Preliminary evaluation of equine umbilical cord blood mesenchymal stromal cells as treatment of synovitis

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Abstract

Preliminary evaluation of equine umbilical cord blood mesenchymal stromal cells as treatment of synovitis

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Joint injury is the leading cause of premature retirement of athletic horses and is associated with significant financial losses to the equine industry. As current therapies for joint injury are only palliative in nature, there is a clinical need for therapies that alter the progression of joint disease. Despite a lack of evidence, mesenchymal stromal cell (MSC) therapy is being pursued for treatment of joint disease in the hope that the MSC will alter disease progression. The twofold purpose of this thesis is to evaluate the effect of allogeneic MSC therapy as treatment for synovitis and to evaluate strategies for improving MSC delivery methods in order to maintain optimal MSC viability.

Intra-articular injection of allogeneic equine umbilical cord blood (CB) MSC with confirmed in vitro lymphocyte suppressive properties into normal joints resulted in a mild self-limiting inflammatory reaction. Co-injection of lipopolysaccharide (LPS) and allogeneic CB-MSC significantly decreased the cellular response associated with the initial inflammatory insult while inducing mild self-limiting inflammation.

We also determined that cryogenic storage did not affect the in vitro lymphocyte suppressive capacity of umbilical cord blood (CB) derived MSC cultures and that injection through various diameter hypodermic needles did not decrease CB or bone marrow (BM) derived
MSC viability in vitro. Aspiration, however, of BM and CB-MSC did significantly decrease MSC viability indicating care is needed where MSC suspensions are subject to negative pressure.

The intravenous safety profile of two carrier solutions proposed for the final product formulation for excipient use of CB-MSC was tested in vivo. Excipient use of HypoThermosol® FRS or Cryostor® alone or in combination with pooled allogeneic CB-MSC resulted in no change in outcome parameters of physical exam, CBC, biochemistry profile, or coagulation profile in healthy ponies. Interestingly, an increase in CD4+ and CD8+ lymphocyte populations were observed 168h post CB-MSC injection indicating a possible allogeneic immune response.

This is the first report of a quantitative effect of IA CB-MSC therapy in a randomized blinded experimental study. Further study is needed to evaluate IA MSC therapy in chronic inflammatory models or naturally occurring osteoarthritis. Questions of interest include evaluating dose, timing of treatment, effect of multiple injections, and conditions responsive to MSC treatment in horses.
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I would also like to acknowledge Dr. Thomas Koch for his role as my advisor. Thank you Thomas, for teaching me cell culture techniques and opening my eyes to the world of regenerative medicine. Thank you for your patience as I began this research. I’m sure it was often apparent during the first year that my focus was on the clinical aspects of my residency rather than my research much of the time. I appreciate your confidence in my skills and your encouragement to present abstracts and submit manuscripts for publication. Of course, none of this could have occurred without the incredible amount of time you spent teaching me about academic writing and helping me edit the manuscripts and figures that I sent you at all hours of the night. I recognize the irony in this since English is my first language yet I seem immune to the influences of spelling and grammar.

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Declaration of Work Performed

I declare that all work in this thesis was performed by Lynn Williams with the following exceptions: CB-MSC isolation and initial cryopreservation was performed by Marianne Stämpfli and various undergraduate students working in the Koch lab. Irradiation of cell suspensions for mixed lymphocyte reactions was performed by Kim Stewart. Flow cytometry used for evaluation of mixed lymphocyte reactions was performed by Laurence Tessier. Some additional assistance with flow cytometry to evaluate CD4+ and CD8+ lymphocyte populations was provided by Carmon Co. Routine cytology of synovial fluid analysis, complete blood counts, serum biochemistry profiles and coagulation profiles were performed by the technicians at the Animal Health Lab, University of Guelph. Interpretation of synovial fluid cytology was provided by Drs. Kristiina Ruotsalo and Emmeline Tan. A significant portion of the ELISA assays associated with synovial fluid biomarkers was performed by Michelle Bedouin-Kimble. Programming associated with statistical analysis was performed by William Sears. Lameness evaluations were performed by Drs. Judith Koenig and Belinda Black.
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**List of Abbreviations**

7-AAD- 7-aminoactinomycin D  
AA-arachidonic acid  
AB- alamar blue  
ACS- autologous conditioned serum  
AIC- Akaike information criterion  
ANOVA- analysis of variance  
AR- autoregressive (1) error structure  
ARH- heterogeneous autoregressive error structure  
AT adipose tissue  
ATP- adenosine triphosphate  
AV- annexin V  
BM- bone marrow  
BM-MSC- bone marrow derived mesenchymal stromal cell  
BMA- bone marrow aspirate  
BMS- bone marrow supernatent  
BrdU- bromodeoxyuridine  
C1,2C- non-specific type I & II collagen cleavage assay  
C2C- type II collagen cleavage assay  
CB-MSC umbilical cord blood derived MSC  
CB- umbilical cord blood  
CBC- complete blood count  
CCAC- Canadian council for animal care  
CI- confidence interval  
xx
ConA-concanavalin A
COX-cyclooxygenase
CP-II-type II collagen synthesis C-propeptide
CS-10-CryoStor® containing 10% DMSO
CS-846-condroitin sulfate CS-846 epitope of aggrecan synthesis
CT-computed tomography
CTL-cytotoxic lymphocyte
DMEM-Dulbecco’s modified eagle medium
DMMB-1,9-dimethylmethylene blue assay
DMSO-dimethylsulfoxide
EDTA-ethylenediaminetetraacetic acid
ELISA-enzyme linked immunosorbent assay
FDA-United States food and drug administration
FITC-flourescein isothiocyanate
FRS-free radical scavenger
Ga-gauge
GAG-glycosaminoglycan
HA-hyaluronic acid
HGF-hepatocyte growth factor
HSC-hematopoietic stem cell
HT-FRS-HypoThermosol® containing free radical scavengers
HT-hypothermosol
IA-intraarticular
IDO-indoleamine 2,3-dioxygenase
xxi
IGF-insulin-like growth factor
IL-1-interlukin-1
IL-8-interlukin-8
IL-6-interlukin-6
IL1Ra-interleukin 1 receptor antagonist
IV-intravenous
LPS-lipopolysaccharide
LTB4-leukotriene B4
MHC-I-major histocompatibility complex type I
MHC-II-major histocompatibility complex type II
MIA-monosodium iodoacetate
MLR-CM-mixed lymphocyte reaction culture media
MLR-mixed lymphocyte reaction
MMP-matrix metalloproteinases
MRI-magnetic resonance imaging
MSC-CM-mesenchymal stromal cell culture media
MSC-mesenchymal stromal cell
MSCx-mitotically arrested (irradiated mesenchymal stromal cells
MTT-colorimetric cell proliferation assay
NK-natural killer lymphocyte
NO-nitric oxide
NSAID-nonsteroidal anti-inflammatory drug
OA-osteoarthritis
OC-osteochondrosis
xxii
OMAF-Ontario ministry for agriculture and food

