters the plasma components for production of synovial fluid. The medium and/or synovial fluid was changed in the pellet cultures every 3 days, but due to this lack of constant nutrient flux of the synovial fluid coupled with the enhanced cellular proliferation observed in the presence of synovial fluid it is possible that there was a lack of adequate nourishment to the cells causing reduced quantity of cells in the micromass pellets during long term culture. Nutrient diffusion could also have been inhibited due to the gel-like matrix that was observed to surround the pellets when cultured in 100%

synovial fluid (Figure 3.3d, arrow). There was a significant increase in total GAG content produced by BMSC micromass pellets cultured in 25 and 50% synovial fluid compared to controls, but a significant decrease in overall GAG content was observed in pellet cultures cultured in 100% synovial fluid. However, when the total GAG production was divided by the total cellular viability, there was a significant increase in GAG production per viable BMSC with increasing synovial fluid concentrations at both 50% and 100% synovial fluid supplementation when compared to controls. GAGs are a key component of the proteoglycan molecules within the extracellular matrix of the articular cartilage, providing compressive stiffness to the articular cartilage, therefore demonstration of GAG formation in the presence of normal allogeneic synovial fluid is vital to support the therapeutic use of BMSCs in articular cartilage regeneration.[23] These findings are similar to a preliminary study that showed one line of equine BMSCs was able to chondrogenically differentiate when exposed to normal autologous synovial fluid with increases in pellet size were noted with increasing concentrations of synovial fluid (up to 50%).[11] These findings support the findings of the current study that synovial fluid supports equine BMSC chondrogenesis and matrix production with increasing synovial fluid supplementation (up to 50%). However, we also demonstrated that even higher concentrations of synovial fluid supplementation enhance production of extracellular matrix (GAGs) despite losses in cellular viability. This is important because it supports the intra-articular use of undifferentiated allogeneic BMSCs in normal joints and suggests that undifferentiated allogeneic BMSCs are able to differentiate toward a chondrogenic lineage after exposure to the synovial environment. Therefore, the practitioner does not need to pre-differentiate cells for them to assist with articular cartilage repair, but rather

the practitioner should allow the synovial environment or niche to provide signals to the cell for chondrogenic differentiation if implanted into an articular cartilage defect or even activation toward an anti-inflammatory phenotype to modulate the inflammatory environment of the synovial membrane.

Normal synovial fluid has been shown to maintain or enhance chondrogenesis of various cell types, but abnormal (osteoarthritic) synovial fluid has been shown to have varying effects on chondrogenesis.[10,11,13,24-28] Multiple studies examining the effects of synovial fluid from degenerative joint disease has shown that osteoathritic[24,27] environments support chondeogenic differentiation of various cell types, but that rheumatoid arthritic conditions are inhibitory.[26] This indicates that the inflammatory environment may play a role in chondrogensis of progenitor cells. Further investigation is warranted into the effects of equine osteoarthritic synovial fluid on BMSC viability, proliferation, chondrogenesis and immunomodulation, however these studies show that MSCs are a promising intra-articular biologic therapeutic for horses with osteoarthritis.

While the results of this study support maintenance of BMSC viability, proliferation, and chondrogenesis after short-term exposure to high concentrations of normal allogeneic synovial fluid *in vitro,* conclusions regarding the clinical relevancy of the study are limited. This study was performed *in vitro* and the alterations of allogeneic BMSCs administered intra-articularly to non-osteoarthritic joints can only be inferred. We have demonstrated survival and integration of allogeneic BMSCs after intra-articular administration of the TCJ up to 10 days prior to euthanasia in 3 horses (unpublished data). This supports that *in vivo* BMSCs are able to survive in the synovial environment after intra-articular administration. Our *in vitro* model also does not incorporate the role

of the environmental niche on BMSC viability, proliferation, and chondrogenesis, meaning that the physical interaction of synoviocytes and chondrocytes with BMSCs was not examined. Co-culture of BMSCs with chondrocytes has been shown to induce chondrogeneic differentiation of BMSCs and therefore the addition of synovial fluid may further enhance this chondrogenesis[1]. A limitation of this study was the classification of "normal" synovial fluid. Synovial fluid was considered normal if the nucleated cell count of the obtained synovial fluid was less than 2500 cells/ μl and total protein was less than 2.5 g/dL. However, a full diagnostic evaluation of the joints from which "normal" synovial fluid was obtained was not performed. Despite these limitations, this study supports further *in vivo* investigation into the safety and efficacy of intra-articular administration of allogeneic BMSCs in normal and osteoarthritic joints.

Our findings demonstrate that allogeneic synovial fluid does not have deleterious effects on BMSC viability, proliferation, and chondrogenesis *in vitro* and may in fact enhance cellular proliferation and chondrogenic differentiation. This supports the intra-articular use of BMSCs in the joints of normal horses, but further work should be done to address the use of allogeneic BMSCs in osteoarthritic joints.

FOOTNOTES

[a] Jamshidi bone marrow biopsy needle, Jorgensen Laboratories, Inc., Loveland, CO

[b] Heparan Sulfate (1000 Units/ml) Hospira, Inc. Lake Forest, IL

[c] DMEM, Cellgro, Mediatech,Inc. Manassas, VA

[d] Fetal Bovine Serum, Atlanta Biologicals, Lawrenceville, GA

[e] Glutamine, Gibco, Invitrogen™, Auckland, AZ

[f] Penicillin/ Streptomycin, Gibco, Invitrogen™, Auckland, AZ

[g] 0.05% Trypsin-EDTA, Gibco, Invitrogen™, Auckland, AZ

[h] Calcein AM Gibco, Invitrogen™, Auckland, AZ

[I] FlowJo version 9.6.2, Tree Star, Inc, Ashland, OR

[j] Hoescht 33342, Thermo Scientific, Inc., Waltham, MA

[K] Clik-iT® cell proliferation assay, Invitrogen, Auckland, AZ

[l] HyClone Advance STEM ,Thermo Scientific, Hampton, New Hampshire

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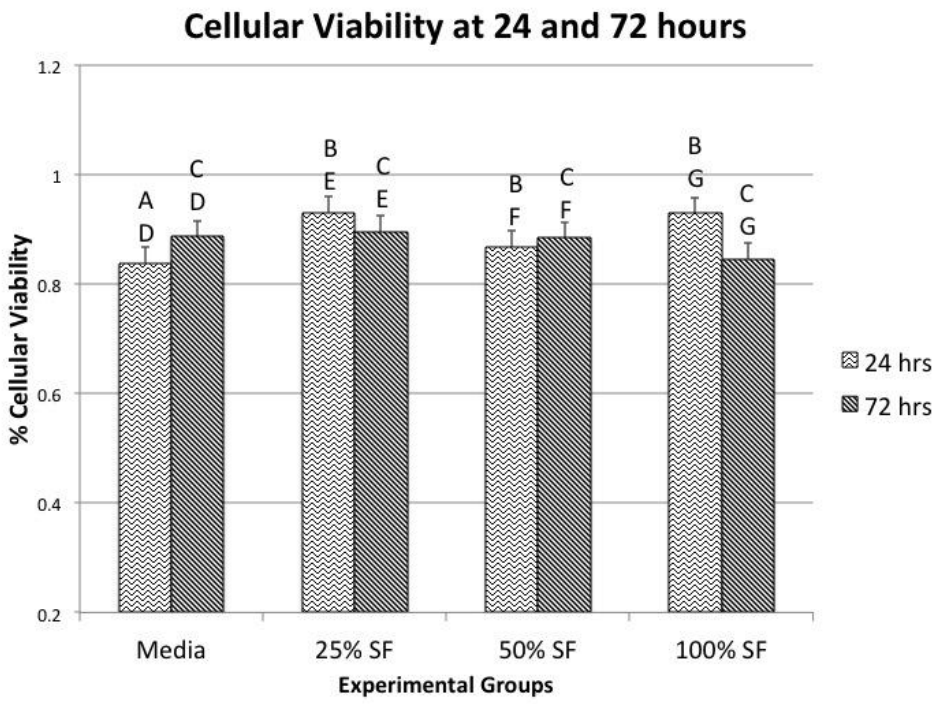

Figure 3.1. Cellular viability of BMSCs in allogeneic synovial fluid. Mean cellular viability (±SEM) of eight equine BMSC lines cultured for 24 and 72 hours in control medium, medium supplemented with 25% synovial fluid, medium supplemented with 50% synovial fluid, or medium supplemented with 100% synovial fluid. Means with different letters are significantly different (Tukey-Kramer, $p < 0.05$).

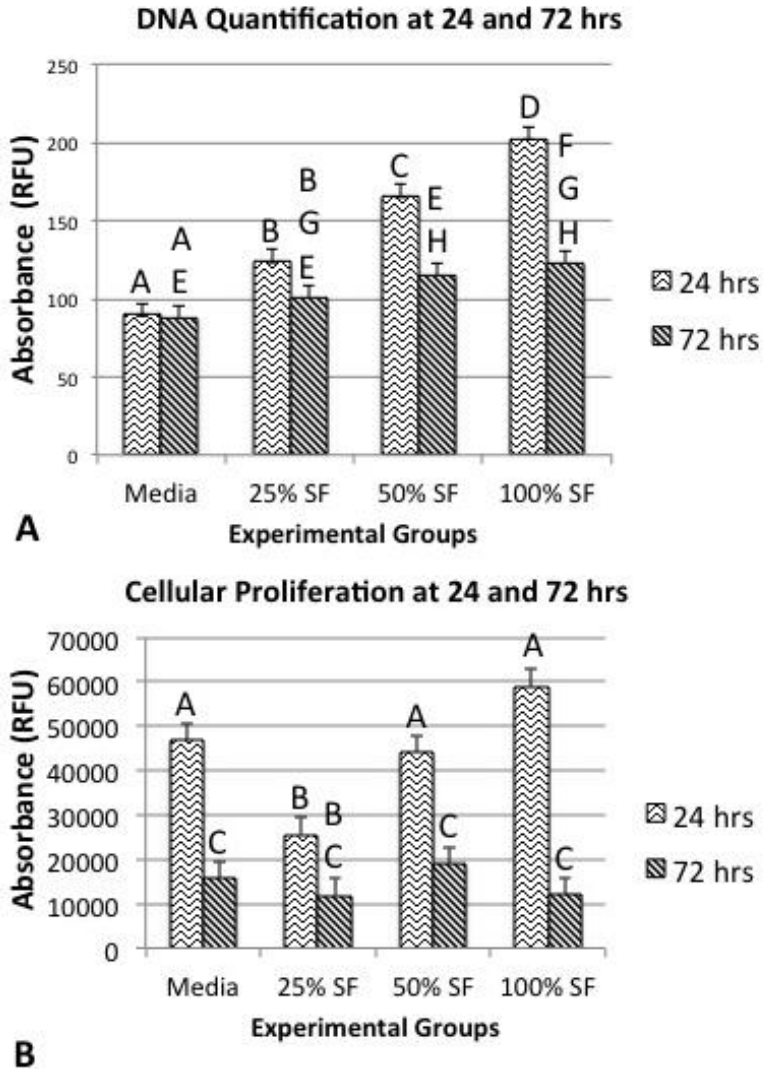

Figure 3.2. Cellular proliferation of BMSCs in allogeneic synovial fluid. (A) Mean absorbance of Hoescht 33342 (±SEM) and (B) mean absorbance of EDU (5-ethylnyl-2-deoxyuridine) (±SEM) for eight equine BMSC lines cultured for 24 and 72 hours in control medium, medium supplemented with 25% synovial fluid, medium supplemented with 50% synovial fluid, or 100% synovial fluid. Means with different letters are significantly different (Tukey-Kramer, $p < 0.05$).

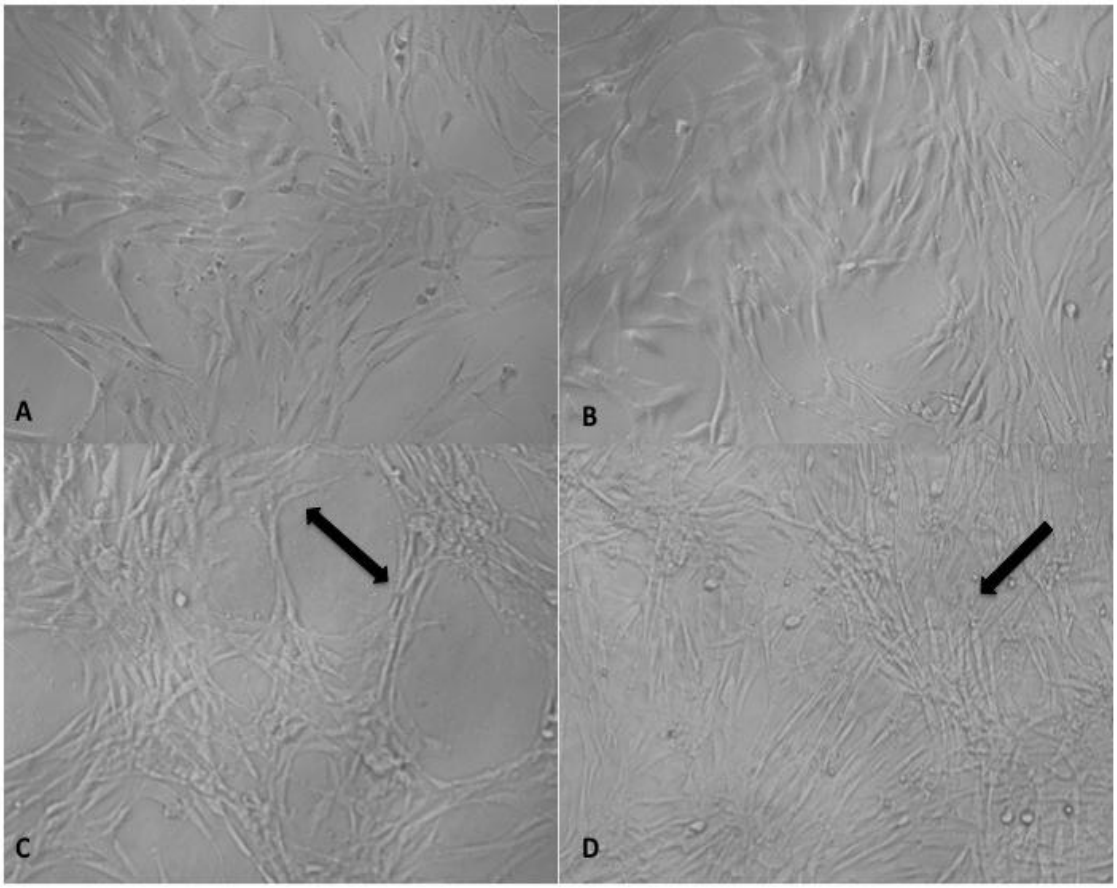

Figure 3.3. Cellular morphology of one BMSC line cultured for 72 hours in allogeneic synovial fluid. Cellular morphology of one BMSC line cultured for 72 hours in (A) medium, (B) 25% synovial fluid, (C) 50% synovial fluid and (D) 100% synovial fluid. Note how cellular number observed per high power field increases with increasing concentration of synovial fluid. With synovial fluid supplementation >25% cells begin to aggregate toward one another forming web-like networks (double headed arrow). These networks begin for form cellular aggregates with cellular stacking (single headed arrow).

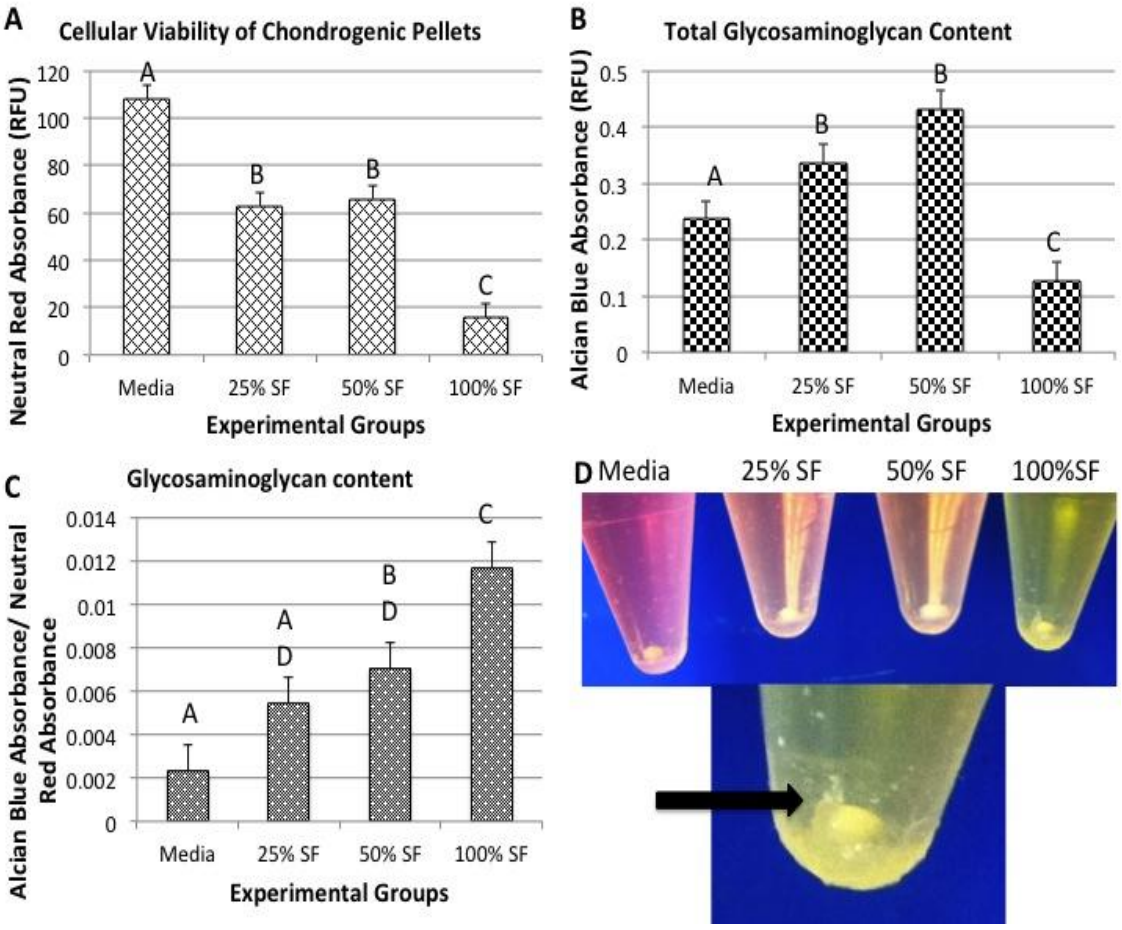

Figure 3.4. Chondrogenesis of BMSCs in allogeneic synovial fluid. (A) Mean neutral red absorbance (±SEM) for assessment of cellular viability, (B) mean absorbance of alcian blue (±SEM) for assessment of total GAG content, and (C) mean alcian blue/ neutral red absorbance (±SEM) for assessment of GAG production per viable BMSC within micromass pellets formed from eight BMSC cell lines and cultured in medium, 25% synovial fluid, 50% synovial fluid, and 100% synovial fluid for 28 days. Means with different letters are significantly different (Tukey-Kramer, $p < 0.05$). (D) Chondrogenic pellets from one BMSC line cultured for 28 days in medium, 25% synovial fluid, 50% synovial fluid, and 100% synovial fluid. Note that the size of the chondrogenic pellets increases with increasing synovial fluid supplementation up to 50%, but that the chondrogenic pellet cultured in 100% synovial fluid is smaller than the other chondrogenic pellets. The lower picture is the chondrogenic pellet in 100% synovial fluid magnified to highlight the gelatin-like matrix that was observed surrounding all micromass pellets cultured in 100% synovial fluid (arrow).