OR-odds ratio

PBL-peripheral blood lymphocyte

PBLx-mitotically arrested (irradiated) peripheral blood lymphocytes

PBS-phosphate buffered saline

PGE\textsubscript{2}-prostaglandin E\textsubscript{2}

PGF\textsubscript{1α}-prostaglandin F\textsubscript{1α}

PHA-phytohemagglutinin

PI-propidium iodide

PPV-positive predictive value

PROCMIXED-notation for mixed model in SAS software

PSGAG-polysulfated glycosaminoglycan

PTC-post-thaw culture

PTNC-post-thaw non-culture

ROS-reactive oxygen species

RPMI-Roswell Park Memorial Institute medium

RT-room temperature

SAS-statistical analysis software

SD-standard deviation

SEM-standard error of the mean

SF-synovial fluid

SVF-stromal vascular fraction

TB-trypsin blue

TB\textsubscript{2}-thromboxane B\textsubscript{2}
TC-tarsal crural joint
TGF-transforming growth factor
Th-T helper lymphocyte
Th1-T helper lymphocyte type 1
Th2-T helper lymphocyte type 2
TNCC-total nucleated cell count
TNF-tumor necrosis factor
TOEP-toeplitz error structure
TOEPH-heterogeneous toeplitz error structure
TP-total protein
Treg-regulatory T lymphocyte
WBC-white blood cell
WST-colorimetric cell proliferation assay
Introduction

The equine industry contributes approximately $19 billion to the Canadian economy, of which $7 billion is within the province of Ontario (1). Approximately 900,000 horses reside within Canada and 400,000 within the province of Ontario. Lameness afflicts more than 50% of racehorses annually and up to 70% of lost training days are a result of lameness (2-5). Joint injuries are the main cause of lameness in the horse. Treatment of joint injuries; however, remains inadequate since current therapies are limited to reducing inflammation and often require continuous administration.

A widely accepted theory of MSC function is that in the presence of disease conditions MSC secrete chemical messengers that act to regulate cellular processes and thereby encourage a return to homeostasis of the host tissue. These properties have lead equine practitioners to administer MSC as treatment for various inflammatory-associated conditions such as joint and tendon injuries. This thesis evaluated the effects of umbilical cord blood (CB) MSC in inflammatory environments in vitro and in vivo. Using in vitro mixed lymphocyte reactions, a lymphocyte suppressive CB-MSC culture was selected for evaluation in-vivo using lipopolysaccharide (LPS) induced synovitis.

This investigation also evaluated several strategies for improving the delivery of MSC in clinical scenarios. The effect of aspiration and injection, through various needle diameters, on MSC viability was evaluated. To investigate a potential final product formulation for allogeneic CB-MSC suspensions two carrier solutions for excipient injection (HypoThermosol®-FRS, CryoStor® 10) and pooled allogeneic CB-MSC were evaluated for safety for IV injection.
Chapter 1: Literature review

Normal Structure and Function of Equine Joints

Synovial joints consist of two or more adjacent bones covered in articular cartilage and supported by fibrous joint capsule, muscles, ligaments and tendons that span the joint (8). Synovial membrane covers the interior of the joint capsule and attaches along with the joint capsule to bone on either side of the joint (8). The intimal layer—usually one to four cells thick—is primarily responsible for filtering and containing the synovial fluid. Beneath the intimal layer lies the subintimal layer—made up of fibrous, areolar, and fatty tissue—which contains vasculature and innervation to the synovial membrane. This arrangement allows containment of synovial fluid and regulation of the synovial environment. The synovial joint has two major functions: allowing near-frictionless movement between opposing bone ends and supporting the musculoskeletal system by transferring loads to weight-bearing surfaces (9). Near frictionless movement results from a thin film of fluid that is exuded from the articular cartilage when pressure is applied, lubricating the cartilage surfaces in contact (10, 11). The remarkable, near-frictionless nature of articular cartilage is a result of “weeping” (10) or “elastohydrodynamic” (8) lubrication that allows a coefficient of friction ten-fold less than ice gliding on ice (12-14). In addition to providing near frictionless movement large forces are transmitted across synovial joints with each stride. Forces equal to 2.1, 4.1, and 4.7 times a horse’s body weight are transmitted across the metacarpal-phalangeal-joint with each stride at walk, trot, and canter; respectively (15). These forces compress the area of cartilage in contact with adjacent cartilage causing fluid movement away from the area into the synovial fluid and surrounding articular cartilage while maintaining a thin fluid film separating opposing cartilage surfaces. The resulting shock absorption increases stability by increasing contact surface area and allows lubrication of
the joint space (9, 10). The loadbearing capability of articular cartilage is primarily attributed to articular cartilage’s ability to remain hydrated under significant pressure. This is attributed to the osmotic pressure associated with high concentrations of proteoglycan and the unique configuration of collagen fibrils that provide great tensile strength.

Articular cartilage consists of hyaline (glass-like) cartilage that is made up of 1-12% chondrocytes, depending on location (9). The remaining non-cellular component is comprised of water, collagen fibrils, and proteoglycan. Water is the most plentiful component of equine cartilage making up 70% and 80% of cartilage in adults and neonates, respectively (9). Collagen fibrils in articular cartilage are primarily type II collagen which is identified as a triple helix of identical alpha chains of amino acid with the sequence of Gly-X-Y with X and Y often being proline and hydroxyproline (9). These collagen fibrils also contain small amounts of types III, VI, IX, X, and XI, XII, XIV collagen (9, 16) and make up 50% of cartilage on a dry weight basis (8). In the body, type II collagen is mainly located in articular cartilage allowing synthesis and breakdown products of type II collagen to serve as important biomarkers of cartilage turnover (17).

Arrangement of collagen fibers differs depending on the distance from the articular surface to where the fiber is located. Superficially collagen fibers are oriented parallel to the joint surface providing strength against shear forces achieved with compression of loadbearing cartilage. The intermediate zone contains fibers aligned in an intricate three-dimensional network allowing compression and absorption of weight bearing forces while stabilizing compressive and shear forces. The deep zone and the calcified cartilage adjacent to the subchondral bone contain collagen fibers aligned perpendicular to the joint surface providing secure attachment to subchondral bone (9).
Proteoglycan, dispersed between collagen fibers, is the third major component of articular cartilage and makes up 35% of articular cartilage dry weight. Proteoglycan molecules are comprised of a protein core covalently bonded to multiple glycosaminoglycan chains in a “bottlebrush” arrangement (9). The glycosaminoglycan chains are hydrophilic due to their large charge density. These proteoglycan molecules provide compressive stiffness to articular cartilage due to their strong attraction to water. Articular cartilage responds to compression by releasing water then and reabsorbing it when pressure is released (9). In addition to shock absorption, this dynamic response provides lubrication when needed in response to compression—allowing near frictionless movement (8, 10, 18).

The combination of collagen fiber arrangement, hydrophilic proteoglycan molecules, and high water content allow cartilage to behave as a heterogeneous, elastic, fiber reinforced, hydrated gel that is able to react to forces as a resilient, self lubricating, near-frictionless tissue ideal for synovial joints.

**Joint Injury Epidemiology and Pathophysiology**

Twenty years after the first epidemiologic studies of wastage in racehorses (3, 4, 8), joint disease remains the most significant cause of poor performance (5). Lameness in thoroughbred racehorses is reported to affect 53-68% of animals in one racing season, with joint injuries (fetlock and carpus) being the predominant cause (3, 4, 8). Lameness in show jumpers accounts for 77% of training days lost (22% acute, 55% chronic) without further elaboration as to the cause of lameness (2). Overall 4-8% of all available training days are lost due to lameness within racing (thoroughbred and standardbred) and show jumping disciplines (2-5). Considering horse owners, on average, invest $7000 annually in each animal and $2.6 billion collectively in the
province of Ontario alone (1)—not to mention time and emotional investments—rapid treatment of lameness is a significant concern for horse owners (19).

Synovitis commonly results from primary physical damage to cartilage due to abnormal loading of joints, inherent abnormalities in cartilage or bone, and septic conditions (8, 20, 21). Secondary cartilage damage often follows as a result of metabolic tissue failure following a disturbance in the balance between cartilage repair and reabsorption. If the reabsorption of cartilage exceeds its slow regenerative capacity, a net loss of cartilage will result due to chondrocyte destruction, type II collagen breakdown, and degradation of the cartilage matrix (8, 17, 22). Re-absorptive pathways are up-regulated through activation of matrix-metalloproteases (MMP)—collagenases, strombelysins, and gelatinases—by inflammatory mediators stimulated by the primary injury. These enzymes cleave type II collagen (collagenase), unwind and degrade cleaved collagen (gelatinases) and break down proteoglycan (stromelysins) (23). Further propagation of the damage occurs as a result of exposed collagen and/or subchondral bone, which initiate further cytokine signaling leading to a cascade of inflammatory events (22). This cytokine “storm” causes migration of inflammatory cells, changes in vascular permeability of the synovial membrane, joint effusion, synovial edema, synovial hyperplasia, and increased synovial fluid total protein (21).

Cytokines associated with propagation and regulation of inflammation in equine joints include interleukin 1, 6, and 8 (IL-1, IL-6, IL-8), Tumor necrosis factor (TNF), insulin like growth factor (IGF), and transforming growth factor (TGF) (8, 22). Study of IL-1 and TNF show these two cytokines have a similar and synergistic effect to regulate catabolic pathways through suppressing proteoglycan and collagen synthesis, and stimulation of prostaglandin and collagenase production (23, 24). IGF and TGF appear to regulate anabolic pathways in cartilage homeostasis. Both stimulate proteoglycan and type II collagen synthesis thereby reducing
catabolic effects in an effort to repair damaged cartilage (25-27). In cases of clinical synovitis anabolic regulation of cartilage homeostasis is down-regulated or exhausted allowing increased production of prostaglandin E2, and MMP, which further compound cartilage damage (9, 17). Overall this metabolic shift towards catabolism results in cleavage of type II collagen and subsequent weakening of the structural integrity of the articular cartilage (21). The resulting complex pathway ultimately causes cartilage thinning, chondrocyte destruction and is clinically observed as lameness, joint effusion, decreased range of motion, and radiographic abnormalities in the horse (8).

**Diagnosis of Synovitis**

*Current Clinical Practice*

As joint injury is the most common cause of lameness, equine veterinarians are challenged with diagnosing synovitis on a daily basis. Clinically synovitis presents as one of two clinical pictures: acute, and chronic inflammatory processes. Although relatively less common acute inflammatory processes involving articular structures commonly present as emergencies to practitioners as severe or non-weight-bearing lameness (28). Often if such lameness is localized to a joint it occurs as a result of intra-articular fractures or septic arthritis. Differentiation between these two conditions requires radiography (for diagnosis of a IA fracture) and synovial fluid analysis (for diagnosis of septic processes) (28). Diagnosis of chronic synovitis (often referred to as osteoarthritis) is often a daily occurrence for equine veterinarians. This requires a clinical lameness examination and often regional or intra-articular anesthesia (21). Once lameness is localized it is often investigated further by taking a series of radiographs to attempt to confirm the
diagnosis by detecting bony abnormalities. Based on clinical experience, synovial fluid analysis does not provide enough relevant information to be performed for the diagnosis of chronic synovitis. Of the many methods discussed below, clinical lameness examinations, regional anesthesia, and radiography remain the cornerstones of synovitis diagnosis due to the limitations, risks, and expense associated with other methods.

**Conventional methods**

Clinical lameness evaluation remains the front-line for detection of synovitis despite a lack of sensitivity, specificity, repeatability, or adequate inter-observer correlation (29, 30). Targeted assessment of joint damage may include diagnostic imaging (radiography (31-33), ultrasound (34-36), CT (37, 38), MRI (32, 39)), arthroscopic visualization (38, 40), synovial membrane biopsies (41), routine cytological evaluation (41), biomechanical/biochemical evaluation of cartilage biopsies (42), and evaluation of specific biomarkers of cartilage damage and joint inflammation (40, 43, 44). A selected panel of these diagnostic tools or even all of them may be utilized in the diagnostic assessment of lameness. These targeted tests, however, must be interpreted with knowledge of their limitations and side effects. A lack of sensitivity/specificity (radiography, ultrasonography), logistical challenges (arthroscopy, synovial membrane biopsy, MRI, CT), the invasive nature of the procedure (arthroscopy, synovial membrane biopsy, cartilage biopsy), risk of iatrogenic morbidity (arthroscopy, synovial membrane biopsy, cartilage biopsy), and expense hinders investigation using many of these modalities in most clinical settings.
**Gold standard of OA diagnosis**

Current gold standard for diagnosis of joint disease is either arthroscopy or MRI because each modality allows direct assessment of articular cartilage. Equine veterinarians do not routinely use a gold standard to diagnose synovitis due to the risk and cost associated with each procedure. Since general anesthesia is required for both procedures, the risk of mortality—approximately 1%(45)—cannot be taken lightly. Additionally, even though a minimally-invasive surgery, arthroscopy is not without complications; for example, septic synovitis is reported to occur in 1% of horses that underwent elective arthroscopy (46). Complication rates from human medicine document 0.5% overall complication rate with up to 5% reported for shoulder arthroscopy (47). For many conditions these potential risks reserve arthroscopy for conditions where surgical intervention is deemed necessary or when anatomy, patient size and specific circumstances necessitate diagnostic arthroscopy.

Despite gold standard status for diagnosing joint disease, MRI and/or arthroscopy are often not feasible due to the significant cost associated with each.Sophisticated equipment is required along with technical skill for administering anesthesia, performing surgery, and/or performing and interpreting an MRI. As a result, despite being considered “gold standard” neither MRI nor arthroscopy are used for routine diagnosis of synovitis; both are reserved for when less invasive, less complicated, and less expensive diagnostic tests have failed to provide conclusive evidence of injury.