CHAPTER 4

EFFECT OF AUTOLOGOUS AND ALLOGENEIC PLATELET RICH PLASMA ON VIABILITY, PROLIFERATION, AND CHONDROGENESIS OF EQUINE BONE MARROW DERIVED MESENCHYMAL STEM CELLS *IN VITRO*[1]

[1] Boone, L., Mumaw, J., Thoresen, M., Peroni, J.
To be submitted to the *American Journal of Veterinary Research*

ABSTRACT

Objective: Regenerative therapeutics in horses are becoming more commonplace, and optimizing treatments allows for further enhancement of the healing process. Platelet rich plasma (PRP), provides both a provisional biological scaffold and enhances growth factor concentration at the site of tissue injury. PRP is often used in combination with mesenchymal stem cells (MSCs) to enhance the biological repair at the site of injury. For these reasons, PRP is used as a cellular suspension for delivery of mesenchymal stem cells (MSCs) for musculoskeletal injury. Depending on the timing of the injury, and time for MSC expansion, allogeneic instead of autologous MSC administration may be pursued. Therefore, interactions of cellular suspensions in allogeneic PRP compared to autologous PRP on MSC viability, proliferation, and chondrogenesis were evaluated.

Animals: Adult horses (n=6).

Procedure: BMSCs were obtained, culture expanded, and characterized through tri-lineage differentiation. BMSCs were cultured for 48-72 hours in Dulbecco's modified eagle's medium (DMEM) supplemented with either 25% or 50% (v/v) autologous or allogeneic PRP. Cellular viability was measured at 48 and 72 hours. Cellular proliferation (and DNA quantification) was assessed at 48 hours. Chondrogenesis was quantified after 28 days in culture using Alcian Blue staining for glycosaminoglycan production.

Results: Autologous and allogeneic PRP supplementation of the culture medium caused a dose dependent decrease in BMSC viability after 24 to 72 hours of culture. Discrepancies in BMSC proliferation were observed between proliferation assays. DNA quantification increased in a dose dependent manner after 24 to 72 hours of culture in both autologous and allogeneic PRP. However, cellular proliferation as measured by EdU

DNA incorporation decreased in a dose dependent manner after 48 hours of culture in autologous and allogeneic PRP. BMSC chondrogenesis was significantly enhanced with exposure to PRP compared to medium, but no differences were seen in the capacity for chondrogenesis between the PRP treatment groups.

Conclusions and clinical relevance: BMSCs cultured in 25% and 50% autologous or allogeneic PRP showed similar responses in cellular viability, proliferation, and chondrogenesis. BMSCs can be suspended in autologous or allogeneic PRP for regenerative therapies. However, exposure to PRP will significantly alter BMSC viability and proliferation, but will enhance BMSC chondrogenesis.

INTRODUCTION

Lameness due to osteoarthritis or tendon and ligament injuries are the leading causes of reduced or lost performance in horses and causing significant economic hardship to the equine industry.[1-4] Currently, the mainstay for therapy of tendon and ligament injuries is directed toward modifying the symptoms of the injury associated with inflammation through local or systemic non-steroidal anti-inflammatory medications and rest followed by an extensive controlled exercise program. For osteoarthritis, therapy focuses on temporary reduction of inflammation with corticosteroids or non-steroidal anti-inflammatory agents and additional viscosupplementation with hyaluronic acid or polysulfated glycosaminoglycans in an attempt to restore articular homeostasis. Although these measures successfully reduce injury related symptoms, they fall short in re-establishing the biomechanical properties of the tissue prior to the injury. This results in high rates of tendon or ligament re-injury and progression of cartilage damage within the joint contributing to reduced or lost performance of the patient. Implementing regenerative medicine therapeutics has the potential to improve the rate and quality of tissue healing, reducing the rate of re-injury.

In equine medicine, cellular enhancement of tendon and ligament or articular cartilage defects is growing in popularity. Cellular enhancement of musculoskeletal injury repair has been achieved with terminally differentiated cells, but problems associated with culture expansion of these “older” more specialized cells coupled with induced morbidity to the patient at the site of tissue harvest makes this method undersirable; Mesenchymal stem cells, however are easily obtainable, expandable, and manipulated cellular source for musculoskeletal repair. The stem cells used in

regenerative therapies are typically obtained from fetal or adult mesenchymal tissue either with or without culture expansion. Both cellular concentrates and culture-expanded stem cells have enhanced tendinous and ligamentous repair as measured by increased cellular integration[6] and tissue matrix production,[7-11] leading to biomechanically superior repair or return to function with reduced re-injury rates.[12,13] Although MSC use in equine injury still requires optimization, it is likely that early treatment may be more beneficial; however, this is often offset by the time required to culture expand stem cells.

In most therapeutic instances, autologous adipose derived MSCs or bone marrow derived MSCs (BMSCs) are used, thus avoiding potential complications associated with introducing cells obtained from a different animal. Despite this, the use of allogeneic cells to treat lesions offers attractive possibilities.[14] Notably, allogeneic cells eliminate the time delay associated with harvesting and culturing autologous cells, a process which can take several weeks delaying prompt therapeutic intervention. Allogeneic cells may be characterized prior to clinical use to ensure that these cells possess appropriate and desirable phenotypic and genotypic characteristics. Furthermore, allogeneic MSCs have not been associated with a significant immune response in part due to differential expression of major histocompatibility complexes (MHC) on the cell surface.[15-18] Encouraging results were obtained in a recent study in which intra-articular administration of placentally derived MSCs in healthy equine joints resulted in no significant differences in the inflammatory response of the joint between allogeneic or autologous cells.[14] Allogeneic use of BMSCs in humans has shown promising results in clinical trials in which allogeneic treatment resulted in subjects being 3.5 times less likely to develop degenerative changes in naturally occurring osteoarthritis.[21] Allogeneic MSCs

have the potential to offer immediate therapeutics to injured horses, and the use of this therapy in combination with biological therapeutics may increase the benefit from these treatments. The increased regenerative capacity of tissues following administration of MSCs makes these therapeutics desirable and has the potential to be further enhanced through the use of growth factor supplementation.

Platelet Rich Plasma (PRP) provides a unique alternative to specific growth factor supplementation because it provides a concentrated mileu of growth factors typically utilized by one's own body during repair as well as providing a scaffold to promote enhanced cellular migration. It can be used to suspend the MSCs for injection and can further enhance regional tissue regeneration. PRP is easily obtained after centrifugation or filtration of venous blood with a three to six fold increase in the baseline platelet concentration resulting in an average platelet concentration of 500,000-1,000,000 cells/µl. The predominant growth factors and associated proteins found in PRP include platelet derived growth factor (PDGF), transforming growth factor- beta (TGF-β), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), vascular endothelial growth factor (EGF), fibrin, fibronectin, and vitronectin.[20] All these growth factors have beneficial properties in enhancing healing and have the potential to replace xenogenic substances currently used for MSC expansion, further increasing the safety of culture expanded MSCs.[21]Effects of autologous or allogeneic platelet products on cellular proliferation, migration, and differentiation have been previously tested; however, few studies directly compare the effects of either autologous or allogeneic PRP on cellular activity. Recently, Creeper and Ivanovski (2012) demonstrated no statistical difference between autologous and allogeneic PRP supplemented from 10-100% (v/v) on cellular

migration and proliferation of human gingival fibroblasts.[20] Given the potential for therapeutic enhancement of MSCs and the sparse literature investigating exposure of MSCs to higher, more clinically relevant concentrations of PRP, assessment of the cellular response to high concentrations of PRP is necessary to support clinical use in musculoskeletal repair.

In our hospital, PRP is frequently used in combination with either autologous or allogeneic BMSCs for tendon or ligament injuries of the distal limb as well as intra-articular therapies. The purpose of this study was to evaluate the effects of autologous or allogeneic PRP on BMSC viability, proliferation, and chondrogenesis. There are numerous studies in the literature evaluating the effect of platelet products on the biological activity of various cells; however, few studies in the literature directly compare the source (autologous vs. allogeneic) of the platelet product on the biological activity of MSCs. In this current study, no differences in viability, proliferation, or chondrogenesis were observed between equine BMSCs cultured with 25% autologous or allogeneic PRP supplementation or 50% autologous or allogeneic PRP supplementation. These finding are consistent with other cell types cultured in PRP.[22]

MATERIALS AND METHODS:

Animals

Bone marrow and blood was aseptically harvested on separate dates from 6 healthy adult horses aged 8-14 years old. All procedures involving collection of samples for this study were done with the approval of the Animal Care and Use Committee at The University of Georgia.

Isolation of BMSCs

Bone marrow was aseptically harvested from the sternum of six healthy adult horses using an 8 gauge x 4 in. Jamshidi bone marrow biopsy needle.[a] Approximately 20 cc of bone marrow were drawn from either the 4^{th}, 5^{th}, or 6^{th} sternebrae into 2-35cc syringes containing 2500 units of heparan sulfate[b] in each syringe. BMSCs were obtained by plating the cells using a plate adherency method. Cells were cultured in defined culture medium consisting of Dulbecco's Modified Eagles Medium with 4.5 g/L glucose and sodium pyruvate without L-Glutamine[c] supplemented with 10% fetal bovine serum[d], 4 mM L-Glutamine[e] and 50 ug/ml Streptomycin, 50 U/ml Penicillin[f] under standard cell culture conditions (37°C and 5% CO_2). Initial BMSC colonies were manually dissociated to allow re-distribution prior to trypsinization. Once the cells reached 70-80% confluency, they were harvested with 0.05% trypsin-EDTA[g],reseeded at 5000 cells/cm^2 for further expansion.

BMSC characterization

For osteogenic and adipogenic differentiation, 36,000 MSCs per cm^2 from each of the 6 lines were plated in 35mm plates. The cells were differentiated using Hyclone AdvanceSTEM osteogenic differentiation medium[h] with 50 ug/ml Streptomycin, 50 U/ml Penicillin, and Hyclone AdvanceSTEM adipogenic differentiation medium[i] with 50 ug/ml Streptomycin, 50 U/ml Penicillin respectively. The medium was changed every third day for 28 days. For osteogenic differentiation, cells were stained with Von Kossa[j] and for adipogenic differentiation cells were stained with 7% Oil Red O[k].
Chondrogenesis was performed in 15 ml polypropylene tubes.1 million cells were

pelleted at 400g and AdvanceSTEM chondrogenic differentiation medium[l] was changed every third day for 28 days. Micromasses were stained with 1% alcian blue[m].

Platelet rich plasma processing

Blood was aseptically harvested from the 6 adult horses for which BMSCs lines were established. Approximately 1 liter of blood was aseptically harvested from the left jugular vein in 2 blood collection bags containing 63 mls of citrate phosphate dextrose adenine solution (CPDA-1).[n] The blood was centrifuged at 1800 rpm at 4°C for 10 minutes without automated deceleration. Plasma above the buffy coat was harvested and then centrifuged at 2400 rpm at 4°C for 5 minutes with automated deceleration. The supernatant was decanted and then centrifuged at 3300 rpm at 4°C for 5 minutes with automated deceleration. The platelet poor supernatant was decanted and discarded. The cellular pellets from the second and third centrifugation cycles were re-suspended in the remaining plasma after decantation and combined to produce PRP. A 200µl sample was obtained from the PRP to perform an automated platelet count. This method of PRP processing yields approximately 10-15 mls of PRP from 1 liter of blood. To allow for analysis, BMSCs were kept separate from the PRP through the use of transwell inserts with a 0.04 µM pore size. BMSC groups were organized so that each BMSC line was exposed to autologous PRP and two allogeneic PRP lines. Treatment groups were as follows: defined culture medium, defined culture medium with 25% autologous PRP, defined culture medium with 50% autologous PRP, defined culture medium with 25% allogeneic PRP, and defined culture medium with 50% allogeneic PRP. The PRP was activated by the addition of 10% $CaCl_2$ to each well.

Proliferation assay

BMSCs were plated at 3500 cells/cm^2. Proliferation of BMSCs in the presence of autologous or allogeneic PRP was assessed using the Clik-iT® EdU Microplate Assay[o]. 5-ethynyl-2'deoxyuridine (Edu) was added at a concentration of 10µM to the medium of all treatment groups and cells were cultured for 48 hours in the presence of Edu. Six hours prior to initiating the Clik-iT® EdU microplate assay the medium was removed from the plates and 150µl defined medium containing type IV collagenase (1 mg/mL)[p] and 50µl of 0.05% trypsin-EDTA were added to each well of the 96 well plate to allow dissolution of cells from the protein matrix that eluded from the transwell. The plates were then centrifuged and the assay was performed according to package insert instructions with the addition of centrifugation between steps. The plate was analyzed using a microplate reader set to read at 495 with correction at 519.

Viability assay

BMSCs were plated at 3500 cells/cm^2. Viability of BMSCs was assessed using the LIVE/DEAD® viability/cytotoxicity kit for mammalian cells[q] after 24 and 72 hour incubation in autologous or allogeneic PRP. DNA quantification was performed with bis-Benzimide H33342 tri-hydrochloride[r]. Six hours prior to performing the assay cells were separated from the protein matrix as described above. The cells were then centrifuged and the assay was performed according to package insert instructions. The plate was then assessed for absorbance at 528 with correction set at 617 nm on a microplate reader.

Chondrogenic assay

BMSCs were seeded at density of 200,000 BMSCs/well in 96 well "V" bottom plates[s] for chondrogenesis. The plates were centrifuged at 400 g for 10 min to form

micromass pellets. 12 hours later the micromasses were transferred to transwell plates for an additional 24 hours prior to exposure to PRP and chondrogneic differentiation medium supplemented with 50 ug/ml Streptomycin and 50 U/ml Penicillin. Treatment groups were as follows: chondrogenic medium, chondrogenic medium supplemented with 25% activated autologous PRP, chondrogenic medium containing 50% activated autologous PRP, chondrogenic medium containing 25% activated allogeneic PRP, and chondrogenic medium containing 50% activated allogeneic PRP 50% medium changes were performed every other day. After 28 days in culture the transwell insert was removed and the micromass pellet was harvested from the protein matrix encasing the micromass pellet on the bottom of the insert and transferred to the correspondent well of the plate. The micromass pellets were rinsed 3 times with PBS and then fixed with 100% methanol for 10 minutes at -20°C. A 0.2% Alcian Blue 8GX in 0.1 M HCl solution was applied to the micromass pellets for 2 hours at room temperature. The pellets were then washed three times with PBS and alcian blue stain that had been taken up by the pellets was extracted by exposing the pellets to 6 M guanidine/HCl[t] overnight at room temperature. The optical density of the extracted alcian blue was measured at 650 nm on a microplate reader. The quantity of viable cells within the pellets was measured by staining the pellets in parallel with Neutral Red[u]. The detection of neutral red content was measured at an optical density of 550 nm. Using this method cell viability and chondrogenesis were simultaneously quantified. The total GAG/cell content was measured by dividing the fluorescence alcian blue by the fluorescence of neutral red for each micromass pellet.

Statistical analysis

All analyses were performed using SAS V 9.2[v]. Control medium values were averaged over the three replicates for proliferation and chondrogenesis data to obtain a single control value for each horse. Control values were subtracted from viability, proliferation and chondrogenesis data prior to analysis to obtain change from control values for analysis.

A repeated measures model that recognized multiple observations as belonging to the same horse was used to test for differences in viability, proliferation and chondrogenesis change from control values between groups and PRP lines. The full model included fixed factors for group, PRP line and an interaction effect of group and PRP line and a random factor of horse. If a significant interaction ($p<0.10$) of PRP line and group was found then paired group differences were examined separately for each PRP line. An unstructured covariance structure was used in all repeated measures models. Student's t-tests were performed to test the hypothesis that change from control values were significantly different than 0, which indicated a significant difference from control. All hypothesis tests were 2-sided and the significance level was $\alpha = 0.05$. Tukey's test was used to adjust for multiple paired comparisons.

RESULTS

BMSC characterization

Equine BMSCs demonstrated tri-lineage differentiation potential. Figure 4.1 is a single representation of one of the six BMSC lines. All cell lines were capable of osteogenic, adipogenic and chondrogneic differentiation.

Platelet counts

Concentrations of platelets from each individual horse are displayed in Table 4.1 in cells/µl for each PRP line. The mean platelet concentration was 2,668 ± 209 x 10^3 cells/ µl with a mean fold increase from baseline platelet concentration of 14.3 ± 1.1.

Cellular viability

Statistical differences between the groups are shown in Table 4.2a and b as well as figure 4.2a. BMSC viability was significantly enhanced after 24 hours in culture with defined culture medium supplemented with 25% allogeneic PRP (p=0.005), but this slight enhancement in viability was not maintained after 72 hours in culture (p=0.08). BMSC viability significantly decreased with increasing concentration of PRP after 24 and 72 hours in culture regardless of whether the PRP was autologous (24 hours p=0.004; 72 hours p= 0.005) or allogeneic (24 hours p=2.5x10^{-4}; 72 hours p=0.002).

A significant interaction ($p<0.10$) was detected between experimental groups and PRP lines for the data obtained after 24 hours of culture meaning that the effects of the different treatment groups on BMSC viability were not consistent among PRP lines. PRP lines were therefore evaluated individually and compared between groups (Table 4.2a and b; Figure 4.2b and c). PRP 4 caused a significant reduction in BMSC viability with increasing concentration (50% vs. 25%) after 24 hours (autologous p=0.001; allogeneic vs. autologous p=4.3x10^{-4} or vs. allogeneic p=0.003), but this reduction in viability was not statistically sustained after 72 hours in culture. Allogeneic PRP 5 increased BMSC viability after 24 hours in culture at a lower concentration (25%) of supplementation compared to both 50% autologous (p=6.5x10^{-4}) and 50% allogeneic PRP 5 (p=5.5x10^{-5}), but this increase in BMSC viability was not sustained after 72 hours in culture.

Additionally, 25% allogeneic PRP 5 significantly increased BMSC viability compared to 25% autologous PRP 5 after 24 hours (p=0.005). It is interesting to note that this PRP 5 line had the lowest platelet concentration yet produced the only significant increase in BMSC viability when supplemented at the lowest concentration (25%) compared to higher supplementation of the same PRP line. When BMSC viability was compared for each individual PRP lines regardless of treatment, PRP 2 was the only PRP line that caused an overall increase in BMSC viability compared to medium, though this increase was not statistically significant (p=0.169) (Data not shown). In contrast to PRP 5, PRP 2 had one of the highest platelet concentrations of the PRP lines used.

Cellular proliferation

DNA quantification

Table 4.3 reports the mean absorbance of BMSCs stained with Hoescht after 24 (a) and 72 hours (b) in culture. There was a significant increase in BMSC DNA quantification with both 25% and 50% autologous or allogeneic PRP supplementation after 24 and 72 hours in culture compared to medium. DNA quantification increased with increasing medium supplementation of PRP compared to medium. DNA quantification also increased with increasing supplementation of autologous or allogeneic PRP (50%) compared to supplementation with 25% autologous or allogeneic PRP after 24 and 72 hours (Figure 4.3a). A significant interaction between the experimental groups and the PRP lines was detected and therefore individual data was evaluated for each PRP cell line.