**Routine synovial fluid (SF) analysis**

SF is obtained by arthrocentesis which is considered minimally invasive, low risk, and inexpensive. Arthrocentesis involves aspiration of SF through a needle inserted through the skin
and into the joint space. A basic SF analysis provides significant information about the synovial environment through total nucleated cell count (TNCC), total protein (TP), differential cell count, and evaluation of the mucin clot. Elevations in TNCC and/or TP as well as a shift from the predominantly mononuclear cell population to a neutrophil population all are signs of an inflammatory condition (21). These changes are particularly evident in septic conditions making arthrocentesis and SF analysis essential under these circumstances. In combination with the clinical picture, SF analysis provides valuable information to allow the veterinarian to make informed treatment recommendations (21, 28, 41).

**Synovial fluid biomarker analysis**

Assessment of SF biomarkers has become popular when evaluating synovitis in a research setting (17). SF biomarkers are substances detected by biochemical or immunochemical methods that provide information on joint health and homeostasis. Evaluation of these biomarkers is intended to add information and increase the accuracy of an assessment of the synovial microenvironment and to evaluate tissue pathology and/or subsequent therapeutic efficacy (17, 40, 42, 43, 48). The definition of biomarkers has been applied to substances detected by immunological and biochemical means and more recently has been applied to genetic markers, microarrays, and abnormalities detected through imaging techniques (17). This broader definition is not, however, well represented in the literature. Biomarkers continue to be divided by their method of detection: biochemical or immunological. Unfortunately, evaluation of synovial fluid biomarkers creates added expense, requires sophisticated equipment and demands technical skills to perform the assays. In addition, a single biomarker has not been identified that indicates reliably both the presence of synovitis and is often not indicative of the degree of damage to
articulart structures. As a result, evaluations of SF biomarkers are generally excluded from clinical use due to added expense, logistical challenges, and a lack of a single marker of joint health.

Use of a biomarker would ideally enable detection of joint disease earlier than conventional methods, assess the progression of disease, and be able to detect subclinical disease, thereby predicting future injury. In veterinary medicine, biomarkers of joint disease evaluated in the horse include substances that are up- or down-regulated in the presence of inflammation and substances that result from cartilage anabolism or catabolism. Targeted development of biomarkers of osteoarthritis (OA) in human medicine has resulted in 5 categories designed to each answer specific diagnostic questions (49). These categories include: 1) diagnostic, 2) burden of disease (acute/chronic), 3) prognostic, 4) efficacy of intervention, and 5) investigative (49). Current biomarkers validated in the horse fall in the diagnostic (43, 48) and burden of disease (40, 48) categories. Inferences can be made as to the efficacy of intervention when pre- and post-treatment diagnostic biomarkers are compared (43, 50, 51); however, prognosis and investigative (predictive) biomarkers of OA are largely undeveloped in veterinary medicine.

Bertone and colleagues (2001) (48) evaluated a panel of potential biomarkers of inflammation, cytokines and eicosanoids, for the ability to detect OA in horses. Of the cytokines evaluated (TNF-α, IL-1β, II-6), TNF and IL-1β had a “good” positive predictive value (PPV>0.5) and IL-6 achieved an “excellent” PPV (>0.75) for detection of joint disease in horses. All three cytokines also correlated highly with SF white blood cell (WBC) count. Likewise, of the eicosanoids evaluated (prostaglandin E₂ (PGE₂), prostaglandin F1-alpha (PGF₁-α), leukotriene B₄ (LTB₄), thromboxane B₂ (TXB₂)), elevated SF PGE₂ appeared to be an “excellent” (PPV>0.75) predictor of joint disease. Elevated levels of the remaining eicosanoids were considered a “good” (0.5<PPV<0.75) predictor of joint disease (48). While elevated levels of a single cytokine or
eicosanoid achieved a PPV of >0.5, only elevated PGE$_2$ was more effective at predicting joint disease than SF WBC count (48).

Biomarkers of changes in cartilage metabolism include type II collagen or procollagen fragments, changes in GAG populations, and detection of activated enzymes capable of damaging articular structures within the synovial fluid. Increased concentrations of carboxypropeptide of type II collagen (CP-II) and chondroitin sulfate (namely the CS-846 epitope) are indicative of up-regulated synthesis of type II collagen and cartilage matrix, respectively (52)—indicating cartilage anabolism. Increases in CP-II and CS-846 have been observed in osteochondral fragmentation (52), endotoxin induced synovitis (43), repeated corticosteroid (triamcinolone acetate) administration (53, 54). Interestingly, significant suppression of CP-II was observed following repeated methylprednisolone acetate administration suggesting this corticosteroid suppresses cartilage anabolic processes thereby inhibiting cartilage repair processes (53).

Cleavage of type II collagen and release of GAG molecules into the synovial fluid are observed as signs of cartilage breakdown. Collagenase specifically cleaves all three strands of the type II collagen triple helix at a specific location ¾ of the length from the N-terminal end (55). Following collagenase cleavage, the new C-terminal end is recognized as a new epitope and can be detected by the monoclonal C2C antibody (53,54) or the polyclonal C1,2C (57) antibody. C2C differs from C1,2C in that C2C only binds to cleaved type II collagen whereas the C1,2C antibody nonspecifically binds to cleaved fragments of both type I and type II collagen (56, 57). Elevated C2C, indicating cartilage damage, has been observed in equine LPS induced synovitis (43) yet not observed in horses with osteochondral fragmentation (58) indicating the inflammatory processes associated with collagenase induced cartilage damage.
Release of GAG into the SF as a result of cartilage matrix degradation is detected through the 1,9 dimethyl methylene blue (DMMB) assay (52). Conflicting reports regarding the usefulness of SF GAG levels have been reported leading authors to disagree on the usefulness of this biomarker (17, 59). Significant elevations in GAG have been observed by some authors in horses with LPS induced synovitis (43), experimental osteochondral fragmentation (60), septic arthritis, (61), osteochondrosis (61), and traumatic arthritis (61). No significant change in GAG SF concentrations has also been observed in OC (58), and GAG levels have been observed to not correlate with the degree of macroscopic cartilage damage (59). These conflicting reports indicate that elevated GAG, although a useful indicator of cartilage matrix degeneration should not be evaluated in isolation.

Another biomarker of joint injury in the horse is increased matrix metalloproteinase (MMP) activity in the SF. Though not a direct marker of damage to articular structures, an increase in total MMP activity indicates an increase in one or more enzymes that cleave and denature type II collagen (17). In adult horses increased MMP concentrations closely correlate with the presence of OA (62, 63) and the severity of cartilage damage observed (59). Elevated MMP activity has been observed in septic arthritis (63), and LPS induced synovitis (43) but not in normal joints and joints with non-inflammatory pathology, ie. osteochondrosis (58, 59, 63). Despite this, MMP activity is not utilized commonly in clinical diagnosis of joint injury due to the complexity of the assay and specialized equipment and expertise required. Additionally, MMP activity is not a useful predictor of preexisting joint damage, just ongoing inflammatory conditions (63, 64).

Currently, evaluation of SF biomarkers is not performed clinically, despite proving effective in vitro (48, 62), in synovitis/OA models (43, 60, 65, 66), and in naturally occurring OA (43, 48, 63, 64). No single biomarker, or panel of biomarkers, provides sufficient information for
diagnosis of joint injury or differentiation between joint injuries. Clinical use is further limited by the sophisticated equipment, required expertise, and added expense associated with analysis of SF biomarkers.

**Summary**

Although there are many potential tools for diagnosis of joint injury, the many risks, several challenges and the expense associated with many of these methods severely limit the tools available to the equine practitioner. As a result, the equine practitioner is often restricted to making diagnostic and treatment recommendations for joint injuries on the basis of clinical examination and radiography. Restriction to these modalities has significantly hindered the diagnosis of joint injury in a clinical setting. There is a distinct need for improvement of the accuracy of existing modalities and/or new modalities with a focus on practicality, cost effectiveness, and reduced risk of iatrogenic morbidity or mortality while seeking a diagnosis.

**Current Synovitis Therapy**

**Introduction**

Currently the focus of systemic and IA therapies involves slowing the progression of synovitis, since reversal of synovitis associated articular damage has yet to be demonstrated (19, 67). Current therapies, including systemic and topical non-steroidal anti-inflammatories (NSAIDs), corticosteroids, hyaluronic acid, polysulfated glycosaminoglycans, and autologous conditioned serum have improved comfort and slowed the progression of disease; resulting in improved performance (3, 19, 60, 65, 68). These therapies provide much of their benefit through
reduction of inflammation associated with synovitis (19, 60, 66, 68-70), however, they are not without deleterious effects on joint health (67) nor do they reverse preexisting articular damage.

**NSAID therapy**

Systemic NSAID administration currently is the first line of treatment for synovitis. NSAIDs act on the arachidonic acid (AA) pathway, inhibiting further inflammatory mediator production. Briefly, AA is released from the phospholipids of cell membranes following cellular injury. AA is metabolized by either cyclooxygenase (COX) into prostaglandins, or by lipoxygenase into leukotrienes (67), both of which stimulate the production of inflammatory molecules. NSAIDs primarily inhibit COX; thereby prevent prostaglandin formation and further activation of the remainder of the inflammatory cascade (71). Beneficial effects of COX inhibition include relief of mild to moderate pain, decreased inflammation, and suppression of fever (67, 68). Two isoforms of COX have been described (71) with COX 1 responsible for maintenance of mucosal barriers, vascular regulation, renal blood flow, and renal tubular function (71). COX 2 regulates inflammation throughout the body (67, 71, 72). Adverse side effects and variable efficacy of different NSAIDS is attributed to differences in the extent to which COX 1 and COX 2 enzymes are inhibited. Undifferentiated COX suppression, or primarily COX 1 suppression, is associated with gastric ulceration, right dorsal colitis, and renal tubular necrosis. COX 2 selective NSAIDs are associated with fewer side effects over longer periods of time while providing analgesia. With the exception of the topical NSAID preparation of diclofenac, all NSAIDs are currently administered systemically. As a result the side effects associated with COX suppression pose a significant risk to patient health especially with chronic treatment that often is required for treatment of chronic synovitis.
**Corticosteroid therapy**

Corticosteroids act on the AA pathway by inhibiting phospholipase A$_2$ preventing the formation of AA (73). This in turn decreases both prostaglandin and leukotriene derived inflammatory molecules. Corticosteroids therapy inhibits production of inflammatory cytokines, retards neutrophil function, and prevents the release of lysosomal enzymes (67). Administration of corticosteroids is a common synovitis therapy despite controversy associated with their use. Corticosteroids are commonly administered by IA injection rather than systemically to maximize efficacy and minimize systemic side effects. The most common side effects reported following IA steroid injection in the horse are steroid induced arthropathy, post injection non-septic inflammation, iatrogenic septic arthritis, and laminitis. Septic arthritis following steroid injection has been reported to have an incidence of 1/10,000 and 7.8/10,000, in humans (73) and horses (74), respectively. Although relatively rare, septic arthritis following intra-articular steroid injection exhibits many similar clinical signs as those during a non-septic post injection inflammation. Acute post injection inflammation or “flare” has been reported following 2-12% of intra-articular injections (73, 75) with the majority being mild and self-limiting. Though there is controversy about the risk of steroid induced arthropathies and laminitis (76), these concerns have resulted in guidelines for cautious and prudent clinical use (19). Despite the potential for adverse effects, the majority of equine practitioners believe that IA corticosteroid administration is beneficial and that side effects can be minimized through strict asepsis and careful selection of steroid type, dose, and frequency of administration (19, 67).
Hyaluronic Acid

Hyaluronic acid (HA), or hyaluron in its uncharged form, is a non-sulfated naturally occurring substance found in SF and articular cartilage made up of repeating disaccharide units in long un-branched chains. HA serves an important role of increasing the viscoelasticity of synovial fluid, facilitating boundary lubrication of articular soft tissues, and steric hindrance of inflammatory mediators and leukocytes in the SF (77). Although its mechanism is not fully understood, HA supplementation has been shown to restore viscoelasticity of SF (77, 78), improve boundary lubrication of SF (79), decrease chondrocyte apoptosis (80), and decrease cartilage fibrillation (60) when administered to damaged joints. Although initially thought to strictly have a role in viscosupplementation (81), anti-inflammatory properties are also attributed to HA as a result of steric hindrance (non-specific exclusion of inflammatory cells and proteins due to the large molecular size of HA) (77). Following intra-articular HA injection significant improvement in subjective lameness examinations (82) and force plate lameness quantification (81) have been observed in racing horses. Following intra-venous administration of HA, decreased SF TP, SF PGE, vascularity within the synovial membrane, and fibrillation of the articular cartilage (66) have been observed. These changes indicate HA possess so-called disease-modifying properties that are beneficial in improving joint health and slowing progression of joint disease.

Polysulfated glycosaminoglycan (PSGAG)

PSGAG is a semi synthetic preparation of repeating disaccharide units containing three sulfate esters per disaccharide unit (77). The principal glycosaminoglycan in commercial PSGAG formulations is chondroitin sulfate, which is naturally found in aggregating proteoglycans that
make up much of the cartilage matrix. Early reports as to the in vitro effects of PSGAG on cultured chondrocytes revealed mixed results. The first report of stimulated proteoglycan production (83) could not be repeated in similar in vitro studies (84, 85). Joints treated with PSGAG intra-articularly (60, 86) showed decreased size of experimentally induced osteochondral defects, decreased lameness, and increased range of motion. Although the exact mechanism is not known, PSGAG are thought to inhibit enzymes such as MMPs and serine proteases that damage articular cartilage during inflammatory insults (19). As a result PSGAGs are used for their disease modifying ability in cases with suspected or confirmed cartilage damage.