When evaluating the individual PRP lines, PRP lines 3-6 caused a significant increase in BMSC DNA quantification compared to medium regardless of the treatment

group after 24 hrs in culture and all PRP lines produced a significant increase in BMSC DNA quantification after 72 hours (Data not shown). Higher supplementation concentrations of autologous or allogeneic PRP 3 significantly increased DNA quantity of BMSCs 24 hours after culture when compared to 25% autologous or allogeneic PRP supplementation. After 72 hours in culture this increase in BMSC viability was only sustained with supplementation of 50% allogeneic PRP compared to 25% autologous or allogeneic PRP. BMSC DNA quantity was also increased in PRP 1 and 5, 24 hours after culture in 50% allogeneic PRP compared to 25% autologous (PRP 1) or allogeneic (PRP 1 and 5), but this increase was not maintained 72 hours after culture. Supplementation with 50% autologous PRP 2 and 6 significantly increased BMSC DNA quantification compared to supplementation with 25% autologous or allogeneic PRP. Therefore, DNA quantification increased with increasing PRP supplementation regardless of the nature of the PRP (autologous or allogeneic) (Figure 4.3 b and c).

EdU DNA incorporation

There was a significant decrease in cellular proliferation of BMSCs cultured in all PRP conditions compared to BMSCs cultured in medium (Table 4.4 and figure 4.5). No significant differences between PRP conditions were reported, but there was a trend for increasing PRP concentration to decrease the quantification of EDU.

Chondrogenesis

There were no differences in the quantity of viable cells contained within chondrogenic pellets cultured in control chondrogenic medium and all PRP conditions as determined by Neutral Red staining (data not shown). Supplementation of medium with 25% allogeneic PRP resulted in significant reduction of BMSC viability compared to

supplementation with 50% autologous (p=0.03) or allogeneic (p=0.0001) PRP. These results indicate that PRP supplementation enhanced viability of BMSCs in micromass in a dose dependent manner. All PRP conditions enhanced GAG production compared to control medium and GAG production was higher when cultured in higher concentrations of PRP (50%). When GAG production was evaluated per viable BMSC, all PRP conditions produced significantly higher GAG per BMSC compared to medium, but no differences were detected between treatment groups (Figure 4.5).

DISCUSSION

PRP is an easily obtainable, autologous blood product that serves as an important biologic scaffold and depot of growth factors shown to variably sustain or enhance the cellular proliferation, migration, and biological activity of progenitor or stem cells from various tissue sources and species. PRP co-treatment with stem cells, can potentially enhance endogenous musculoskeletal repair and both of these treatments are rapidly gaining popularity for of tendonopathies and desmopathies as well as synovial-related pathologies of the equine patient. Applications of allogeneic stem cells with autologous PRP have been demonstrated with success in a small cohort of naturally occurring tendonitis with 87.5% being capable of returning to an undisclosed level of work.[24] Despite this success, understanding the beneficial and deleterious effects of each regenerative therapeutic component in these treatments is warranted to ensure that the optimal therapeutic is being used. Additionally, the source (autologous or allogeneic) of each of these components is an important interaction that should be considered prior to application. The purpose of this study was to investigate the *in vitro* effect of autologous or allogeneic PRP on BMSCs for clinical translation.

In the current study the platelet counts (average of 14.3 fold increase from a baseline platelet count of 150,000 cells/ul) used are much higher than what has been investigated in the literature (2-10 fold increase from baseline). The ideal concentration of platelets in PRP for therapy is unknown, but therapy if often delivered in the range of a 3-8 fold increase in platelet concentration over baseline values. Growth factor concentration has been correlated with platelet number and growth factor analysis of the PRP lines used in this study may have enhanced the information gained on the individual responses of the BMSC lines to PRP lines.[33,34] On the other hand, standardization of the concentration of platelets could have accounted for the variability of the results, but the authors wanted to reflect the clinical scenario of PRP and BMSC use in which the clinician is typically unaware of the platelet count or growth factor concentration.

There are numerous studies in the literature evaluating the effect of platelet products on the biological activity of various cells; however, few studies in the literature directly compare the source (autologous vs. allogeneic) of the platelet product on the biological activity of MSCs. In this current study, no differences in viability, proliferation, or chondrogenesis were observed between equine BMSCs cultured with of 25% autologous or allogeneic PRP supplementation or 50% autologous or allogeneic PRP supplementation. These finding are consistent with other cell types cultured in PRP.[22]

Few studies addresses cellular viability after the application of PRP or PL *in vitro*.[27] In the current study, at 24 hours there was an obvious shift in cellular viability with 25% PRP having comparable (autologous) or slightly improved (allogeneic) cellular viability compared to control medium where as medium supplementation with 50% PRP

caused a significant reduction in cellular viability at 24 hours. This time period reflects the initial adjustment period of the cells to the PRP and indicates that potentially the viscosity of the PRP or the influx of soluble factors affected the initial viability. This indicates that high concentrations of PRP if left in direct contact with MSCs has the potential to reduce viability and cellular potential for tissue repair.

Chondrogenesis was improved through the addition of PRP in comparison to chondrogeneic medium, however a significant dose-dependent effect was not observed. Chondrogenic capacity of MSCs has been shown to be maintained or enhanced with 5-10% platelet product supplementation of culture medium,[27,29-32] but the effect of higher supplementation is rarely evaluated. In the current study, both 25% and 50% autologous or allogeneic PRP was shown to enhance GAG production of BMSCs over medium. These higher supplementation volumes would reflect clinical implantation of BMSCs in an articular cartilage defect and indicates that there is potential that PRP and BMSCs in combination may be more beneficial than either therapy alone.

The contamination of stem cell culture with xenogenic factors such as fetal bovine serum or fetal calf serum is a potential cause for inflammatory reactions in horse. With 5-10% activated PRP or platelet lysate being comparable to or superior to FBS in supporting cellular proliferation in human cells, [28] equine PRP could be used to increase the safety of equine therapeutics. In the current study, due to significant interactions between experimental groups, a significant difference among 25% and 50% autologous or allogeneic PRP could not be concluded for all PRP lines. There does not appear to be a generalized dose dependent effect of PRP on equine BMSC proliferation. There was not one "superior" PRP line that produced a consistent, significant response. PRP 3 did cause

a significant effect for autologous PRP after 24 hours and allogeneic PRP after 24 and 72 hours. The differences in results are likely due to the variability among individual BMSC lines and PRP lines. Standardization of platelet numbers across PRP lines may have helped to account for this difference, but the authors wanted to reflect the clinical scenario of PRP and BMSC use in which the clinician is typically unaware of the platelet count or growth factor concentration. Similar or enhanced cellular proliferation with higher percentages of PRP supplementation (>20%) compared to medium supplemented with 10-20% FBS or FCS has sparsely been reported and according to one report in human cells, may resulted in reduction in cellular proliferation.[28] Equine studies have shown enhanced cellular proliferation of equine MSCs with >10% PRP or PL supplementation.[23,29] Equine MSCs in comparison to other species derived MSCs could have enhanced cellular proliferation with increasing PRP supplementation, but further studies are warranted.

Our findings indicate that there is no differential effect in viability, proliferation, or chondrogenesis of BMSCs treated with either 25% or 50% autologous or allogeneic PRP and higher supplementation of PRP (25-50%) promotes BMSC proliferation and chondrogenic differentiation, but in the acute exposure period (24 hours) affects cellular viability. Suspension of equine BMSCs in 25-50% autologous or allogeneic PRP for injection may cause an acute decrease in cellular viability, but promote cellular proliferation and chondrogenesis. Extrapolating this data to a clinical setting where autologous or allogeneic BMSCs are mixed or re-suspended in nearly 50-100% autologous PRP that is subsequently activated immediately prior to, during, or after injection calls into question maintenance of viable cells within the wound after injection.

This *in vitro* cellular culture methodology falls short of the *in vivo* cellular interaction of BMSCs and PRP with the wound environment, but this information obtained within this study is inherently sparse in the equine literature. This study reinforces the application of allogeneic BMSCs in autologous PRP. While studies have shown increased pain and edema[23] over autologous concentrate[24], the complication rate is well within the ranges seen in other studies using autologous PRP[25,26] and all complications resolved within 48 hours[23] Further studies are warranted to evaluate the effects of autologous or allogeneic BMSCs and PRP in musculoskeletal (tendonitis/desmopathy or articular cartilage defect) wound environment. Further investigation into cellular viability after injection with PRP *in vivo* is warranted to elucidate the contribution of BMSCs and PRP to the repair tissue.

FOOTNOTES

[a] 8 gauge Jamshidi bone marrow biopsy needle, Jorgensen Laboratories, Inc., Loveland, CO

[b] Heparan Sulfate (1000 units/ml) Hospira, Inc. Lake Forest, IL

[c] DMEM, Cellgro®, Mediatech,Inc. Manassas, VA

[d] Fetal Bovine Serum, Atlanta Biologicals®, Lawrenceville, GA

[e] L-Glutamine, Gibco®, Invitrogen™, Auckland, AZ

[f] Penicillin/Streptomycin, Gibco®, Invitrogen™, Auckland, AZ

[g] 0.05% Trypsin-EDTA, Gibco®, Invitrogen™, Auckland, AZ

[h] Hyclone AdvanceSTEM Osteogenic Differentiation Medium, Thermo Scientific, Hampton, Newhampshire

[i] Hyclone Advance STEM Adipogenic Differentiation Medium,Thermo Scientific, Hampton, New Hampshire

[j] BD Biosciences, San Jose, CA

[k] AdvanceSTEM Chondrogenic Differentiation Medium, Thermo Scientific, Hampton, New Hampshire

[l] Alcian Blue 8 GX, Sigma-Aldrich®, St. Louis, MO

[m] Teruflex® Blood Bag System, Terumo Corporation, Tokyo Japan

[n] Clik-iT® EdU Microplate Assay, Invitrogen Auckland, AZ

[o] LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells, Gibco®, Invitrogen™ Auckland, AZ

[p] Hoescht, Invitrogen™, Auckland, AZ

[q] NUNC™, Roskilde, Denmark

[r] SAS V 9.2, Cary, NC

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Figure 4.1 BMSC tri-lineage differentiation. Tri-lineage differentiation of one representative BMSC line. BMSCs stained positive for Oil Red O (A), Von Kossa (B), and Alcian blue (C).

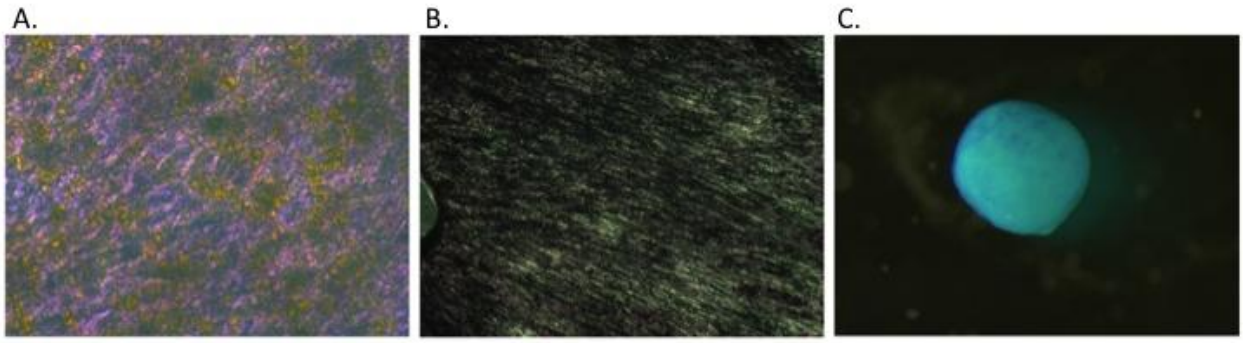

Table 4.1 Platelet counts. Platelet counts (cells/µl) for each individual PRP line.

PRP Line	Platelet Count (x 10^3 platelets/µl)	Fold increase over baseline platelet count (150,000 platelets/µl)
1	3,185	17.1
2	3,150	16.9
3	2,235	12.0
4	3,015	16.2
5	2,000	10.8
6	2,420,	13.0

Table 4.2 BMSC viability after 24 (A) and 72 (B) hours of culture. First column of the table represents mean BMSC viability (%) after 24 (A) and 72 (B) hours of culture in defined culture medium, 25% autologous PRP, 50% autologous PRP, 25% allogeneic PRP, and 50% allogeneic PRP. The remaining table columns represent the mean ± SEM differences between treatment groups. P values are displayed below the mean differences between groups. Significant differences are ($p < 0.05$) bordered in bold and the p value is displayed in bold font.

A	**PRP**	**% Viability**	**25% Autologous PRP**	**50% Autologous PRP**	**25% Allogeneic PRP**	**50% Allogeneic PRP**
Medium		96.8	0.008±0.005 (0.15)	-0.020±0.005 **(0.004)**	0.016±0.004 **(0.005)**	-0.026±0.004 **($2.5x10^{-4}$)**
25% Autologous PRP	1	98.9		-0.007±0.014 (1)	-0.005±0.013 (1)	-0.002±0.013 (1)
	2	100		0.001±0.014 (1)	-0.005±0.013 (1)	-0.017±0.013 (1)
	3	99.8		0.018±0.014 (1)	0.004±0.013 (1)	0.010±0.013 (1)
	4	101		0.121±0.014 **(0.001)**	0.059±0.014 (0.09)	0.136±0.013 **($4.3x10^{-4}$)**
	5	96.1		0.032±0.014 (0.74)	-0.100±0.013 **(0.005)**	0.036±0.13 (0.54)
	6	89.8		0.005±0.14 (1)	-0.004±0.013 (1)	0.041±0.13 (0.39)
	Total	97.6		0.028±0.006 **(0.004)**	-0.008±0.005 (0.411)	0.034±0.005 **(0.0005)**
50% Autologous PRP	1	99.7			0.002±0.013 (1)	0.005±0.013 (1)
	2	100.0			-0.007±0.013 (1)	-0.019±0.013 (0.99)
	3	98.0			-0.014±0.013 (1)	-0.007±0.013 (1)
	4	89			-0.062±0.013 (0.07)	0.015±0.013 (1)
	5	92.9			-0.129±0.013 **($6.5x10^{-4}$)**	0.004±0.013 (1)
	6	89.3			-0.009±0.013 (1)	0.036±0.013 (0.54)
	Total	94.8			-0.036±0.005 **(0.0003)**	0.006±0.005 (0.65)
25% Allogeneic	1	99.5				0.003±0.010 (1)
	2	101.2				-0.01±0.009 (1)
	3	99.5				0.006±0.01 (1)

	4	86.2				0.0777±0.01 **(0.003)**
	5	95.9				0.133±0.01 **($5.5x10^{-5}$)**
	6	94				0.045±0.01 (0.08)
	Total	96.0				0.042±0.004 **($2.5x10^{-5}$)**
50% Allogeneic PRP	1	99.8				
	2	100.6				
	3	98.7				
	4	94.4				
	5	93.5				
	6	93				
	Total	96.6				
B	**PRP**	**% Viability**	**25% Autologous PRP**	**50% Autologous PRP**	**25% Allogeneic PRP**	**50% Allogeneic PRP**
Medium		85.31	-0.001±0.23 (0.691)	-0.086±0.023 **(0.005)**	-0.038±0.019 (0.080)	-0.083±0.019 **(0.002)**
25% Autologous PRP	1	94.08		0.124±0.065 (0.908)	0.100±0.062 (0.972)	0.110±0.062 (0.939)
	2	91.57		0.77±0.065 (0.561)	0.026±0.422 (1.00)	0.017±0.062 (1.00)
	3	79.75		-0.000±0.065 (1.00)	0.034±0.062 (1.00)	0.058±0.062 (1.00)
	4	73.61		0.107±0.065 (0.968)	-0.049±0.062 (1.00)	0.023±0.062 (1.00)
	5	77.92		0.024±0.065 (1.00)	0.079±0.062 (0.997)	0.142±0.062 (0.764)
	6	89.24		0.027±0.065 (1.00)	-0.026±0.062 (1.00)	0.091±0.062 (0.988)
	Total	84.36		0.077±0.027 (0.080)	0.0280±0.023 (0.634)	0.073±0.023 (0.052)
50% Autologous PRP	1	81.64			-0.024±0.062 (1.00)	-0.013±0.062 (1.00)
	2	73.82			-0.0151±0.062 (0.690)	-0.016±0.062 (0.618)
	3	79.78			0.038±0.062 (1.00)	0.058±0.062 (1.00)
	4	62.9			-0.156±0.062 (0.657)	-0.084±0.062 (0.994)
	5	75.55			0.056±0.062 (1.00)	0.118±0.062 (0.910)
	6	86.55			-0.053±0.062 (1.00)	0.064±0.06 (1.00)
	Total	76.71			-0.048±0.023 (0.233)	-0.003±0.023 (0.999)

25% Allogeneic PRP	1	85.23		0.011±0.046 (1.00)
	2	89.63		-0.009±0.046 (1.00)
	3	80.83		0.020±0.046 (1.00)
	4	72.74		0.072±0.046 (0.980)
	5	71.91		0.062±0.046 (0.995)
	6	87.29		0.117±0.046 (0.651)
	Total	81.28		0.045±0.019 (0.153)
50% Allogeneic PRP	1	76.00		
	2	82.19		
	3	81.93		
	4	66.48		
	5	86.76		
	6	70.34		
	Total	77.28		

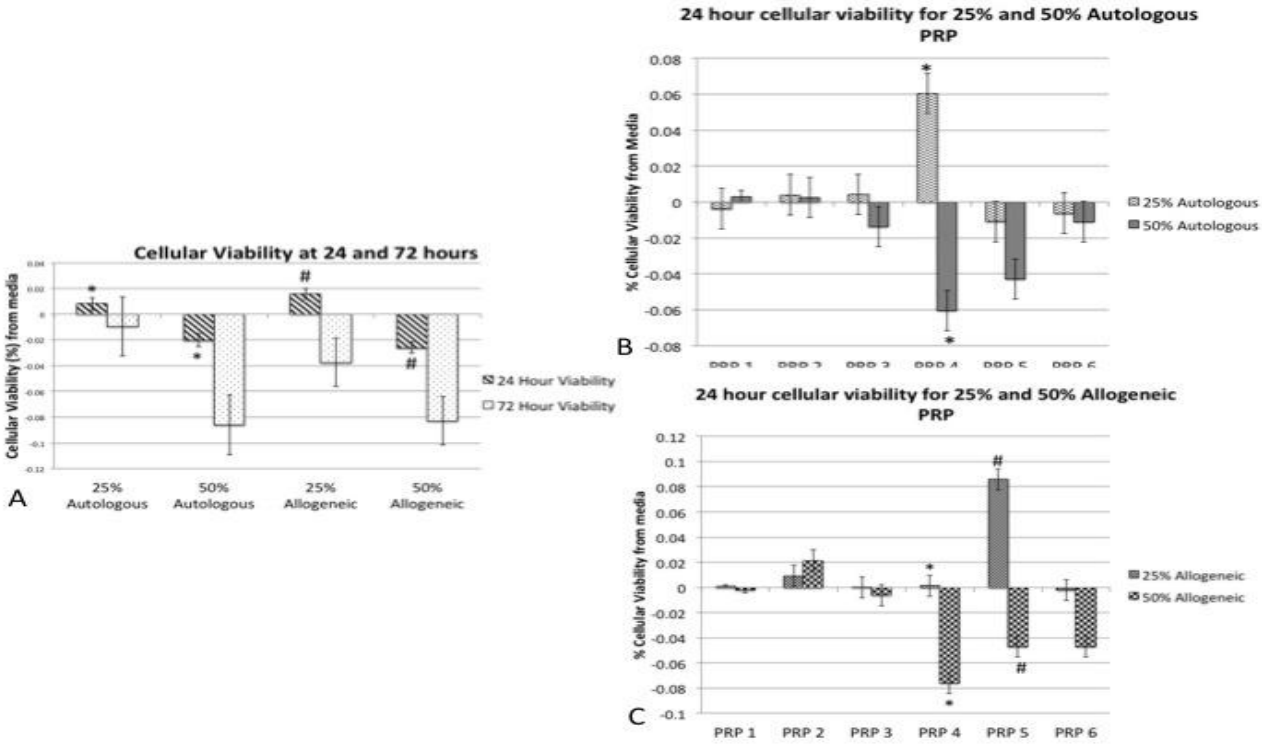

Figure 4.2. BMSC viability after 24 and 72 hours of culture (A) and BMSC viability after exposure to individual PRP lines after 24 (B) and 72 (C) hours of culture. Mean percent of live BMSCs cultured for 24 and 72 hours in 25% autologous PRP, 50% autologous PRP, 25% allogeneic PRP, and 50% allogeneic PRP subtracted from mean percent of live BMSCs cultured in control medium (Baseline) (A). Mean percent of live BMSCs cultured for 24 hours in 25% autologous PRP and 50% autologous PRP separated by exposure to individual PRP lines (B). Mean percent of live BMSCs cultured for 24 hours in 25% allogeneic PRP and 50% allogeneic PRP separated by exposure to individual PRP lines. Significant differences ($p < 0.05$) are depicted among groups by *, #.

Table 4.3 BMSC DNA quantification after 24 (A) and 72 (B) hours of culture. First column of the table represents mean BMSC proliferation (RFU) after 24 (A) and 48 (B) hours of culture in defined culture medium, 25% autologous PRP, 50% autologous PRP, 25% allogeneic PRP, and 50% allogeneic PRP. The remaining table columns represent the mean ± SEM differences between treatment groups. P values are displayed below the mean differences between groups. Significant differences are ($p < 0.05$) bordered in bold and the p value is displayed in bold font.

A.	PRP	Proliferation (RFU)	25% Autologous PRP	50% Autologous PRP	25% Allogeneic PRP	50% Allogeneic PRP
medium		57.17±6.64	10.78±4.01 **(0.009)**	48.05±4.01 **($1.49x10^{-19}$)**	14.08±3.10 **($1.89x10^{-5}$)**	47.11±3.10 **($2.45x10^{-25}$)**
25% Autologous PRP	1	55.33±1.45		-27.00±12.47 (0.875)	-6.41±11.53 (1.00)	-54.74±11.53 **(0.002)**
	2	51.67±0.33		-19.00±12.47 (0.997)	9.84±11.53 (1.00)	-4.33±11.53 (1.00)
	3	57.00±7.0		-73.67±12.47 ($2.06x10^{-5}$)	-12.26±11.53 (1.00)	-53.43±11.53 **(0.003)**
	4	84.33±4.41		-24.33±12.47 (0.950)	-11.91±11.53 (1.00)	-39.58±11.53 (0.123)
	5	75.67±1.76		-34.67±12.47 (0.467)	5.84±11.53 (1.00)	-28.00±11.53 (0.722)
	6	83.67±4.84		-45±12.47 (0.078)	-4.93±11.53 (1.00)	-37.92±11.53 (0.174)
	Total	67.94±8.64		-37.28±5.09 **($1.01x10^{-9}$)**	-3.31±4.41 (0.876)	-36.33±4.41 **($1.72x10^{-11}$)**
50% Autologous PRP	1	82.33±6.23			20.59±11.53 (0.980)	-27.74±11.53 (0.737)
	2	70.67±2.60			28.84±11.53 (0.671)	14.67±11.53 (1.00)
	3	130.67±18.19			61.40±11.53 **(0.0002)**	20.24±11.53 (0.984)
	4	108.67±6.06			12.42±11.53 (1.00)	-15.25±11.53 (1.00)
	5	110.33±8.67			40.50±11.53 (0.100)	6.67±11.53 (1.00)
	6	128.67±7.31			40.08±11.53 (0.110)	7.08±11.53 (1.00)
	Total	105.22±14.03			33.97±4.41 **($1.83x10^{-10}$)**	0.944x4.41 (0.996)
25% Allogeneic	1	47.33±2.06				-48.33±8.82 **(0.0001)**
	2	62.00±4.43				-14.17±8.82 (0.994)

	3	63.50±5.30				-41.17±8.82 **(0.003)**
	4	100±5.76				-27.67±8.82 (0.243)
	5	75.67±0.76				-33.83±8.82 **(0.040)**
	6	83.83±1.54				-33±8.82 (0.054)
	Total	71.25±12.21				-33.03±3.60 **($1.23x10^{-12}$)**
50% Allogeneic PRP	1	95.67±16.71				
	2	76.17±4.36				
	3	104.67±11.19				
	4	122.83±6.62				
	5	109.50±5.30				
	6	116.83±1.78				
	Total	104.28±14.20				
B.	**PRP**	**Proliferation (RFU)**	**25% Autologous PRP**	**50% Autologous PRP**	**25% Allogeneic PRP**	**50% Allogeneic PRP**
medium		46.67±1.67	32.44±3.86 **($1.26x10^{-12}$)**	71.28±3.86 **($1.34x10^{-30}$)**	37.11±3.07 **($9.6x10^{-20}$)**	64.08±3.07 **($3.99x10^{-34}$)**
25% Autologous PRP	1	55.00±1.45		-23±11.45 (0.934)	-3.17±10.74 (1.00)	-6.34±10.74 (1.00)
	2	63.33±4.10		-57±11.45 **(0.0008)**	-5.71±10.74 (1.00)	-23.04±10.74 (0.884)
	3	81.00±5.00		-29.67±11.45 (0.606)	-9.62±10.74 (1.00)	-58.12±10.74 **(0.0002)**
	4	86.00±6.11		-18.00±11.45 (0.996)	-7.21±10.74 (1.00)	-36.21±10.74 (0.142)
	5	91.33±3.38		-41.67±11.45 (0.071)	-3.61±10.74 (1.00)	-32.95±10.74 (0.280)
	6	98.00±2.65		-63.67±11.45 **($8.51x10^{-5}$)**	1.33±10.74 (1.00)	-33.17±10.74 (0.269)
	Total	79.11±6.80		-38.33±4.67 **($1.28x10^{-11}$)**	-4.67±4.05 (0.658)	-31.64±4.05 **($1.11x10^{-10}$)**
50% Autologous PRP	1	78.00±5.51			19.83±10.74 (0.971)	16.66±10.74 (0.966)
	2	120.33±4.26			51.29±10.74 **(0.002)**	33.96±10.74 (0.231)
	3	110.67±12.72			20.05±10.74 (0.968)	-28.46±10.74 (0.563)
	4	104.00±5.29			10.79±10.74 (1.00)	-18.21±10.74 (0.989)

	5	133.00±6.94		38.05±10.74 (0.093)	8.72±10.74 (0.093)
	6	161.67±16.19		64.99±10.74 **($1.13x10^{-5}$)**	30.49±10.74 (0.425)
	Total	117.94 ±11.53		34.17±4.05 **($7.48x10^{-12}$)**	7.19±4.05 (0.292)
25% Allogeneic PRP	1	68.5±7.24			-3.17±8.09 (1.00)
	2	70.00±4.92			-17.33±8.09 (0.885)
	3	79.33±4.25			-48.50±8.09 **($1.45x10^{-5}$)**
	4	102.29±7.51			-29.00±8.09 (0.083)
	5	91.17±3.24			-29.33±8.09 (0.075)
	6	95.33±3.19			-34.5±8.09 (0.075)
	Total	83.78±3.88			-26.97±3.30 **($2.41x10^{-11}$)**
50% Allogeneic PRP	1	71.67±4.67			
	2	87.33±9.35			
	3	127.83±7.18			
	4	127.83±10.31			
	5	120.50±3.21			
	6	129.83±5.51			
	Total	110.75±25.30			

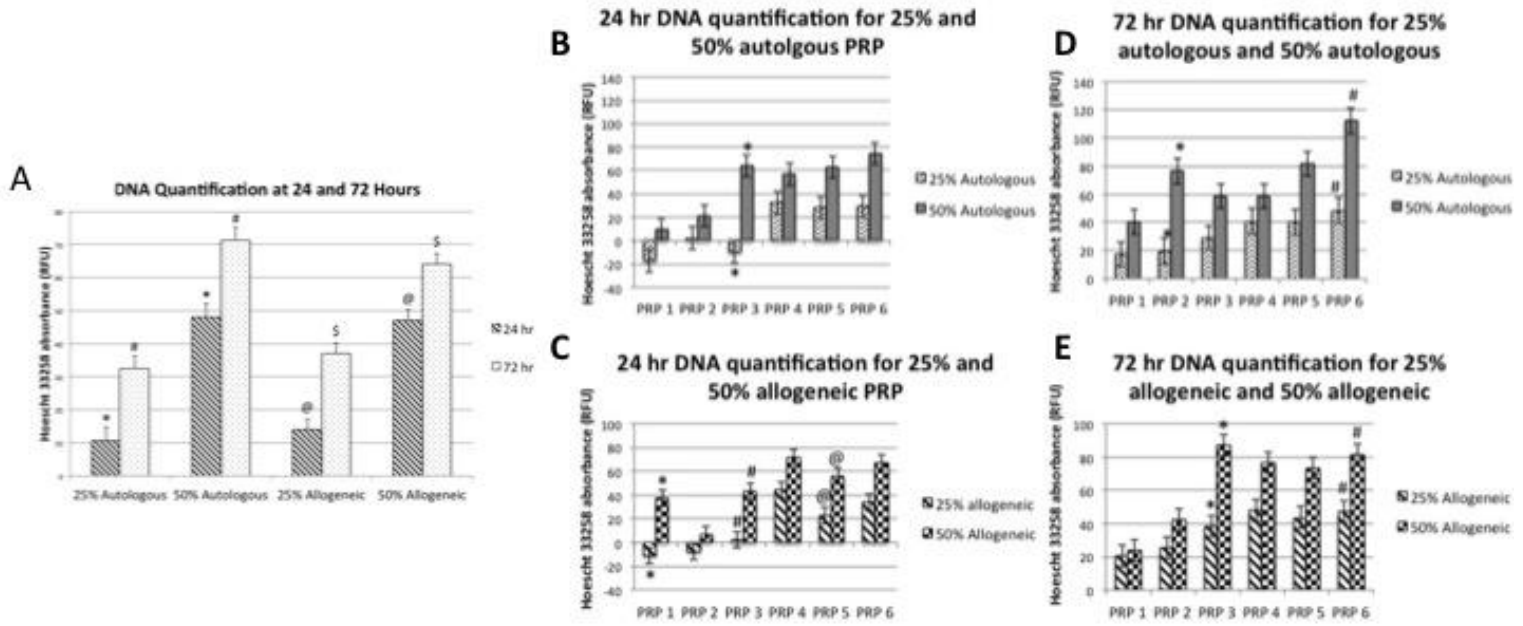

Figure 4.3. BMSC DNA quantification after 24 and 72 hours of culture (A) and BMSC viability after exposure to individual PRP lines after 24 (B) and 72 (C) hours of culture. (A) Mean Hoescht RFU of BMSCs cultured for 24 and 72 hours in 25% and 50% autologous or allogeneic PRP after subtraction of mean Hoescht 33258 absorbance of BMSCs cultured for 24 and 72 hours in control medium. *, #, $ represent significant ($p<0.05$) differences between the two experimental groups marked with the same symbol. Mean absorbance of Hoescht in BMSCs cultured for 24 hours (B and C) and 72 hours (D and E) in 25% and 50% autologous PRP (B and D) and 25% and 50% allogeneic PRP (C and E) separated for individual PRP lines with control values subtracted. *,#,$ represent significant differences ($p<0.05$) between the two experimental groups marked with the same symbol.

Table 4.4 Cellular proliferation after 48 hours of culture. Mean EDU RFU ± SEM for BMSCs after 48 hours of culture in medium, 25% autologous PRP, 50% autologous PRP, 25% allogeneic PRP, and 50% allogeneic PRP. Signficant differences between groups are outlined in bold with significant P values also displayed in bold font.

	EDU RFU	**25% Autologous PRP**	**50% Autologous PRP**	**25% Allogeneic PRP**	**50% Allogeneic PRP**
Medium	22241.56 ±1885.92	-10429.6± 3643.51 **(0.005)**	-13075.3± 3643.51 **(0.0006)**	-9069.33± 3480.48 **(0.011)**	-11143.9± 3480.48 **(0.002)**
25% Auto PRP	14541.39 ±1075.34		2645.72± 2155.37 (0.611)	-1360.22± 1866.60 (0.885)	714.39± 0.981 (0.981)
50% Auto PRP	11895.67 ±882.96			-4005.94± 1866.60 (0.148)	-1931.33± 1866.60 (0.730)
25% Allo PRP	14586.82 ±883.675				2074.61± 1524.08 (0.527)
50% Allo PRP	13273.07 ±994.51				

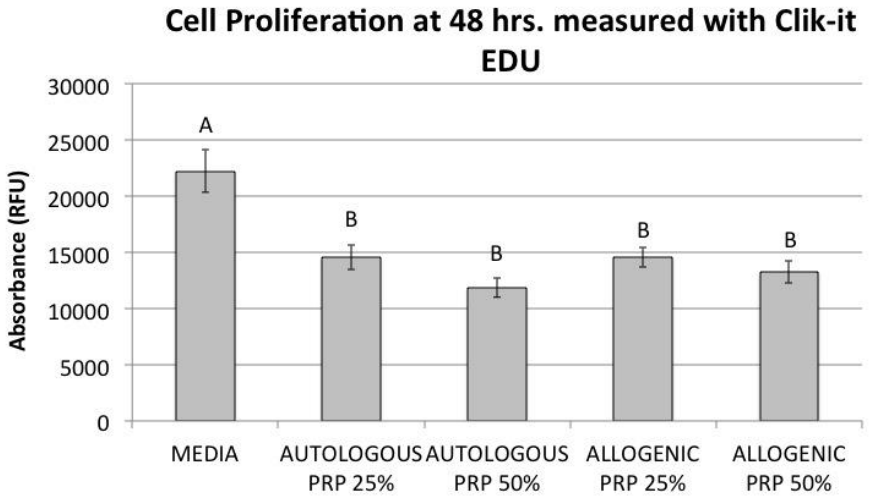

Figure 4.4 Cellular proliferation after 48 hours in culture. Mean RFU for Edu for BMSCs cultured for 48 hours after subtraction of control values. Treatment groups with different letters are significantly different ($p<0.05$).

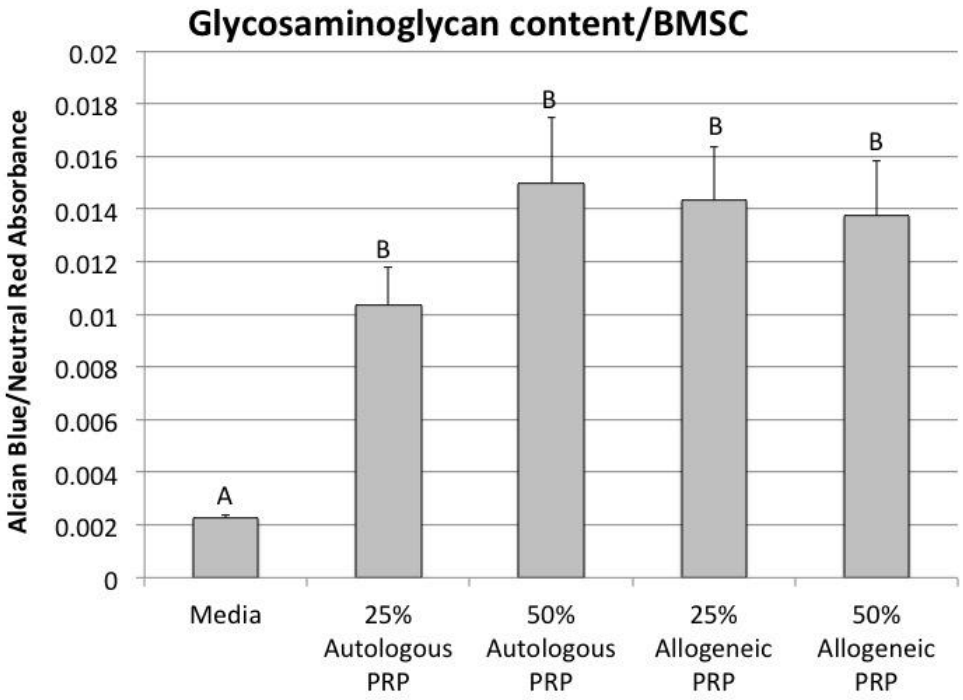

Figure 4.5 Glycosaminoglycan content per viable BMSC. Ratio of alcian blue absorbance (glycosaminoglycan content) to neutral red absorbance (cell number) for BMSC micromass pellets cultured for 28 days in 25% autologous PRP, 50% autologous PRP, 25% allogeneic PRP, and 50% allogeneic PRP. Treatment groups with different letters are significantly different ($p<0.05$).

CHAPTER 5

INTRA-ARTICULAR ADMINISTRATION OF EQUINE ALLOGENEIC BONE MARROW DERIVED MESENCHYMAL STEM CELLS[1]

[1] Boone, L. Mumaw, J, Thoresen, M, Brown, H, Uhl, E, Peroni, J
To be submitted to *Veterinary Comparative Orthopedics and Traumatology*.

ABSTRACT

Objective: Evaluate the local and systemic effects of intra-articular administration of equine allogeneic bone marrow derived mesenchymal stem cells (BMSCs).

Study Design: Randomized blinded experimental study

Animals: Adults (n=20)

Methods: BMSCs were harvested and culture expanded from ten horses. These 10 BMSC lines were then used for allogeneic administration of BMSCs into 1 radiocarpal joint of nine horses and 1 tarsocrural joint of ten horses. The contralateral joint served as a control joint and was injected with phosphate buffered saline. Horses were evaluated for changes in physical exam parameters; lameness; joint circumference, heat, and effusion daily. Synovial fluid was evaluated at 0, 24, 38, and 108 hrs after injection. Horses were euthanized after 5 days, the joints were evaluated grossly and synovial membrane was evaluated microscopically.

Results: Allogeneic BMSC treatment did not result in an adverse systemic response in any of the study horses. Allogeneic BMSC treatment resulted in moderate, but transient changes in palpable heat, effusion, and circumference of the joint, but significant changes in lameness were not detected. There was marked, but transient increases in the nucleated cell count and total protein of joints after treatment with allogeneic BMSCs. The radiocarpal joint was more sensitive to allogeneic BMSC injection compared to the tarsocrual joint. TNFα was not detected in the synovial fluid of any of the horses before and after allogeneic BMSC administration, but induction of mRNA expression of inflammatory and catabolic cytokines after allogeneic BMSCs was not significant due to high variability between horses.