*Autologous conditioned serum*

Autologous conditioned serum (ACS) represents a recent approach to synovitis treatment though specific-cytokine inhibition. This is achieved through up-regulation of interleukin-1 receptor antagonist (IL-1Ra) protein that is produced by monocyte and macrophage cell populations. IL-1Ra down-regulates IL-1, a major pro-inflammatory cytokine associated with synovitis resulting in decreased inflammatory stimulus. ACS is prepared using commercially available kits (87) or in glass “serum” vials (88) from autologous whole blood by activation of the monocyte population and used to treat mild to moderate synovitis. It is important to note that although increased IL-1Ra is observed in ACS, the composition of growth factors and cytokines present in ACS is not entirely known. In a comparison of three methods (two commercially available kits and glass serum vials), IL-1Ra was observed in different quantities by all systems when compared to non-incubated blood (88). In the same study, presence of multiple anti-inflammatory growth factors in the SF suggests IL-1Ra is not solely responsible for improvement observed in clinical cases (65). Clinically, ACS was evaluated in the horse in one clinical trial using an osteochondral fragment model for inducing OA (65). In this trial, improvement in
lameness and decreased synovial hyperplasia was achieved in the treated animals. To confirm the role of IL-1Ra in modulating inflammation associated with OA, two studies evaluated the effect of adenoviral vector based gene transfer of IL1-Ra coded DNA in an osteochondral fragment model (89) and a full thickness cartilage defect model (90). These studies report clinical and histological improvement as well as improvement in biomarkers of inflammation and cartilage catabolism in treated animals (89, 90). Increased IL-1Ra levels were detected in SF of horses in both studies for several weeks at similar levels to animals receiving ACS (65, 89, 90). In summary, these findings indicate that although antagonism of IL-1 is beneficial in decreasing inflammation, the beneficial effects of ACS cannot be attributed to IL-1Ra alone as many other growth factors and cytokines in this heterogeneous mixture could also be responsible for the improvement observed in clinical cases (88).

**Summary**

Existing treatments commonly used for treatment of synovitis in the horse are useful for decreasing clinical signs and slowing progression of underlying pathology. Conventional synovitis treatments, however, have been unable to do more than modify the progression of disease. New therapies are needed to halt and ideally reverse the progression of the clinical, biochemical, and histological changes associated with synovitis.

**Induced synovitis in experimental models**

Experimentally, treatment of synovitis has been evaluated using numerous animal models (91-97). Equine synovitis experimental models can be grouped into two categories: injection of inflammatory substances and attempts to replicate specific disease processes. Inflammatory
substances that have been injected IA include monosodium iodoacetate (MIA) (94), live *S. aureus* bacterial cultures (42), *E. coli* derived LPS (93), and IL-1 (95). These models—with the exception of MIA—cause a temporary, mild synovitis that allows the researcher to evaluate treatments in the acute inflammatory phase of disease. Although MIA injection results in acute inflammation, significant cartilage erosion occurs resulting in prolonged inflammation and lameness (94).

Attempts at replicating specific disease processes have lead to the development of models involving joint instability, mechanical trauma, overuse, and immobilization. Instability, mechanical trauma and overuse models of synovitis differ only in the mechanism that trauma is inflicted, whereas immobilization of joints results in pathological changes as a result of chondrocyte nutrient deprivation. Trauma occurs gradually in instability models as a result of abnormal forces placed upon a joint (96). These models replicate certain naturally occurring disease processes, i.e. cruciate or collateral ligament pathology, and vary in the amount of articular damage that occurs. Mechanical trauma models initiate synovitis through deliberate surgical damage of articular structures (60, 66, 89). As such, controlled uniform lesions are created with decreased variability between animals in the study population. These lesions, however, may not replicate a naturally occurring pathological condition. Overuse models of synovitis often involve animals adhering to a strict athletic routine usually involving timed exercise on a treadmill or track (97). Although overuse models better replicate normal forces, they result in greater variability in severity of disease due to physiologic and conformational differences within the study population. Strict immobilization of a joint using a cast or splint results in chondrocyte apoptosis due to nutrient deprivation. Cartilage, being an avascular tissue, relies upon synovial fluid for nutrient delivery. Immobilization has been observed to cause
lameness, in the horse and laboratory animals, resulting in significant cartilage degeneration that is attributed to inadequate cartilage nutrition (98-101).

Although these models replicate a specific disease process, the trauma or instability induced is often only appropriate in studies where research animals are to be euthanized because the animals remain permanently lame. Similarly, inflammation resulting from MIA injection causes significant cartilage damage and residual lameness (94). Models involving IA use of S. aureus live cultures (42), LPS (93), or IL-1 (95) have induced transient reversible synovitis in the horse. These models result in transient synovitis while avoiding many of the long-term debilitating effects of other experimental models. Compared to other models, the LPS and IL-1 models are ideal from an animal welfare perspective as they transiently mimic the inflammatory process that occurs during OA yet do not contain an infectious agent or result in permanent articular damage. Despite this benefit, LPS and IL-1 induced synovitis only allow evaluation of various interventions in the first few days after induction. After this time has passed the joint has recovered spontaneously regardless of intervention being assessed. This timeline does not fully reflect that which occurs in a clinical setting as spontaneous joint injuries are sometimes not treated immediately and the period of inflammation often persists for longer periods.

As previously reported, IA injection of 0.5ng LPS produces a reliable, transient, acute synovitis without causing clinical signs of endotoxemia (93). Synovial fluid parameters (total nucleated cell count, total protein, and differential cell count) appear similar to those observed in many severe septic and non-septic inflammatory conditions for up to 48 hours (43, 93). Following this time period, synovial fluid parameters, biomarkers of inflammation, and clinical signs of lameness rapidly return to baseline values with joint effusion subsiding within seven days (43).
Mesenchymal Stromal Cells as Emerging Synovitis Therapies

Clinical use of cell based therapies for treatment of orthopedic injuries in horses is common despite a lack of scientific evidence (102). The desire to reverse the damage associated with injury has resulted in significant research interest and efforts in the emerging fields of mesenchymal stromal cell (MSC) therapy and regenerative medicine. The key feature that differentiates MSC from other cells is their adipogenic, osteogenic, and chondrogenic differentiation capacity under the right conditions (103). This unique ability theoretically allows utilization of MSC as progenitor cells that differentiate and become part of the repair tissue (104). A second theoretical ability of MSC that is receiving equal or greater interest is their possible function in a non-progenitor fashion where the MSC direct cellular repair cellular repair without themselves integrating into the repair tissue (105, 106).

Despite the ability to isolate and culture MSC from many different tissues, the majority of research has been performed using culture expanded bone marrow (BM) derived MSC or non-culture expanded adipose tissue derived nucleated cell fractions, so-called stromal vascular fraction (SVF) (103). Umbilical cord blood (CB) has also been shown to be a viable source of MSC in the horse (107-109).