Conclusions: Intra-articular administration of allogeneic equine BMSCs causes moderate, but transient inflammation of normal equine joints.

Clinical Relevance*:* Allogeneic administration of normal equine BMSCs appears safe, but further work is needed to investigate allogeneic administration of equine BMSCs in OA joints.

INTRODUCTION

Joint-related injury due to trauma or chronic abnormal biomechanical wear leads to the development of osteoarthritis (OA). Joint related injury is a significant cause of lameness in horses leading to reduced or loss of performance.[1-3] This reduced performance leads to a significant economic loss to the horse industry.[4] Current therapeutics for OA primarily modify the symptoms of the disease by temporarily reducing disease-related inflammation, but fall short in modifying the underlying progressive cartilage degeneration that perpetuates the inflammatory cycle of OA. Therefore clinicians seek a therapeutic that can promote biomechanical restoration and regeneration of degenerated articular cartilage as well as modulate the inflammatory environment of osteoarthritic joints.

Mesenchymal stem cells (MSCs) are stem cells derived from mesenchymal tissue capable of self-renewal. MSCs maintain a multipotent state with the ability to differentiate into a variety of connective tissues such as cartilage, bone, and adipose. Due to this capacity to differentiate, these cells are capable of contributing to the repair of connective tissue in musculoskeletal injury. MSCs are considered to be immunomodulatory and can exert anti-inflammatory effects upon activation.[5] For these reasons, treatment strategies utilizing MSCs for joint-related injuries are gaining popularity in both human and veterinary orthopedics.[6-16]

Practitioners often harvest bone marrow or adipose from horses for concentration of the mononuclear cell population containing a small proportion (0.001-0.01%) of MSCs or use culture-expansion of MSCs followed by therapeutic injection. Culture expansion of MSCs can take up to 4-6 weeks, delaying therapeutic intervention. Additionally, the

proliferation rate and chondrogenic differentiation capacity of MSCs can be altered due to certain patient-related factors, such as the age and disease state of the patient.[17-19] For these reasons, allogeneic administration of MSCs has gained interest, but studies evaluating their safety and efficacy after intra-articular administration in horses are lacking.[13]

Allogeneic administration of MSCs would allow practitioners a more uniform, quality controlled product for therapeutic delivery in a shorter period of time. Allogeneic MSCs produce little immunogenicity due to the absence of major histocompatibility complex (MHC) class II molecules and T-cell co-stimulatory molecules.[5,13] When activated, equine MSCs have been shown to decrease lymphocyte proliferation, decrease production of certain pro-inflammatory cytokines (TNFα and IFNγ), and increase certain anti-inflammatory cytokines (PGE_2 and IL-6).[5] Though scientific research into the immunomodulatory properties of murine and human MSCs are numerous, characterization of the immunomodulatory functions of equine MSCs has lagged behind the characterization of their regenerative functions and more research into the dual action (regeneration and immunomodulation) of these cells for joint related injury or OA is warranted.

Few studies have examined the safety of intra-articular injection of allogeneic MSCs. A study using placentally derived MSCs showed that a one-time injection of allogeneic placentally-derived MSCs induced similar synovial immune responses as a one-time injection of autologous placentally-derived MSCs, and that repeated intra-dermal injection of allogeneic umbilical cord tissue-derived MSCs and found no adverse systemic or local response following the initial intra-articular injection.[13,20] MSCs

obtained from fetal tissue are not as easily obtained by equine practitioners as adult-derived tissues, such as adipose or bone marrow, and until commercialization of characterized allogeneic MSC lines from various tissue sources are available, allogeneic administration of adipose-derived (ADMSCs) and bone marrow derived MSCs (BMSCs) is more likely to be sought by the practitioner, however there are no studies examining the inflammatory effects of these cells in equine joints. Allogeneic administration of BMSCs into surgically created superficial digital flexor tendinopathies of the forelimbs of two horses was shown to induce leukocyte migration to the site of injury, but the density of leukocyte migration was no different than induced leukocyte migration following autologous BMSC injection.[21] Administration of allogeneic ADMSCs suspended in autologous platelet rich plasma (PRP) in 16 horses with naturally occurring tendinopathy resulted in a local inflammatory response characterized by pain and swelling of the associated injection site in 8 of 16 horses, however these horse were not compared to autologous controls.[22] Safety and efficacy studies evaluating intra-articular administration of allogeneic BMSCs are yet to be published. Therefore, the purpose of this study was to evaluate the systemic and local effects of intra-articular administration of equine allogeneic BMSCs. We hypothesized that intra-articular administration of BMSCs would not result in adverse changes to physical examination parameters or parameters of complete blood counts. However, intra-articular administration of BMSCs would result in minimal adverse changes to the local synovial environment, but adverse changes would be transient in nature and resolve by the conclusion of the study period.

MATERIALS AND METHODS

Animals

Twenty healthy, vaccinated adult horses were used for this study. Ten horses aged 2 – 15 years old were used for bone marrow collection and BMSC culture expansion. Ten horses aged 5 - 20 years old were used for intra-articular injection of allogeneic BMSCs (alloBMSCs). Horses used for BMSC collection and culture were donated to the University of Georgia for teaching of fourth year veterinary students and bone marrow was harvested after varying lengths of hospitalization of these horses. Horses used for intra-articular administration of alloBMSCs underwent a minimum acclimatization period of 24 hours prior to study enrollment. Horses were confined to stall rest, but were handwalked or grazed for 10 minutes once daily during the study period. Non-steroidal anti-inflammatory medications were not administered and limbs were not bandaged during the study period. All procedures were performed in accordance with the University of Georgia Institutional Animal Care and Use Committee.

Study inclusion criteria

Horses were included in the study if they were >2 years of age, systemically healthy as determined by physical examination and complete blood count (CBC), and free of musculoskeletal abnormalities of the radiocarpal (RCJ) or tarsocrural (TCJ) joints determined by joint palpation, upper limb flexion, lameness, and radiographic examination. Horses were excluded from the study if there was a history of non-steroidal anti-inflammatory administration 72 hours prior to the initiation of the study. Due to the nature of horses donated to the University of Georgia, horses were not free of lameness prior to inclusion in the study.

Study design

One RCJ (10 horses) and one TCJ (9 horses) were randomly assigned to serve as the control joint and the contralateral limb was assigned to serve as the treatment joint. Control joints were intra-articularly administered phosphate buffered saline (PBS) and treatment joints were intra-articularly administered 10-20 million allogeneic BMSCs (alloBMSCs). Two of the 10 horses were administered 20 million alloBMSCs transduced with an adeno-associated viral vector containing the green fluorescent protein gene (scAAV-GFP) into treatment joints. AlloBMSCs were suspended in the same volume of PBS (4 mls) as the volume of PBS administered to control joints.

BMSC isolation and characterization

Bone marrow was aseptically aspirated from two sternebrae using an 8 gauge Jamshidi bone marrow biopsy needle[a]. Approximately 20 cc of bone marrow were drawn from either the 4^{th}, 5^{th}, and/or 6^{th} sternebrae into 2-35cc syringes containing 2500 units of heparan sulfate[b]. BMSCs were obtained by directly plating the cells and establishment of plate adherency. BMSCs were cultured in defined culture medium consisting of Dulbecco's Modified Eagles Medium with 4.5 g/L glucose and sodium pyruvate without L-Glutamine[c] supplemented with 10% fetal bovine serum[d], 0.05% L-glutamine[e], and 1% penicillin/streptomycin[f] under standard cell culture conditions (37°C and 5% CO_2). Initial BMSC colonies were allowed to reach confluency and then manually separated to allow re-distribution of lower passage cells for greater expansion. Once cells had reached 70-80% confluency, cells were harvested with 0.05% trypsin-EDTA[g], reseeded at 5000 cells/cm^2, allowed to reach 70-80% confluency harvested with trypsin and cryopreserved for later use.

Transduction of BMSCs: BMSCs were plated at 32,000 cells/cm^2 Cells were transduced with scAAV-GFP (Addgene plasmid 21893). The virus was produced using the three plasmid transfection system as previously described in HEK293 cells.[23] The cells were lysed 3 days following transfection and filtered supernatant was used to transfect the BMSCs. BMSCs were purified for GFP positive cells using FACs sorting.[h]

Cellular preparation:

Cryopreserved BMSCs were thawed and plated for culture expansion. BMSCs were allowed to reach 70-80% confluency then harvested from the plate with 0.05% trypsin and manually counted by staining a subset of BMSCs with 0.4% Trypan Blue[i]. Twenty to 40 million BMSCs were then re-suspended in PBS and centrifuged at 1200 rpm for 4 minutes. The supernatant was discarded and the BMSCs were again re-suspended in PBS and centrifuged. This process was repeated for three cycles, at the end of the third cycle the supernatant was discarded. BMSCs were re-suspended in PBS to a concentration of 2.5-5 million BMSCs/ml of PBS. Four milliliters of the cell suspension were placed in 2, 50 ml conical tubes and 4 mls of PBS were placed in 2 additional 50 ml conical tubes. Two investigators (JM and MT) not responsible for the clinical evaluation of horses were responsible for cellular preparation. The conical tubes used for transport of the treatments (PBS or alloBMSCs) were marked such that tubes could only be identified by these un-blinded investigators.

BMSC administration:

Horses were sedated with either 0.1-0.2 mg/kg of xylazine hydrochloride[j] or 0.003-0.005 mg/kg detomidine hydrochloride[k] and 0.003-0.005 mg/kg butorphanol tartrate[l] intravenously. The hair over the dorsal aspect of the RCJ and/or TCJ was clipped

and aseptically prepared with three alternating applications of 4% chlorihexidine gluconate and 70% isopropyl alcohol using a total application time of 5 minutes. The contents of each conical tube were aspirated into sterile 6 milliliter syringes and the blinded investigator was instructed as to which syringe was to be injected into which joint. Synoviocentesis of the RCJ was performed with the limb in flexion. A 1 inch, 20 gauge needle was directed proximally and caudally into the dorsolateral pouch of the RCJ. Synoviocentesis of the TCJ was performed with the limb weight bearing. A 1.5 inch 20 gauge needle was inserted 1-1.5 cm distal to the medial malleolus, medial to the saphenous vein and directed caudolaterally into the dorsomedial pouch of the TCJ.

Synovial fluid analysis

Synoviocentesis was perfomed at 0, 24, 48, and 108 hours during the study period. The same investigator (LB) performed all synoviocenteses throughout the study period (LB). Approximately 1-2 mls of synovial fluid was aspirated from both RCJ (n=20) and TCJs (n=18). 500µl of synovial fluid was aliquoted into an EDTA tube for clinicopathological analyses. Synovial fluid was subjectively graded for synovial fluid color (1=yellow, 2=orange, 3=red) and opacity (1=clear, 2=opaque or hazy, 3=cloudy). The aliquoted synovial fluid was diluted 1:1 with hyaluronidase[m] and a nucleated cell count (NCC)(cells/µl) was obtained using an automated cell counter[n]. Total protein (g/dL) was measured using a chemistry analyzer[o]. Synovial fluid was applied to slides and stained with hematoxylin and eosin for cytological assessment by a boarded clinical pathologist (HM) blinded to treatment. The remaining synovial fluid was aliquoted into microcentrifuge tubes (500µl/tube) centrifuged at 10,000 x g for 10 minutes and the

supernatant was subsequently frozen at -80°C for ELISA analysis of tumor necrosis factor-α (TNFα).

Synovial fluid obtained from two horses treated with alloBMSCs transduced with scAAV-GFP was processed as described above, but an additional aliquot was obtained for enumeration of green fluorescent cells that remained within the synovial fluid.

Clinical evaluation:

All clinical assessments were performed by the same blinded investigator (LB). Physical examinations were performed every 24 hours at 0, 24, 48, 72, 96, and 108 hours Temperature, pulse, and respiration were recorded as well as any observed changes to the horses overall attitude and/or appetite. Lameness examinations were performed at the walk and trot on hard ground every 24 hours at 0, 24, 48, 72, 96, and 108 hours. Lameness was subjectively graded (0-5) according to the AAEP lameness scale. Joints were subjectively graded every 12 hours for heat (0 = none, 1= mild, 2 = moderate, 3 = severe) and effusion (0 = none, 1= mild, 2 = moderate 3 = severe). Joint circumference (cm) was measured every 12 hours with a standard cloth measuring tape. The site for circumferential measurement was marked prior to initiation of the study by clipping a circumferential line in the hair overlying the joint of interest. This ensured that joint circumference was consistently measured at the same site on the limb during the study period.

Blood sample collection:

Ten milliliters of blood was obtained via jugular venipuncture by use of an 18 gauge needle and 12 mL syringe. Blood was obtained for a complete blood count (CBC) from 8 horses at 0, 48, and 120 hours and from 2 horses 48 hours after treatment. Total

white blood cells ($x10^3$cells/µl), neutrophils ($x10^3$cells/µl), banded neutrophils ($x10^3$cells/µl), and fibrinogen (mg/dL) were recorded.

Tissue harvest

Horses were euthanized five days (120 hrs) after treatment, with an overdose of sodium pentobarbital (20 mg/kg IV). Each joint was examined grossly for evidence of articular cartilage damage and/or abnormalities of the synovial membrane. Synovial membrane was harvested from all joints. A 2 x 2 cm portion of synovial membrane was harvested and preserved in 10% buffered neutral formalin for histopathological examination by a boarded anatomic pathologist (EU) blinded to treatment. The preserved specimens were processed routinely, paraffin embedded, and 5µm sections were stained with hematoxylin and eosin. Sections were subjectively graded for inflammation (1=no inflammation, 2=mild inflammation, 3=moderate inflammation). Another portion (1 x 1cm) of harvested synovial membrane was snap frozen in liquid nitrogen for real time RT-qPCR analysis.

TNFα ELISA

Synovial fluid was incubated for one hour with 2 mg/ml hyaluronidase[24] and concentration of TNFα protein was measured by use of an equine TNFα ELISA using a recombinant TNFα standard as previously described[25].

Real-time RT-qPCR

Synovial membrane was homogenized and RNA extracted using a commercial kit[p] in accordance with the manufacturer's protocol. A digestion step with deoxyribonuclease I to remove contaminating genomic DNA was included in the manufacturer's protocol. RNA was assessed for concentration and quality with a

spectrophotometer[q]. Complementary DNA was synthesized by use of a thermal cycler[r] and commercial kit[s]. RT-qPCR was performed with SYBR green using a sequence detection system, with 18S rRNA used as an endogenous control sample.

Expression of five genes associated with synovial inflammatory response (TNFα, IL-1β, IL-6, IL-8, and IL-10) and four genes associated with catabolic enzymatic degradation of the extracellular matrix of the articular cartilage (MMP-1, MMP-3, MMP-13, and ADAMTS4) were examined. The RT-qPCR oligonucleotide primers were designed with commercial software by use of sequences obtained from GenBank. The primer sequences that were used are presented in table 5.1.

Full validation of the SYBR green RT-qPCR assays of 5 of the 9 genes had previously been performed with RNA isolated from LPS-stimulated equine leukocytes. Validation assays were performed for the remainder of the genes analyzed. The housekeeping gene 18S was used as the internal control. ΔCT was calculated as $CT_{gene} - CT_{18S}$.

To interpret the effect of intra-articular administration of alloBMSCs gene expression of synovial membrane, the ΔΔCT approach was used, with gene expression of synovial membrane obtained from joints administered PBS serving as the calibrator. ΔΔCT was calculated as follows:

$$\Delta\Delta CT_{RCJ/TCJ} = \Delta CT_{RCJ/TCJ\ administered\ alloBMSCs} - \Delta CT_{RCJ/TCJ\ administered\ PBS}$$

The mean values of ΔΔCT were used to calculate fold change in gene expression produced after administration of alloBMSCs (fold change = $2^{-mean\ \Delta\Delta CT}$).

Statistical analysis

All analyses were performed using statistical software. Repeated measure analyses of variances (ANOVA) that recognized multiple observations as belonging to the same horse were used to test for differences in local effects between treatments, joints and times. The full model included fixed factors for treatment, joint, time and all two- and one three-way interaction effect and a random factor of horse. Tukey's test was used to adjust for multiple paired comparisons. A repeated measures ANOVA was also used to test for differences in systematic effect between baseline and other time-points. The full model included a fixed factor of time and a random factor of horse. Dunnett's test was used to adjust for multiple paired comparisons.

A repeated measures ANOVA was also used to test for differences in real time quantitative PCR between treatments and joints for ΔCT data and between joints for ΔΔCT data. The full model for the ΔCT data included fixed factors for treatment and joint and a treatment by joint interaction effect and a random factor of horse. Tukey's test was used to adjust for multiple paired comparisons. For ΔCT values, an approximate t-test was used to test if treatment means for each joint separately and pooled were significantly different than 0. The full model for the ΔΔCT data included a fixed factor for joint and a random factor of horse. For ΔΔCT values, a 95% confidence interval around each treatment mean was evaluated to test if 1 was included in each confidence interval for each joint separately and pooled over both joints. An unstructured covariance structure was used in all repeated measures models. All hypothesis tests were 2-sided and the significance level was $\alpha = 0.05$.

RESULTS

Physical Examination

There were no significant differences in temperature (p=0.542), pulse (p=0.526), and respiration (p=0.148) between baseline parameters and parameters obtained 24, 48, 72, 96, and 108 hours after treatment. No changes in horses attitude or appetite were observed during the study period.

Complete blood count (CBC*)*

No significant differences in total white blood cell count (p=0.391), segmented neutrophil count (p=0.650), banded neutrophil count (p=0.435) and fibrinogen concentration (p=0.493) between baseline measurements and measurements obtained 48 and 108 hours after treatment were observed (Table 5.2). Horse 5 and 7 had marked decreases in total white blood cell count and segmented neutrophil counts 48 hours after treatment.

Lameness

No significant difference in lameness grade were detected between treatments (p=0.567), joints (p=0.906), or times evaluated (P=0.997). Four of 10 horses (horse 1, 2, 4, and 9) showed an increase of 1-2 lameness grades 24-72 hours after injection of the RCJ with alloBMSCs. Horse 8 showed a 2-grade increase in lameness 24 hours after injection of the TCJ with alloBMSCs and horse 4 showed a 1 grade increase in lameness 24 hours after injection of the TCJ with PBS.

Clinical Assessment

Surface Heat

Significant differences in palpable surface heat were observed between treatments ($p<0.0001$), joints ($p<0.0001$) and times evaluated ($p<0.0001$). A significant interaction was detected between treatment and joint ($p=0.0001$) indicating that the effect of treatment was not consistent between joints (RCJ vs. TCJ). Overall, detected changes in surface heat were higher for TCJs treated with alloBMSCs than for RCJs treated with either PBS or alloBMSCs, though this difference was not statistically significant. There was a significant increase in surface heat 24 ($p=5.0 \times 10^{-4}$), 36 ($p=5.0 \times 10^{-4}$), and 84 hours ($p=0.014$) after injection of TCJs with alloBMSCs compared to surface heat of TCJs prior to injection. Table 5.3 displays mean palpable surface heat for RCJ and TCJs treated with either PBS or alloBMSCs 0-108 hours after injection.

Joint effusion

Significant differences in joint effusion were detected between treatments ($p<0.0001$), joints ($p<0.001$), and times of evaluated ($p<0.0001$). A significant interaction between treatment and joint ($p=0.0001$) was detected, meaning that the treatment effect on joint effusion was not consistent between joints (RCJ vs. TCJ). Table 5.3 reports mean joint effusion scores for RCJ and TCJs treated with either PBS or alloBMSCs. Joint effusion increased during the initial 24-72 hours of the study period and then decreased during the remainder of the study period for joints treated with alloBMSCs. Changes in joint effusion were more prominent in TCJs compared to RCJs treated with alloBMSCs. Though this difference was not statistically significant for all time points, there was a significant increase in joint effusion of TCJs compared to RCJs 24 ($p=0.025$) and 48

hours (p=0.025) after intra-articular administration of alloBMSCs. A significant increase in joint effusion was observed in RCJs, 60 (p=8.3 x 10^{-4}) and 72 hours (p=0.016) after intra-articular injection of alloBMSCs compared to joint effusion of RCJs prior to injection. There was a significant increase in joint effusion of TCJs 24 (p=0.001), 36 (p=0.007), 48 (p=0.001), 60 (p=3.6 x 10^{-5}), 72 (p=2.4 x 10^{-4}) and 84 hours (p=0.028) after intra-articular injection of alloBMSCs compared to joint effusion of TCJs prior to injection. Joint effusion was significantly greater in TCJs 60 hours (P=0.028) after intra-articular administration of alloBMSCs compared to intra-articular administration of PBS.

Joint circumference

Significant differences were detected between treatments (p<0.001) and joints (p<0.001), but not times evaluated (p=0.383). A significant interaction between joint and treatment was detected (p=0.031). Measured joint circumference was significantly lower in RCJs compared to TCJs for all time points regardless of treatment (p =1 x 10^{-12}). No significant increase in joint circumference was detected 0-108 hours after intra-articular injection of RCJs or TCJs with alloBMSCs compared to injection of PBS (p=1.0). Mean joint circumferences of both RCJs and TCJs 0-108 hours after injection are displayed in table 5.3.

Synovial fluid assessment

Synovial fluid color and opacity

No significant differences of synovial fluid color were detected between treatments (p=0.203) and joint (p=0.838), but differences were detected in synovial fluid color with time (p=0.0005). Synovial fluid tended to become orange to red with time

regardless of the joint that the synovial fluid was obtained from or treatment that was administered.

No significant differences in synovial fluid opacity were detected between joints (p=0.136), but significant differences were detected between treatments (p=0.003) and with time (p<0.0001). A significant increase in synovial fluid opacity was observed in RCJs 24 (p=0.013), 48 (p=0.003), and 108 hours (p=0.010) after intra-articular injection of PBS compared to synovial opacity of RCJs prior to injection of PBS. A significant increase in synovial fluid opacity was observed in RCJs 48 hours (p=0.046) after intra-articular injection of alloBMSCs compared to RCJs prior to injection.

Nucleated cell counts (NCC)

There was a significant difference in the NCC of synovial fluid between treatments (p=0.0004), joints (p=0.0002), and time (p<0.0001). There was a significant interaction between treatment and time (p=0.018) and between joint and time (p=0.019). Table 5.4 displays mean NCCs for RCJ and TCJs 0, 24, 48, and 108 hours after intra-articular injection of PBS and alloBMSCs. A significant increase in the NCC of synovial fluid obtained from RCJs 24 (p=2.77 x 10^{-6}) and 48 hours (p=0.002) after alloBMSC injection was detected compared to RCJs prior to injection. There was a significant increase in the NCC of synovial fluid obtained from RCJs 24hrs after intra-articular injection of alloBMSCs (p=0.011) compared to synovial fluid obtained 24 hours after injection of PBS. There was a significant increase in NCC of synovial fluid obtained from RCJs 24 hours after intra-articular injection with alloBMSCs compared to the NCC of synovial fluid obtained from TCJs 24 hours after intra-articular administration of alloBMSCs (p=0.013). Overall, RCJs had a greater increase in NCC after treatment with

either PBS or alloBMSCs compared to TCJs. NCCs increased to a maximum cell count in the majority of horses 24 hours after injection, decreasing 48 and 120 hrs after injection. 120 hours after injection, NCCs had returned to near normal values for RCJs injected with PBS (1240 ± 1228cells/μl) and TCJs injected with PBS (989 ± 1004 cells/μl) or alloBMSCs (1600 ± 1094 cells/ μl). The NCCs of RCJs 120 hours after treatment with alloBMSCs decreased dramatically (3780±4460 cells/ μl), but remained higher than normal values and other joint/treatment combinations. One RCJ injected with alloBMSCs (Horse 1) did have an increase in NCC 120 hours after injection. Horse 7 had the greatest increase in NCCs of all horses after injection of PBS and alloBMSCs after injection of the RCJs with both PBS and alloBMSCs. After injection with alloBMSCs, the NCC of horse 7 reached 61,200 cells/ μl 24 hours after injection and 88,000 cells/μl 48 hours after injection, but returned to near normal (1000 cells/ μl) 108 hours after injection. Horse 7 showed a significant increase in NCC of the RCJ treated with PBS as well, with the NCC increasing to 23,000 cells/ μl 24 hrs after injection and 25,200 cells/μl after injection, but returning to near normal (800cells/μl) 108 hours after injection. Horse 7 developed severe joint effusion of the RCJ treated with alloBMSCs and moderate effusion of the RCJ treated with PBS 12 hours after injection and this degree of effusion was maintained through the remainder of the study in both of these joints. Despite this dramatic increase in NCC and increased palpable joint effusion, the investigator noted no changes to the baseline lameness. However, the systemic total white blood cell count and neutrophil count of horse 7 had one of the most dramatic changes of all 8 horses. The total white blood cell count at baseline was 11,400 cells/μl and this total

white blood cell count dropped to 6,600 cells/µl 48 hours after injection and then began to increase to 9,400 cells/µl by 108 hours after injection.

The NCCs of two horses (Horse 9 and 10) treated with alloBMSCs transduced with scAAV-GFP were similar to NCCs of horses injected with untransduced alloBMSCs. Horse 9, had one of the second highest increases in NCC of RCJs injected with PBS, but one of the lowest increases in NCCs of RCJs treated with alloBMSCs. When synovial fluid was evaluated from these two horses, green fluorescent cells were not detected 24 and 48 hours after injection indicating that these cells had already integrated into synovial tissue, either the synovial membrane or articular cartilage or were cleared. Figure 5.1 is synovial membrane obtained from a horse treated with alloBMSCs transduced with scAAV-GFP 5 days after intra-articular injection.

Total protein

A significant difference in total protein of synovial fluid was detected between treatments ($p<0.0001$), joints ($p=0.031$) and time ($p<0.0001$). A significant interaction between treatment and time (P=0.0003) for total protein of obtained synovial fluid was detected meaning that the treatment effects were not consistent over time. Like the NCCs, total protein increased to a maximum total protein 24 hours after injection with decreasing total protein values 48 and 108 hours after injection in both joints and treatments, but with joints treated with alloBMSCs having higher increases in total protein values compared to joints treated with PBS. RCJs had higher increases in total protein compared to TCJs. Table 5.5 displays the mean total protein values for horses 0, 24, 48, and 108 hours after injection of the RCJ or TCJs with either PBS or alloBMSCs. There was a significant difference in total protein of synovial fluid obtained from RCJs

prior to injection with alloBMSCs and the total proteins of RCJs 24 (p=1x10-12) and 48 (p=0.003) hours after alloBMSC injection, but no difference 108 hours (p=0.089) after alloBMSC injection. There were significant difference in total protein of synovial fluid obtained from TCJs prior to injection of alloBMSCs and the total protein of synovial fluid obtained from TCJs 24 (p=0.005) and 48 hours (p=.010) after alloBMSC injection, but no difference 108 hours (p=0.543) after alloBMSC injection. Horse 4 and 7 had the highest increases in total protein values of RCJs treated with alloBMSCs.

Synovial fluid cytology

There was a significant difference in neutrophil % and mononuclear % of synovial fluid detected between treatments (p=0.027; P=0.030) and time (p<0.0001; p<0.0001), but no difference was detected between joints (p=0.775; p-0.793). Table 5.6 displays the mean proportions of cells within the synovial fluid that were mononuclear and neutrophilic for RCJ and TCJs 0, 24, 48, and 108 hours after injection of PBS or alloBMSCs. The proportion of the synovial fluid cell population that was classified as mononuclear decreased 24-48 hours after injection while the neutrophilic population increased 24-48 hours after injection and both percentages returned to baseline values 108 hours after injection. A significant increase in neutrophil % was detected between synovial fluid obtained from RCJs prior to alloBMSC treatment and synovial fluid obtained from RCJs 24 (p=5.21 x 10^{-6}) and 48 hours (p=0.008) after injection. A significant decrease in mononuclear % was detected between synovial fluid obtained from RCJs prior to alloBMSC treatment and synovial fluid obtained from RCJs 24 (p=1.42 x 10^{-5}) and 48 hours (p=0.013) after injection. No significant differences in mononuclear and neutrophil populations were seen between RCJs and TCJs treated with

PBS and alloBMSCs for all time points. Horse 3 had the most dramatic change in the proportion of mononuclear cells and neutrophils 24 hours after injection (19% mononuclear vs. 81% neutrophilic) of the RCJ with alloBMSCs. This dramatic change corresponded to a dramatic increase in total protein (4.4g/dL), but only a moderate increase in total NCC (16,400 cells/µl). Interestingly, changes in cellular population (31% mononuclear and 69% neutrophils at 24 hrs; 34% mononuclear and 66% neutrophils at 48 hours) were the lowest in Horse 7 despite horse 7 having the highest NCC 24 and 48 hrs after injection of the RCJ with alloBMSC

TNFα ELISA

TNFα was not observed in any of the synovial fluid samples for all ten horses. No significant differences in TNFα were observed between treatments, joints, or times evaluated.

Synovial membrane histopathology

A significant difference was detected in inflammation of synovial membrane between treatments (p=0.0002), but no difference between joints (p=0.129). A significant interaction between treatment and joints was detected meaning that the treatment effects were not consistent for the different joints (p=0.032). This was observed as a trend for greater inflammation of synovial membrane obtained from TCJs treated with alloBMSCs compared to RCJs treated with alloBMSCs (p=0.050). Table 5.7 displays the synovial membrane inflammatory score for eight horses with additional comments made by the blinded pathologist regarding specific pathology noted at the time of histopathologic assessment. There was significantly greater inflammation of the synovial membrane obtained from TCJs treated with alloBMSCs compared to TCJs treated with PBS

(p=0.001). However, there was no significant difference in inflammation of the synovial membrane observed between RCJs treated with alloBMSCs and RCJs treated with PBS (p=0.345). Joints injected with alloBMSCs had greater hemorrhage and edema of the synovial membrane as well as perivascular aggregations of lymphocytes compared to joints injected with PBS.

RT-PCR of synovial membrane

No differences in ΔΔCt were detected between synovial membrane of the RCJ after treatment with PBS and alloBMSCs or TCJ after treatment with PBS and alloBMSCs for IL-1β (RCJ p=0.995; TCJ p=0.699), IL-6 (RCJ p=0.936; TCJ p=0.159), IL-8 (RCJ p= 0.956; TCJ p=0.373), IL-10 (RCJ p=0.676; TCJ p=0.637); TNFα (RCJ p= 0.693; TCJ p=0.367); MMP 1 (RCJ p=0.797; TCJ p=0.196); MMP 3 (RCJ p= 0.052; TCJ p=0.236), MMP 13 (RCJ p=0.197; TCJ p=0.648); ADAMTS 4 (RCJ p= 0.584; TCJ p=0.751), and ADAMTS 5 (RCJ p=0.929; TCJ p=0.240). There was a trend for decreased ΔΔCt values of synovial membrane after injection of alloBMSCs compared to injection of PBS. Table 5.8 displays the ΔΔCt and fold change in gene expression after alloBMSCs injection compared to PBS injection for the genes of interest. Biologically relevant fold changes include less than a 0.5-fold change consistent with down regulation of expression of the gene of interest or greater than a 2-fold change that would be consistent with up regulation of expression of the gene of interest. Down regulation of catabolic enzymes, MMP1 (0.33 fold change) and MMP 13 (0.25 fold change) after alloBMSC injection of the RCJ were present. This was in contrast to up regulation of catabolic enzymes, MMP 1 (5.37 fold change) and MMP 3 (2.88 fold change) after

alloBMSC injection of the TCJ. Inflammatory genes were neither up nor down regulated at biologically relevant levels after injection of alloBMSCs of the RCJ or TCJ.

DISCUSSION

Allogeneic administration of equine BMSCs would allow practitioner's the ability treat equine musculoskeletal injuries during the acute phases of wound healing with a more uniform and characterized cellular product. The results of this study supported our hypotheses that intra-articular administration of equine alloBMSCs would result in no adverse systemic changes, but would result in minimal yet transient clinical and pathologic changes of the local synovial environment

Adverse changes to horse's attitude, appetite, or physical examination parameters were not observed through the course of the study. Additionally, no significant changes were seen systemically in the clinically relevant parameters of CBCs obtained from eight horses after intra-articular injection of alloBMSCs. However, Horse 7 did have a marked decrease in his total white blood cell count (~42% drop) and segmented neutrophil count (~43% drop) 48 hours after treatment. Interestingly, this horse was the most responsive to intra-articular injection of the RCJ with PBS and alloBMSCs. 48 hours after injection of the RCJ with alloBMSCs, horse 7 achieved a NCC of 88,000 cells/µl. Therefore, it is presumed that the inflammation present within the joint after PBS or alloBMSC injection caused chemotaxis of white blood cells, predominantly neutrophils, in systemic circulation to the joint(s) causing a rise in the NCC of the joint(s) and a decrease in the systemic white blood cell count as well as the segmented neutrophil count. In contrast, horse 5 also had a moderate decrease (~30%) in the total white blood cell count 48 hours after treatment, but only a moderate change in the NCC of the RCJ that was injected with

alloBMSCs. This change in total white blood cell count could be a result of transient stress related to hospitalization, but due to the concurrent and substantial increase in the NCC, migration of peripheral white blood cells due to chemotactic signals remains a plausible cause for the substantial decrease in the peripheral white blood cell count. This would suggest that some horses may experience mild systemic changes as a result of intra-articular administration of allogeneic BMSCs. However, it is important to note that these transient changes were only observed in 2 of 10 and were not accompanied with changes to their physical examination parameters that would indicate a systemic response. The degree of systemic response observed is related to variability of the recipient as well as variability of the donor BMSC line. To decrease the variability of the donor BMSC line, donor BMSCs would need to be characterized phenotypically, functionally, and immunogenically for the "best" donor BMSC line for intra-articular administration.

No statistically significant difference in lameness scores were detected between treatments, joints, and times evaluated. However, several horses did show an increase of 1-2 lameness grades after administration of alloBMSCs indicating that administration of alloBMSCs causes a minimal increase in lameness grades compared to limbs injected with PBS(for how long?). This is in contrast to another study that did not see significant increases in lameness between RCJs injected with autologous and allogeneic placentally-derived MSCs.[13] It could be inferred that differences were not observed in lameness between RCJs because the resultant lameness from allogeneic placentally derived MSCs was balanced by the resultant lameness from treatment of the contralateral RCJ with autologous placentally derived MSCs causing the horse to appear sound. This "lack of

gait alteration" was then compared to the horse's own TCJ that served as a control joint. This comparsion would be inadequate according to this study in which differences between the RCJ and TCJ related to their clinical and pathological responses to alloBMSC and PBS injection were observed. Therefore, alloBMSC administration causes minimal changes to a horse's baseline lameness, but a greater increase in lameness compared to administration of PBS.

Equine BMSCs and placentally-derived MSCs (umbilical cord blood or tissue) do not differ significantly in immunogenicity.[5] In fact, the only difference that was observed between MSCs derived from these different tissue sources, was the ability of BMSCs to inhibit T cell proliferation both in the absence and presence of activation, where as placentally-derived MSCs could only inhibit T cell proliferation in the after activation.[5] Therefore differences in immunogenicity of the tissue source do not account for the differences in observed changes in lameness between studies.

The current study used a larger treatment dose of MSCs (10-20 million/joint) compared to study that evaluated intra-articular administration of placentally derived MSCs (7.5 million/joint).[13] The majority of equine studies that evaluate the use of MSCs empirically administer a dose of 5-20 million MSCs, but an effective treatment dose has not been established for equine musculoskeletal injuries. Nor have different treatment doses in the equine been evaluated for treatment efficacy. The contribution of the increased treatment dose to minimal changes in lameness grade is unknown.

Significant changes in clinical assessment of each joint were evident after both treatments, but injection with alloBMSCs produced the most profound changes. Also, the TCJ had the most clinically evident and therefore the most significant changes after both

alloBMSC and PBS injection with alloBMSCs causing the greatest increases in surface heat and joint effusion. The discrepancies in the clinical assessments of the RCJ and TCJ are attributed to the anatomical differences of the synovial compartments of the RCJ compared to the TCJ. The TCJ has a more prominent, therefore more easily palpable, dorsal and plantar synovial compartment making changes in joint effusion and surface heat more easily detected by an observer. Joint circumference was significantly different between treatments, with joints treated with alloBMSCs having a 1-2 cm increase in joint circumference after treatment. This increase in joint circumference subsided in RCJs treated with alloBMSCs over the course of the study, but the increase in joint circumference observed after injection of TCJ with alloBMSCs persisted through the remainder of the study period. This change in joint circumference is consistent with findings of the study injecting RCJ of horses with both autologous, allogeneic placentally-derived MSCs.[13] In contrast to the previously mentioned study, the current study does suggest that there is a significantly greater inflammatory response after intra-articular injection of allogeneic BMSCs compared to PBS. This detected difference in inflammatory response in our study compared to the previously mentioned study is likely due to the use of the TCJ as a control joint. [13] In our study, the TCJ had a greater detected difference in clinical parameters compared to the RCJ, therefore, if one compares the differences in the RCJ to the TCJ a difference may not be detected because the TCJ will have a greater increase in joint circumference when treated with PBS compared to treatment of the RCJ with PBS.

Significant changes in synovial fluid color were only observed over time, and significant differences in synovial fluid opacity, NCC, and total protein were observed

between both treatments and times evaluated. Differences between joints were observed for synovial NCC and total protein. Joints increased in color from yellow to red (hemorrhagic) with time regardless of initial treatment due to marked synovial inflammation that is induced with repeated synoviocenteses. Synovial fluid opacity, NCC, and total protein were significantly increased in joints treated with alloBMSCs compared to joints treated with PBS. Horse 7, had the most significant increase in NCC of RCJs 48 hours after treatment with alloBMSCs reaching a NCC of 88,000 cells/µl. This increase in synovial NCC corresponded to a decline in the peripheral white blood cell and neutrophil count indicating a systemic response. This degree of cellular influx into the synovial compartment is remarkable and clinically the NCC would be consistent with synovial sepsis. However, this cell count returned to near normal (1000 cells/µl) by five days without treatment indicating an inflammatory rather than infectious process. The total protein within this joint was also markedly elevated 24 hours after treatment (4.4 g/dL), but declining 48 hours after treatement (3.3 g/dL) despite the increase in NCC. This horse also had the second most dramatic shift in the RCJ's mononuclear/neutrophil population. Despite this marked degree of inflammation, an increase in lameness was not observed in the limb for which the RCJ was treated. Additionally, only a moderate degree of inflammation (score of 1.5) was observed in the synovial membrane after euthanasia. In contrast, horse 8 had a minimal increase in NCC with minimal alteration to the mononuclear/neutrophilic population and a moderate increase in total protein, but had one of the highest inflammatory scores recorded for synovial membranes of RCJs treated with alloBMSCs. These horses highlight the variability in response between recipients despite similar treatment doses (Horses 7 and 8

received 20 million alloBMSCs/joint) as well as the variability that is introduced due to the donor BMSC line.