MSC Nomenclature

The term MSC is commonly used to refer to both mesenchymal stem cells and mesenchymal stromal cells. Despite interchangeable use in some instances it is important to clearly differentiate between the two. The key difference between these two terms is that mesenchymal stem cells are capable of long-term self-renewal whereas mesenchymal stromal cells undergo senescence following prolonged culture. It is commonly agreed upon that plastic adherent cells capable of multi-lineage differentiation should be referred to as stromal cells while
the term stem cell should be reserved for cell populations with a high proliferative capacity and capable of serial in-vivo transplantation (110). In this thesis the acronym MSC is used to refer mesenchymal stromal cells exclusively.

*Proposed MSC Mechanisms of Action*

**MSC Differentiation and Tissue Integration**

One of the first hypotheses of the role that MSC have in tissue repair is that the exogenously administered undifferentiated or naïve MSC differentiate into the principal cell of the target tissue in response to local cues at the injury site. The hope being that MSC in this manner would provide a new pool of healthy resident cells that could proliferate and regenerate the damaged tissue. This hypothesis of MSC function was adopted from the field of hematopoietic stem cells (HSC) where donor HSC are able to repopulate bone marrow devoid of cells following radiation therapy and subsequently re-establish all erythroid and myeloid cell populations in the patient (111, 112). Serial in vivo transfusion experiments in mice demonstrated that subpopulations transfused HSC retain their “stemness” and capacity to repopulate the hematopoietic system of upon transfusion to a second recipient (113). Such stringent serial in vivo experiments have not been performed for MSC to-date and their true “stemness” remains controversial. In vitro (114) and ex vivo (115) evaluation of MSC integration confirms that integration into cartilage defects can occur. Studies that involve tracking labeled MSC within joints have suggested that only a small proportion of MSC permanently integrate into a healing cartilage defect (116, 117). MSC integration has not been detected following IA injection at 60 days in normal horse joints (118). These findings indicate that functional integration—although
likely in low numbers—into damaged tissue is a possible mechanism of action for MSC mediated repair of articular structures.

**Cell-to-Cell Fusion**

The theory of cell-to-cell fusion refers to a MSC fusing with a differentiated cell forming a hybrid bi-nucleate or quadraploid cell (119). This has been observed in vivo in skeletal muscle, hepatocytes and cardiac muscle and under favorable conditions has been observed in vitro (120-122). In vitro observation of cell-to-cell fusion (123, 124) occurs as an extremely rare event and only under specific conditions that do not have a physiological equivalent (119). Although interesting in vitro, cell-to-cell fusion likely has little role in MSC mediated repair of articular and periarticular tissues.

**Transfer of Organelles, Enzymes, and Vesicles Between Cells**

Transfer of organelles via microtubular pathways has been suggested as a mechanism to which MSC populations could “revive” a “sick” differentiated cell or cell populations. Proteins, organelles, and vesicles, have been observed to be transferred between differentiated cells (125, 126) and from MSC to differentiated cells (127). The importance of this mechanism in articular/periarticular tissue regeneration is unknown, however, due to the rarity (119) that it occurs the effect is thought to be minimal (128).

**Reactive Oxygen Scavenge**

Reactive oxygen species (ROS) cause oxidative stress and cell damage. In vitro and in vivo models of cellular injury have shown ROS reduction following MSC administration (129-
Further study is needed to evaluate if scavenging ROS is a significant role of MSC in preventing further cellular damage.

**Anti-inflammatory/Anti-proliferative**

Di Nicola and colleagues (2002) (106) evaluated human BM-MSC co-cultured with autologous lymphocytes that were stimulated with allogeneic lymphocytes in mixed lymphocyte reaction (MLR) under various spatial and temporal conditions. They found that BM-MSC suppress lymphocyte proliferation in four different ways. First, MSC were associated with lymphocyte suppression in a dose-dependent manner with maximal suppression occurring at MSC to lymphocyte ratios greater than 1:1. Second, MSC suppressed lymphocyte proliferation independent of the time that they were added. Regardless of whether MSC were added on day 0, or 5; lymphocyte proliferation was suppressed when compared to time matched controls. Third, removing MSC from the culture conditions ablated lymphocyte suppression that had previously occurred. Finally, separation of MSC from lymphocytes using a porous interface that allowed MSC to share culture media but prevented cell-to-cell contact resulted in similar suppression of lymphocyte proliferation suggestive of MSC action through secreted soluble factors. MSC are reported to induce specific changes in several lymphocyte populations including CD4+ T-helper lymphocytes (Th), CD8+ cytotoxic T-lymphocytes (CTL), regulatory T-lymphocytes, (Treg), and B-lymphocytes. These effects are thought to be mediated through secretion of soluble factors such as PGE$_2$, TGF-β, nitric oxide (NO), hepatocyte growth factor (HGF), and indoleamine 2,3-dioxygenase (IDO) (133). These factors result in decreased proliferation of CD4+ and CD8+ lymphocyte populations, decreased B-cell proliferation/antibody production, and increased Treg proliferation (106, 134, 135).
The discovery of MSC mediated lymphocyte suppression resulted in a significant paradigm shift in the suspected mechanisms to which MSC act in an inflammatory environment. Currently, MSC are thought to respond to pro-inflammatory molecules by secreting numerous anti-inflammatory molecules that induce conversion of Th1, Th2, natural killer (NK) T-lymphocytes, Treg, and dendritic cells to a more anti-inflammatory phenotype (105, 135, 136). Recently Carrade-Holt et al (2014) (137) reported that MSC mediated lymphocyte suppression could be fully or partially blocked by PGE₂ blockade in BM, CB, adipose tissue (AT) and umbilical cord tissue derived MSC. MSC mediated lymphocyte suppression could be partially blocked by nitric oxide blockade in BM and CB-MSC. Potential mechanisms of lymphocyte suppression may also vary between source tissues considering MSC derived from solid tissues such as AT or umbilical cord tissue decrease proliferation by inducing apoptosis; whereas, MSC derived from cell suspensions such as BM and CB induce cell cycle arrest at the G₀ phase (137).

In vitro assays for immune function of MSCs

Evaluation of the proliferative behavior of lymphocytes has resulted in the development of various assays used to approximate the immune-stimulating or immune-inhibiting properties of various cells, tissues, or chemicals in vitro. First described in 1960 by Weksler et al. (138), these assays were developed following observations that lymphocytes could be stimulated to proliferate in culture when stimulated with the plant mitogen phytohemagglutinin (PHA). Elves & Israels (1965) (139) later reported that small lymphocytes in culture could be stimulated with allogeneic cells or foreign antigens and transformed into large basophilic blast cells. Cells with similar morphology had previously been observed in rejected transplant tissues resulting in rapid recognition of lymphocyte proliferation as an in vitro correlate of the physiologic allograft reaction (138). Additional methods of lymphocyte stimulation have been developed including
concanavalin A (ConA), and anti-CD3 or anti-CD3/CD28 monoclonal antibodies in order to approximate physiologic immune reactions in-vivo (140-143).