Cells expressing GFP were not observed in synovial samples obtained from horses 9 and 10 during any time points. This indicates that the alloBMSCs had homed and integrated into synovial tissue within 24 hours after intra-articular injection or were already cleared. The majority of these cells are presumed to have integrated into the synovial membrane (Figure 5.1), but post-mortem samples of the articular cartilage were not assessed for GFP positive cells.

There was a significant increase inflammatory score of the synovial membrane of joints treated with alloBMSCs compared to PBS. The inflammatory scores of TCJs were higher than RCJs treated with alloBMSCs. Joints that were treated with alloBMSCs had more focal edema, hemorrhage, and perivascular lymphocytic aggregates. Therefore, alloBMSCs induced greater inflammation of the synovial membrane compared treatment of PBS. After intra-articular injection, MSCs were shown to implant in the synovial membrane. Given the reported immunomodulatory role of MSCs integration of MSCs with the synovial membrane would presumably be the site for MSCs to exert their greatest immunoregulation of the synovial environment through cell-cell contact with synovial macrophages. However, interaction of the synovium resulted in greater synovial inflammation compared to PBS. There are currently no studies that have evaluated the synovial membrane after intra-articular administration of autologous let alone allogeneic MSC administration. Studies evaluating the effects of intra-articular administration of allogeneic or autologous ADMSCs in an osteoarthtis model and showed a reduction in synovial membrane thickness and inflammatory infiltrate in both rabbits and mice [26]

Until recently, the synovial membrane was not considered to be a key component of the pathophysiology of osteoarthritis. However, recent studies have shown that the synovial membrane is an integral contributor to the maintenance and progression of OA.[27] Further evaluation of the interaction of both autologous and allogeneic MSCs with synovial membrane is warranted to understand the effect of MSCs on synovial inflammation.

The inflammatory cytokine, TNFα was not observed in any of the synovial fluid samples from all ten horses. No differences in inflammatory cytokine and catabolic enzymatic gene expression were observed, meaning that alloBMSCs neither upregulated or downregulated gene expression profiles of the synovial membrane.

While the results of this study suggest minimal changes to the local synovial environment after intra-articular administration of allogeneic BMSCs, this study had several limitations. Horses used in the study were not free of lameness and pre-existing osteoarthritis of the RCJ or TCJs could not be ruled out with lameness and radiographic evaluation alone. Therefore, these joints could not have been considered normal joints without further diagnostic imaging. However, all joints did have baseline NCC (<2500 cells/ul) and TP (<2.5 g/dL) values consistent with values previously obtained from normal, healthy joints.[28] None of the horses used were free of lameness and therefore, lameness was assessed only if there was a change to the baseline lameness. Therefore if the baseline lameness was severe, a minimal increase in lameness of the contralateral or ipsilateral joint due to treatment may not have been detected because of the severity of the baseline lameness. Lameness evaluation is also a subjective diagnostic evaluation with limited inter-observer agreement. Therefore, this study could have been

strengthened by providing a more objective assessment of lameness such as the use of force plate analysis or computer assisted motion detector analysis (lameness locator). All ten horses were treated with ten different alloBMSC lines. This study design was chosen to evaluate the overall safety of intra-articular injection of multiple alloBMSC line. However, to decrease variability of the donor BMSC lines, one or two alloBMSC lines could have been chosen. This would have allowed greater assessment of recipient responses to the same alloBMSC line. Lastly, the efficacy of alloBMSC treatment in joint-related injury was not assessed. Further research is warranted into the characteristics of the ideal alloBMSC line and the efficacy alloBMSC treatment in musculoskeletal injury.

The results of this study suggest mild to moderate transient clinical and pathologic changes of the local synovial environment after intra-articular administration of alloBMSCs. Further studies are warranted to investigate the efficacy of alloBMSC administration in joints with early synovitis, focal articular cartilage defects, and osteoarthritis (acute and chronic).

FOOTNOTES

[a] Phosphate buffered saline,

[b] Jamshidi bone marrow biopsy needle, Jorgensen Laboratories, Inc., Loveland, CO

[c] Heparin, Hospira, Inc. Lake Forest, IL

[d] Dulbecco's Modified Eagles Medium with 4.5 g/L glucose and sodium pyruvate without L-Glutamine, Cellgro®, Mediatech,Inc. Manassas, VA

[e] Fetal bovine serum, Atlanta Biologicals®, Lawrenceville, GA

[f] L-glutamine, Gibco®, Life Technologies Corporation, Grand Island, NY

[g] Penicllin/Streptomycin, Gibco®, Life Technologies Corporation, Grand Island, NY

[h] MoFlo XDP, Beckman Coulter Brea, CA

[h] 0.05% Trypsin-EDTA, Gibco®, Life Technologies Corporation, Grand Island, NY

[I] Trypan Blue, Sigma Aldrich, St.Louis, MO

[j] Anased®, xylazine hydrochloride

[k] Dormosedan®, detomidine hydrochloride, Pfizer Animal Health, Exton PA

[l] Torbugesic, butorphanol tartate, Fort Dodge Animal Health, Fort Dodge, IA)

[m] Hyaluronidase from bovine testes, Sigma Aldrich, St.Louis, MO

[n] Heska HemaTrue™ CBC analyzer, Heska, Des Moines, Iowa

[o] Roche Hitachi P Module chemistry analyzer (biuret method),

[p] Qiagen RNeasy Fibrous Tissue Mini or Midi Kit depending on RNA yield, Qiagen, Inc. Germantown, MD

[q] DNAase

[r] ND-1000 UV-Vis Spectrophotometer, NanoDrop Technologies Inc., Wilmington, Del.

[s] Mastercycler Gradient, Eppendorf Inc. Westbury, NY

[t] Quanta Biosciences qScript cDNA Super mix, Gaithersburg, MD

[u] SAS V 9.2, Cary, NC.

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Table 5.1 Primer sequences for genes of interest

Gene of Interest	Forward	Reverse
IL-1β	ATGACTTACTGCAGCGGCAAT	GTCTTGGAAGCTGCCCTTCA
IL-6	TGCTGGCTAAGCATTCA	GGAAATCCCTCAAGGCTTCGAA
IL-8	TTGGCCGTCTTCCTGCTT	GGTTTGGAGTGCGTCTTGATG
IL-10	GCCTTGTCGGAGATGATCCA	TTTTCCCCCAGGGAGTTCAC
TNFα	AAAGGACATCATGAGCACTGAAAG	GGGCCCCTGCCTTCT
MMP-1	GGTGAAGGAAGGTCAAGTTCTGAT	AGTCTTCTACTTTGGAAAAGAGCTTCTCT
MMP-3	GCAAGGGACGAGGATAGCAA	GTCTCATTTCTTTTCCAAGGTCGTAGT
MMP-13	GAGATGCGCATTTTGATGATGATGA	TCGTGTGCAGCGACAAGAA
ADAMTS 4	GGCTATGGGCACTGTCTCTTAGA	CCTTGCCAGGGAAAGTCACA
ADAMTS 5	GAGATGACCATGAGGAGCACTAC	GGCCATCGTCTTCAATCACAG

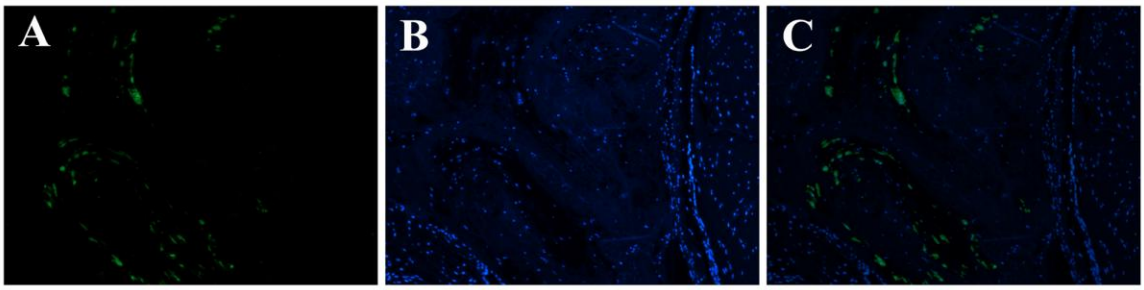

Figure 5.1 GFP positive BMSCs 5 days after intra-articular injection. Presence of GFP positive cells in synovial membrane 120 hours after injection. A) Fluorescence of GFP positive MSCs, B) DAPI nuclear staining C) overlay of GFP positive cells and DAPI nuclear staining.

Table 5.2 Complete blood counts of eight horses after intra-articular allogeneic BMSC administration. Total white blood cell count (x 10^3 cells/µl), segmented neutrophil count (x 10^3 cells/µl), banded neutrophil count (x 10^3 cells/µl) and fibrinogen concentration (mg/dL) for horses 0, 48, and 108 hours after injection.

	Total white blood cell count (x 10^3 cells/µl)			**Segmented neutrophils (x 10^3 cells/µl)**			**Bands (x 10^3 cells/µl)**			**Fibrinogen (mg/dL)**		
	Time (hrs)			**Time (hrs)**			**Time (hrs)**			**Time (hrs)**		
Horse	**0**	**48**	**108**	**0**	**48**	**108**	**0**	**48**	**108**	**0**	**48**	**108**
1	9.2	9.2	7.7	6.9	6.44	5.313	0.276	0	0	300	100	300
2	9.4	7.6	7.4	7.05	4.94	4.218	0	0	0.074	300	300	100
3	6.5	6.8	5.7	5.07	5.168	4.503	0	0	0	200	400	200
4	5.2	4.4	7.5	3.38	2.596	6.375	0	0	0	200	300	200
5	7.0	4.9	5.8	3.64	3.283	4.176	0	0	0	300	200	300
6	4.2	5.3	5.5	1.974	3.339	3.41	0	0	0	200	300	300
7	11.4	6.6	9.4	8.778	5.016	6.486	0	0	0	300	200	200
8	8.1	7.6	5.6	5.832	5.624	3.64	0	0	0	200	300	300
9		9.4			5.546			0			300	
10		13.6			8.568			0			400	

Table 5.3. Physical joint parameters 0-108 hours after intra-articular administration of allogeneic BMSCs. Mean ± SD of surface heat, palpable joint effusion, and joint circumference (cm) detected 0, 12, 24, 36, 48, 60,72, 84, 96 and 108 after treatment with either phosphate buffered saline (PBS) or allogeneic bone marrow derived mesenchymal stem cells (alloBMSCs). Values reported are separated by joint (radiocarpal and tarsocrural joint) and treatment (PBS or alloBMSCs).

		Heat		Effusion		Circumference	
Joint	**Time**	**PBS**	**alloBMSCs**	**PBS**	**alloBMSCs**	**PBS**	**alloBMSCs**
Forelimb (Radcarpal)	**0**	0.3±0.7	0.2±0.4	0.1±0.3	0.0±0.0 **A**	31.3±2.2	31.5±2.1
	12	0.5±0.5	0.9±0.7	0.7±0.7	0.8±0.6	31.7±2.0	31.7±2.1
	24	0.9±0.6	0.9±0.7	0.8±0.6	0.9±1.0*	31.6±2.2	31.9±2.4
	36	0.6±0.5	1±0.8	0.7±0.7	1.0±0.8	31.8±2.0	32.0±2.4
	48	0.6±0.5	1±0.8	0.5±0.5	0.9±0.7 #	31.6±2.0	31.9±2.3
	60	0.6±0.5	0.9±0.6	0.5±0.5	1.4±0.8 **B**	31.6±2.0	31.9±2.4
	72	0.7±0.7	0.8±0.6	0.7±0.8	1.2±0.9 **B**	31.6±2.0	32.0±2.5
	84	0.8±0.9	1±0.6	0.8±0.8	1.0±0.9	30.6±4.0	32.0±2.4
	96	0.7±0.7	0.9±0.7	0.5±0.5	0.9±0.7	31.7±2.0	32.1±2.5
	108	0.7±0.7	0.8±0.6	0.5±0.5	0.5±0.5	31.6±2.2	31.8±2.3
Hindlimb (Tarsocrural)	**0**	0.4±0.5	0.4±0.5 **A**	0.3±0.5	1.0±0.5 **A**	36.9±2.5	37.3±2.8
	12	1.1±0.8	0.9±0.8	0.9±0.9	0.7±0.7	37.2±2.9	37.5±3.0
	24	1.1±0.6	1.9±0.6 **B**	1.0±0.7	1.7±1.1***B**	37.0±2.7	37.4±3.3
	36	0.9±0.7	1.8±0.6 **B**	1.1±0.8	2.1±1.0 **B**	36.7±2.7	38.0±3.0
	48	0.7±0.5	1.4±0.9	1.0±0.7	2.1±1.1 **#B**	37.1±2.7	38.0±2.7
	60	0.9±0.6	1.2±1.0	1.1±0.8@	2.3±0.9**@B**	36.7±2.8	38.3±3.0
	72	0.9±0.6	1.5±0.7	1.2±0.4	2.2±0.7 **B**	37.7±2.8	38.4±3.0
	84	0.8±0.4	1.7±0.9 **B**	1.1±0.6	1.9±0.8 **B**	36.8±2.7	38.1±3.0
	96	1.0±0.5	1.3±0.7	1.2±0.6	1.7±0.7	37.1±2.5	37.9±2.7
	108	0.8±0.6	1.1±0.8	1.0±0.5	1.3±0.9	37.1±2.6	37.9±2.7

Cells within the same column with different letters are significantly different ($p<0.05$) from one another. Cells with the same symbol are significantly different ($p<0.05$) from one another.

Table 5.4 Nucleated cell counts of RCJ and TCJs 0-108 hours after intra-articular injection of allogeneic BMSCs. Nucleated cell counts (cells/µl) of synovial fluid obtained from the radiocarpal (RCJ) and tarsocrural joints (TCJ) 0, 24, 48, and 108 hrs after intra-articular injection of either phosphate buffered saline (PBS) or allogeneic bone marrow derived mesenchymal stem cells (alloBMSCs). Total is the mean ± SD for horses 0, 24,48 and 108 hrs after treatment (PBS or alloBMSCs) of the RCJ and TCJ.

		Nucleated cell count (cells/µl) after PBS injection				**Nucleated cell count (cells/µl) after allogeneic BMSC injection**			
		Time after injection (hrs)				**Time after injection (hrs)**			
	Horse	**0**	**24**	**48**	**108**	**0**	**24**	**48**	**108**
Forelimb (RCJ)	**1**	500	2,400	1,800	500	500	12,400	8,000	16,000
	2	500	2,200	1,000	800	500	22,800	13,400	3,600
	3	500	5,000	1,400	1,200	500	16,400	7,200	1,200
	4	500	4,000	2,200	1,000	500	20,800	10,800	4,400
	5	500	3,600	800	500	500	19,800	8,800	2,800
	6	500	1,200	1,000	600	500	28,600	9,000	800
	7	500	23,000	25,200	800	500	61,200	88,000	1,000
	8	500	13,200	5,600	1,600	500	21,200	8,400	3,600
	9	600	20,600	10,400	4,600	600	2,000	1,000	2,000
	10	800	800	600	800	500	12,200	7,200	2,400
	Mean± SD	540 ± 97	7600 ± 8278 **A**	5000 ± 7729	1240 ± 1228	511 ± 33 **BC**	21,740 ± 15,658 ***AB**	16,180 ± 25,430 **C**	3780 ± 4460
Hindlimb (TCJ)	**1**	500	500	500	500	500	800	500	500
	2	500	2,000	800	600	500	8,200	5,600	2,800
	3	500	5,800	2,800	600	500	8,600	5,200	600
	4	500	1,000	600	1,200	1,400	16,800	13,600	2,600
	6	500	500	600	500	500	2,600	2,000	500
	7	500	500	600	500	500	16,200	10,000	1,200
	8	500	1,800	800	600	500	5,800	10,400	3,200
	9	600	500	1,000	3,600	800	6,600	2,200	800
	10	600	2,600	1,400	800	500	2,200	3,200	2,200
	Mean± SD	522 ± 44	1689 ± 1731	1011 ± 725	989 ± 1004	633 ± 304	7533 ± 5751*	5856 ± 4504	1600 ± 1094

Cells within the same row that have the same letter are significantly ($p<0.05$) different from one another. Cells within the same column that have the same symbol are significantly different ($p<0.05$) from one another.

Table 5.5 Total protein of RCJ and TCJs 0-108 hours after intra-articular injection of allogeneic BMSCs. Total protein (g/dL) of synovial fluid obtained from the radiocarpal (RCJ) and tarsocrural joints (TCJ) 0, 24, 48, and 108 hrs after intra-articular injection of either phosphate buffered saline (PBS) or allogeneic bone marrow derived mesenchymal stem cells (alloBMSCs). Total is the mean ± SD for horses 0, 24,48 and 108 hrs after treatment (PBS or alloBMSCs) of the RCJ and TCJ.

		Total Protein (g/dL) after PBS injection				Total Protein (g/dL) after allogeneic BMSC injection			
		Time after injection (hrs)				Time after injection (hrs)			
	Horse	0	24	48	108	0	24	48	108
Forelimb (RCJ)	1	N/R	1.9	1.2	1.0	N/R	3.8	2.8	2.2
	2	1.0	1.2	0.8	0.6	1.0	2.9	2.6	1.0
	3	0.5	1.1	0.4	0.6	0.5	4.4	2	1.7
	4	0.3	1.8	1.1	0.9	0.5	4.5	3.3	2.3
	5	0.4	2.0	0.5	0.5	0.4	3.7	2.6	3.2
	6	0.4	0.7	1	0.5	0.4	3.6	1.9	1.2
	7	0.4	1.8	0.8	0.5	0.3	4.4	3.3	2.6
	8	1.5	1.7	1.3	0.6	1.3	3.7	2.8	1.9
	9	0.9	4.6	3.9	3.7	0.9	1.2	0.9	1.0
	10	0.7	0.5	0.4	0.3	0.7	1.0	1.4	0.9
	Mean ± SD	0.7±0.4 CDE	1.7±1.1AC	1.1±1.0 BD	0.9±1.0E	0.7±0.3	3.3±1.3 A	2.4±0.8 B	1.8±0.8
Hindlimb (TCJ)	1	N/R	1.3	0.0	0.9	N/R	1.3	1.2	0.9
	2	1.3	1.5	1.1	0.8	1.2	2.3	2.9	2.1
	3	0.2	1.9	0.8	0.6	0.7	3.4	1.9	2.4
	4	0.2	0.6	0.7	0.6	1.0	4.0	3.5	2.0
	6	0.8	0.6	0.5	0.2	0.5	1.7	1.5	0.8
	7	0.3	0.3	0.4	0.6	0.4	2.3	3.3	2.7
	8	1.1	1.0	0.9	0.6	1	2.2	2.6	1.8
	9	0.9	0.9	0.5	3.1	1.1	2.7	2.6	1.4
	10	0.5	0.9	0.9	0.6	0.7	1.4	1.1	1.2
	Mean ± SD	0.7± 0.4	1±0.5 F	0.6±0.3 G	0.9±0.9	0.8±0.3 HI	2.4±0.9 FH	2.3±0.9 GI	1.7±0.7

Cells within the same row that have the same letter are significantly different ($p<0.05$) from one another.

Table 5.6 Synovial fluid cellular differential of RCJ and TCJs 0-108 hours after intra-articular injection of allogeneic BMSCs. Total mononuclear and neutrophil cellular differential counts (mean ± SD) of synovial fluid for all horses 0, 24, 48 and 108 hours after intra-articular treatment.

		PBS Injection								AlloBMSCs Injection							
		% Mononuclear				% Neutrophil				% Mononuclear				% Neutrophil			
		Time															
	Horse	**0**	**24**	**48**	**108**	**0**	**24**	**48**	**108**	**0**	**24**	**48**	**108**	**0**	**24**	**48**	**108**
Forelimb (RCJ)	**1**	-	82	89	95	-	18	11	5	99	59	59	99	1	41	41	1
	2	100	97	91	87	1	3	9	13	-	68	75	95	-	34	25	5
	3	96	64	89	92	4	36	11	8	99	19	52	88	1	81	48	12
	4	95	94	92	100	5	6	8	0	55	46	80	92	44	54	20	8
	5	100	74	54	98	0	26	45	2	99	48	62	79	1	52	38	21
	6	100	91	89	100	0	9	11	0	99	57	85	100	1	43	15	0
	7	59	61	62	91	41	39	38	8	97	31	34	47	3	69	66	53
	8	99	47	78	97	1	53	22	3	99	85	72	99	1	15	28	1
	9	97	66	78	81	3	34	21	19	79	50	75	91	21	50	25	9
	10	88	67	68	99	2	33	32	1	100	71	58	96	0	29	42	4
	Mean ± SD	92.7 ±13	74.3 ±16	79 ±14	94 ±6	6.3 ±13	25.7 ±16	20.8 ±13	5.9 ±6	90.9 ±16 **AB**	53.4 ±19 **A**	65.2 ±15 **B**	88.6 ±16	9 ±15.8 **CD**	46.8 ±19 **C**	34.8 ±15 **D**	12.3 ±12
Hindlimb (TCJ)	**1**	31	34	-	100	64	65	-	0	96	59	63	99	4	40	37	1
	2	-	90	90	88	-	10	10	12	-	90	86	95	-	10	14	5
	3	96	75	87	78	4	25	23	22	51	17	57	90	46	83	43	10
	4	95	87	95	88	5	13	5	12	47	88	77	97	47	12	23	3
	6	85	88	90	99	15	12	10	1	93	47	90	98	7	53	10	2
	7	98	18	26	70	2	82	74	30	85	51	56	56	15	49	44	44
	8	88	81	84	100	12	19	15	0	100	82	84	98	0	18	16	2
	9	100	68	52	71	0	32	48	29	100	77	81	89	0	23	18	11
	10	93	89	86	95	7	11	15	5	97	72	90	92	3	28	10	8
	Mean ± SD	85.8 ±23	70 ±26	76 ±24	87.7 ±12	13.6 ±21	29.9 ±26	24.9 ±24	12.3 ±12	83.6 ±22	64.8 ±23.7	76 ±13.7	90.4 ±13.4	15.3 ±20	35.1 ±24	23.9 ±14	9.6 ±13

Cells within the same row that have the same letter are significantly different ($p<0.05$) from one another.

Table 5.7 Histopathology findings of synovial membrane 108 hours after intra-articular injection of allogeneic BMSCs. Inflammatory score of synovial membrane harvested from radiocarpal (RCJ) or tarsocrural joints (TCJ) 5 days after intra-articular administration of phosphate buffered saline (PBS) or allogeneic bone marrow derived mesenchymal stem cells (alloBMSCs). Specific, but ungraded histopathological findings are recorded in parentheses.

		PBS	AlloBMSCs
	Horse	**Inflammatory score** *(Comments of Pathologist)*	
Forelimb (RCJ)	**1**	**1.5** *(Mild perivascular infiltrate, mild hemorrhage)*	**1.5** *(Mild lymphocytic infiltrate)*
	2	**1**	**2** *(Subepithelial perivascular lymphocytic aggregates)*
	3	**2**	**1**
	4	**1.5** *(Focal aggregate of plasma cells)*	**1.5** *(Focal edema and hemorrhage)*
	5	**1**	**1.5** *(Focal hemorrhage mild diffuse perivascular infiltrate of lymphocytes)*
	6	**1**	**2** *(Multifocal mild perivascular lymphocytic infiltrates)*
	7	**1.5** *(Locally extensive hemorrhage)*	**1.5** *(Locally extensive hemorrhage with scattered small perivascular lymphocytes)*
	8	**1**	**3** *(Moderate multifocal perivascular lymphocytic infiltrates)*
	Mean ± SD	**1.3 ± 0.4**	**1.8 ± 0.6**
Hindlimb (TCJ)	**1**	**1**	**1.5**
	2	**1.5**	**3** *(Moderate scattered subepithelial perivascular lymphocytic aggregates)*
	3	**1.5**	**2.5** *(Scattered aggregates of lymphocytes, macrophages with fibrin, fibrosis, focal necrosis)*
	4	**1**	**2** *(Locally extensive hemorrhage with perivascular lymphoid aggregates)*
	6	**1**	**2** *(Scattered perivascular aggregates of lymphocytes)*
	7	**1**	**3**
	8	N/R	**3.5** *(Moderate multifocal coalescing perivascular lymphocytic infiltrates)*
	Mean ± SD	**1.2 ± 0.3 A**	**2.5 ± 0.7 A**

The same letters within the same row of the table are significantly different ($p<0.05$) from one another.

Table 5.8. Delta Delta CT, fold change, and *P* values for pro-inflammatory (IL-1β, IL-6, IL-8, TNFα), anti-inflammatory (IL-10), and catabolic enzymes (MMP-1, MMP-3, MMP-13, ADAMTS 4, and ADAMTS 5) as measured by RT-PCR of synovial membrane. Synovial membrane was harvested 5 days after intra-articular administration of the radiocarpal (RCJ) or tarsocrural (TCJ) joints. Delta Delta CT and fold change values were normalized to the control (PBS injected) joints.

	Radiocarpal Joint			Tarsocrural Joint		
Genes of Interest	**ΔΔ CT (SE)**	**Fold Change (95% CI)**	***P* Value**	**ΔΔ CT (SE)**	**Fold Change (95% CI)**	***P* value**
IL-1β	0.06 (1.00)	1.0 (0.19, 5.1)	0.995	-0.15 (0.374)	1.11 (0.61, 2.02)	0.699
IL-6	-0.08 (0.98)	1.06 (0.21, 5.28)	0.936	0.475 (0.31)	0.72 (0.44, 1.17)	0.159
IL-8	0.06 (0.99)	0.96 (0.19, 4.88)	0.956	-0.29 (0.31)	1.22 (0.75, 2.01)	0.373
IL-10	0.39 (0.90)	0.76 (0.17, 3.34)	0.676	0.34 (0.70)	0.79 (0.26, 2.40)	0.637
TNFα	-0.35 (0.84)	1.27 (0.32, 5.06)	0.693	-0.50 (0.52)	1.41 (0.61, 3.26)	0.367
MMP 1	-0.50 (1.85)	1.41 (0.68, 29.41)	0.797	-2.43 (1.72)	5.37 (0.34, 83.88)	0.196
MMP 3	1.6 (6.87)	0.33 (0.11, 1.01)	0.052	-1.52 (1.19)	2.88 (0.43,19.27)	0.236
MMP 13	1.98 (1.39)	0.25 (0.03, 2.47)	0.197	-0.92 (1.95)	1.89 (0.08, 42.88)	0.648
ADAMTS 4	-0.60 (1.05)	1.52 (0.27, 8.52)	0.584	-0.24 (0.74)	1.18 (0.36, 3.83)	0.751
ADAMTS 5	0.05 (0.53)	0.97 (0.41, 2.30)	0.929	-0.61 (0.48)	1.52 (0.71, 3.26)	0.240

Values in parentheses represent the standard error for the delta delta CT values and the 95% confidence interval for gene expression fold. Fold change was calculated as $2^{-mean\ \Delta\Delta CT}$. *The* $\Delta\Delta CT$ *was calculated as follows:* $\Delta\Delta CT_{RCJ\ or\ TCJ} = \Delta CT_{alloBMSC} - \Delta CT_{PBS}$

CHAPTER 6

CONCLUSION

Osteoarthritis affects a large proportion of both the human and veterinary population. The end result of osteoarthritis is debilitating pain resulting in disability of human patients or reduced athletic performance in veterinary patients. The clinical manifestation of pain is a result of a perpetuated inflammatory cycle that degrades the overlying articular cartilage. The articular cartilage can repair itself, but the tissue that is used to repair articular cartilage defects, fibrocartilage, is biomechanically inferior and is not tough enough to withstand the hostile osteoarthritic environment. This inflammatory cycle within the local tissue environment also contributes to the formation of an inadequate repair tissue. Therefore, the use of a biological therapeutic that can treat the inflammatory cycle and promote regeneration of tissue in a fast "off the shelf", non-immunogenic manner would be highly sought after for treatment of both human and veterinary species. As demonstrated in chapter 2, mesenchymal stem cells possess both immunomodulatory and regenerative capabilities that make them a unique and attractive disease modifying biologic therapeutic. There has been very little work in the equine field, to establish the safety and efficacy of intra-articular allogeneic MSC treatment in normal and abnormal (osteoarthritic) joints. It is for these reasons that these preliminary studies investigating the intra-articular use of equine BMSCs were performed.

Chapter 3 evaluated the effects of normal allogeneic synovial fluid on BMSC viability, proliferation, and chondrogenesis. Surprisingly, 100% allogeneic synovial fluid

was able to support BMSC viability and proliferation for 72 hours of culture. Additionally, 100% allogeneic synovial fluid was able to support BMSC chondrogenesis as measured by production of glycosaminoglycan. This is a novel finding, as such high concentrations of synovial fluid to support *in vitro* BMSC culture has not been previously reported. This finding supports the maintenance of BMSC viability and proliferation after short term exposure to 100% synovial fluid, representing clinical application of BMSCs within the synovial environment. This finding also supports the intra-articular use of undifferentiated BMSCs because exposure to 100% synovial fluid enhanced BMSC chondrogenesis compared to control chondrogenic medium.

To strengthen the clinical relevancy of this study, exposure of BMSCs to the same concentrations of osteoarthritic synovial fluid should be conducted. The synovial fluid should be obtained after owner consent prior to arthroscopic evaluation of joints presumed to be osteoarthritic based on diagnostic imaging (radiography, ultrasonography, and/or MRI). An inflammatory profile (TNFα, IL-1 β, and PGE2) of the synovial fluid would be encouraged to accurately assess the degree of synovial inflammation, but if appropriate synovial fluid volume could not be obtained the arthroscopic scoring of the articular cartilage could be used to place the synovial fluid in a category of mild, moderate, or severe osteoarthritis for clinical assessment of the degree of synovial inflammation. If enough synovial fluid could not be obtained for the same supplementation concentrations, the BMSCs would be exposed to 50% or 100% allogeneic osteoarthritic synovial fluid and only viability and proliferation would be assessed with individual synovial fluid. For assessment of chondrogenesis, the synovial fluid would again have to be pooled and should be pooled based on cytokine or clinical

analysis such that the effects of mild, moderate, and severe osteoarthritic synovial fluid is assessed. To more accurately reflect the interaction of BMSCs and chondrocytes in the face of an osteoarthritic environment, micromass pellet culture would also be performed with BMSCs alone, chondrocytes alone, and 50% BMSCs/50% chondrocytes in 25%, 50%, and 100% allogeneic osteoarthritic synovial fluid. If adequate osteoarthritic synovial fluid volume could not be obtained, set concentrations of IL-1β and/or TNFα could be added to culture medium or normal allogeneic synovial fluid.

The immunogenicity of the BMSCs after exposure to mild, moderate, and severe osteoarthritic synovial fluid should also be assessed by mixed lymphocyte reactions and/or exposure to conditioned medium. This would provide confirmation that "activation" of MSCs toward an immunomodulatory cell does occur in the presence of allogeneic osteoarthritic synovial fluid. The author is currently conducting a study evaluating the direct and indirect (conditioned medium) effects of equine BMSCs on gene expression of allogeneic synovial membrane after exposure to lipopolysaccharide (LPS). This study will provide preliminary insight into the effects of allogeneic BMSCs on experimentally inflamed equine synovial membrane.

Chapter 4 evaluated the effects of short-term exposure to autologous or allogeneic PRP on the viability, proliferation, and chondrogenic capacity of BMSCs. The results of this study showed that short-term exposure of BMSCs to high concentrations of PRP caused significant reduction in BMSC viability and proliferation regardless of whether the BMSCs were exposed to autologous or allogeneic PRP. Interestingly, no difference in exposure to autologous or allogeneic PRP was observed. This finding has not been reported previously and is clinically relevant for situations in which allogeneic BMSC

administration would be pursued, but autologous PRP would be utilized for cellular delivery. The finding of reduced cellular viability in the face of high concentrations of PRP is important because BMSCs are often suspended in 100% PRP (autologous or allogeneic) for intra-lesional injection of equine tendon or ligament injuries as well as arthroscopic implantation into a focal chondral defects. However, the effects of high concentrations of PRP on BMSC viability and proliferation have not been previously evaluated despite its clinical use an agent for cellular delivery. Higher concentrations of PRP (>50%) were not evaluated in this study because of the need to keep the platelets and BMSCs physically separated for the assays that were performed. Even so, the results of this study showed a slight dose dependent decrease in cellular viability when PRP was supplemented at 50% compared to 25% PRP and this dose dependent effect would likely be observed after exposure of BMSCs to higher concentrations of PRP such as 100% PRP. This decrease in cellular viability may not be clinically relevant and not be significant for the duration of time that the cells are in suspension prior to injection (<2 hrs), but this study does call into question the composition of the PRP and its possible deleterious effects on BMSCs. Certainly, the interaction of the local tissue environment with the BMSCs and PRP suspension could play a significant role in whether the reduction in BMSC viability and proliferation is sustained *in vivo* compared to *in vitro*. The proliferation assays that were used did show discrepancies in their output concerning the effect of PRP on BMSC proliferation. These discrepancies could be a result of the need to provide dissolution of the clot that eluded from the transwell insert and subsequent cellular centrifugation that could have resulted in cell death and/or cell loss. To overcome this concern, platelet lysate rather than platelet rich plasma could have been

used, but the author chose to more accurately represent a clinical scenario in which BMSCs are exposed to high concentrations of PRP.

To improve the clinically relevancy of the study, standardization of the platelet concentration to which the BMSCs were exposed. BMSCs would have been exposed to 3, 6, and 10 fold increases in baseline platelet concentrations. These concentrations would have reflected the platelet concentration that is achieved with commercially available equipment for centrifugation or filtration of equine plasma. Additionally, these platelet concentrations are considered to be the "target" platelet concentration for musculoskeletal regeneration. Studies have not been performed to assess differences in platelet concentration on BMSC viability, proliferation, and chondrogenesis despite the arbitrary dose of PRP with a 3 to 6 fold increase in baseline platelet concentration. Further characterization of the PRP including the pH, growth factor concentration (i.e. TGFβ, IGF-1, VEGF, PDGF**)**, leukocyte concentration, and red blood cell concentration would have enhanced the conclusions that could have been made regarding the composition of PRP that is best suited for cellular suspension of BMSCs.

Additionally, evaluation of the immunogenicity of BMSCs after exposure to autologous or allogeneic PRP using a mixed lymphocyte reaction would have added to the study because PRP does contain a certain amount of red blood cells and leukocytes that could stimulate an immunogenic response and possibly activate the BMSCs capacity for immunomodulation prior to injection..

Chapter 5 evaluated the intra-articular administration of equine allogeneic BMSCs in non-ostoearthric joints. Adverse, systemic responses were not observed in 10 horses after intra-articular administration, but moderate, transient inflammatory changes

to the local synovial environment were observed. The adversity of this inflammation is unknown, meaning that if MSCs are activated towards an anti-inflammatory phenotype in the face of inflammation, this transient inflammation of the local synovial environment may activate the MSCs and ultimately result in greater reduction of the osteoarthritic inflammatory cycle. This observation could be supported by the absence of TNFα in the synovial fluid samples of joints treated with allogeneic synovial fluid. Further cytokine analysis of the synovial fluid would have contributed to this finding, but given the limited synovial fluid volume was not performed. The contribution of allogeneic BMSC administration to mRNA expression of pro-inflammatory cytokines, anti-inflammatory cytokines, and catabolic enzymes is unknown given that punch biopsies of the synovial membrane were not obtained prior to injection.

A major flaw in this study, is the lack of an autologous BMSC control. There are several reasons that the author was unable to use an autologous control. First, culture expansion of autologous BMSCs would have required hospitalization of study subjects for an additional four weeks that would have added substantial constraints to the number of study subjects that were treated and evaluated due to finances. Secondly, as demonstrated in the study, different joints can respond differently to an inflammatory stimulus. Therefore, the only joint that would have been able to be used to compare the synovial response of allogeneic BMSC administration to autologous BMSC administration (positive control) as well as administration of phosphate buffered saline (negative control) would have been the metacarpophalangeal or metatarsophalangeal (fetlock) joints. The reason these joints were not chosen by the author, were due to concerns that the volume of synovial fluid required for analysis would not be obtained

consistently from the fetlock joint. Despite this comparison, the local inflammatory changes observed after intra-articular administration of equine allogeneic BMSCs were transient and consistent with findings of previous studies that evaluated intra-articular administration of equine placentally-derived MSCs. Another limitation of the study was the use of ten different allogeneic BMSC lines. The author chose to perform the study in this manner to more accurately reflect the current clinical scenario of BMSC allogeneic use in which an ideal donor has not been established and clinicians are forced to use BMSC lines that have been previously expanded, but not evaluated for efficacy. This study could have been organized such that one or two donor lines previously characterized with tri-lineage differentiation, immunophenotyping, and *in vitro* immunogenicity assays were administered. This would have decreased the variability among the donor lines so that a more accurate assessment of the recipient response could have been made. However, to make an accurate assessment of the recipient response, the status of the recipients joint (no osteoarthritis vs. mild, moderate, or severe osteoarthritis) would have also been conducted utilizing more advanced diagnostic imaging that was financially not feasible for the scope of this study. Additionally, adding a more objective measurement of lameness such as force plate or computer gait analyses would have strengthened the study particularly considering that the patients were not sound prior to inclusion.

An experimental study in which mechanical induction of osteoarthritis is performed and intra-articular allogeneic BMSCs are administered is warranted. However, more work is needed to identify the proper dose and timing of BMSC administration as

well as whether the cells should be pre-activated toward an anti-inflammatory phenotype or injected in an inactivated state.

Future work should be directed toward establishing an *in vitro* culture system for equine synovial membrane so that the interaction of BMSCs on the innate and adaptive immune systems of the synovial membrane can be studied The importance of this interaction (BMSC and synovial membrane) has been ignored, for the most part, in the literature, but based on these and other studies it is this interaction that may hold the key to the therapeutic effect of MSCs.

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