One-way lymphocyte reaction refers to co-culturing peripheral blood lymphocytes (PBL), known as the responder cells, with a given cell population (stimulator cells) after they have been mitotically arrested. Autoreactivity of PBL, e.g. baseline or negative control, is tested by co-culturing responder and stimulator cells from same individual. Alloreactivity or positive response is determined by co-culturing responder and stimulator cells from different individuals or by use of chemical agents such as ConA and PHA for stimulation of PBLs. MSC originating from a variety of sources have been shown not to induce lymphocyte proliferation through one way MLR. This lack of lymphocyte stimulation following one way MLR has resulted in MSC being referred to as having an “immune privileged” status (109). Interferon-γ stimulation—causing increased MHC-II expression—of equine BM-MSC resulted in one-way MLR proliferation (144). Considering many reports of relatively low MHC-II expression on MSC from a variety of tissue sources (145-149), MSC may achieve their “immune-privileged” status by expressing very low levels of MHC-II molecules and thereby not be recognized as “foreign” for this reason.

Two-way lymphocyte reactions refer to alloreactive one-way lymphocyte reaction as described with addition of mitotically arrested test cells, e.g. MSC in this context. Lymphocyte suppressive properties of the test cells are concluded if the expected responder cell proliferation does not occur. The responding T-lymphocytes proliferate due to recognition of foreign antigens on the allogeneic cells in a dose dependent fashion (138). The MLR differs only from PHA, ConA, anti-CD3, and anti-CD3/CD28 stimulated lymphocyte reactions in the mechanism of stimulation. Allogeneic cells are recognized as foreign and the T-lymphocyte population proliferates in response. PHA and ConA in contrast induce cross-linking of the T-cell receptor resulting in T-cell specific proliferation. Anti-CD3/CD28 antibody stimulation utilize monoclonal
antibodies that partially mimic lymphocyte stimulation by antigen presenting cells, and their co-stimulatory CD28 effect. Allogeneic B-lymphocytes are reported to cause the most significant stimulation while PHA and ConA stimulate PBL proliferation to a lesser extent (138).

Lymphocyte proliferative reactions were used to evaluate autologous (106), allogeneic, and xenogeneic (150) MSC for their effect on lymphocyte suppression. When allogeneic and autologous MSC were evaluated in one-way MLR reactions no significant effect was observed. The MSC were not recognized as foreign (no increase in PBL proliferation) nor did they appear to decrease baseline lymphocyte proliferation (109). MSC evaluated using two way mixed lymphocyte reactions in which (allogeneic (106, 109, 151), PHA (109), Con-A (109), anti-CD3 (152), or anti-CD3/CD28 antibodies (143), have all resulted in suppression of lymphocyte proliferation has been suppressed regardless of the method of stimulation. These findings suggest that MSC suppress proliferation regardless how the stimulation occurs.

**Future MLR directions for MSC evaluation**

Cell proliferation is the current outcome assessment of the lymphocyte proliferation assays. This has been assessed using radiolabeled tritiated (\(^3\)H) thymidine (138) and bromodeoxyuridine (Brd-U) flow cytometry (109). Additional colorimetric assays that have been validated against \(^3\)H thymidine as a measurement of cell proliferation include the MTT (153-155), Alamar blue (154, 156, 157), Brd-U ELISA (154, 158), and WST (159). Although not routinely used to evaluate MLR, these colorimetric assays have the potential to provide similar accuracy and increased efficiency in sample analysis to previous methods while avoiding the challenges of working with radioactive isotopes.

Evaluating cellular metabolic activity rather than relying on various methods of establishing cell numbers could provide a more sensitive evaluation of the lymphocyte
suppressive effects of MSC. Martinez-Florez et al (2013) (160) compared three methods of assessing lymphocyte proliferation (MLR variations) with the ImmuKnow® assay (Cylex Inc.) in post-organ-transplant patients. The goal of this comparison was to assess different methods of functional immunosuppressive therapy evaluation with the goal of developing more sensitive monitoring techniques than immunosuppressive drug levels. Only the ImmuKnow® assay was able to distinguish stable post-transplant (stimulation suppressed) patients from healthy controls (non-stimulated) (160). The ImmuKnow® assay measures intracellular ATP concentration in CD4+ lymphocytes allowing the investigator to assess metabolic function of the responding lymphocytes rather than strictly relying on cell numbers (160).

Although MSC mediated lymphocyte suppression has not been evaluated using ImmuKnow®, this assay may be able to distinguish between MSC cell cultures, all with apparently equal lymphocyte suppressive properties, and allow selection of a cell culture with greater anti-inflammatory properties. Currently the ImmuKnow® assay is not available commercially; however, post-MLR purification of CD4+ lymphocytes followed by measurement of intracellular ATP levels could possibly replicate similar results.

**Lymphocyte suppressive properties of MSC in the horse**

Evidence of anti-inflammatory properties of MSC has been obtained in vitro where equine CB-MSC were shown to have greater lymphocyte suppressive properties than either BM- or AT-MSC in two-way MLR using pooled allogeneic lymphocytes as stimulator cells (personal communication Koch, TG. October 2012). However, similar work by Carrade et al. (2012) (109) found MSC from all sources (CB, BM, SVF, cord tissue) had similarly lymphocyte suppressive properties in vitro using two-way MLR, PHA, or ConA as the stimulating agents. The differences
observed may be related to different CB-MSC isolation protocols, MSC expansion conditions and passage numbers of the MSC.

Equine Cell-Based Therapies for Synovitis

Two experimental studies evaluating IA use of MSC or SVF have shown little benefit to overall lameness and progression of synovitis (50, 161). In contrast, anecdotal reports involving large numbers of horses with clinical joint injuries treated with IA SVF or MSC have shown 50-70% improvement in horses non-responsive to conventional therapies (6, 162-164). Head to head comparison of culture expanded BM-MSC and SVF revealed no clinical difference in a carpal osteochondral fragment model (50). Differences between two biomarkers of inflammation resulted in a claim of superiority for BM-MSC; however, this comparison does not account for actual MSC numbers or specific selection—plastic adherence and culture conditions—associated with culture expansion (50). A possible explanation for these results is the lack of standardized dose, timing of treatment, or case selection, which makes direct comparison unreliable. These knowledge gaps highlight the need for more mechanistic insights into MSC function as well as controlled prospective clinical studies in order to develop evidence-based treatment recommendations.

Cell-Based Therapies for Joint Injury in Other Species

In dogs, one randomized blinded clinical trial and one case series have shown improvement in hip (165) and elbow (166) joint OA after SVF injection. Potential errors, however, in methodology, study design, blinding, and controls, as well as potential conflicts of interest, raise questions about the validity of these findings. These studies relied exclusively on subjective assessment of lameness, pain, mobility, and range of motion for outcome assessment.
Radiographic follow-up was not performed, and confirmation of intraarticular injection (as opposed to periarticular) was not obtained. The study evaluating SVF as treatment for elbow OA (165) lacked a control group resulting in subjective evaluations not being performed blinded to treatment. Improved healing of articular/peri-articular lesions has been observed following MSC therapy in several other species. In the goat, MSC were detected in partially regenerated medial menisci when MSC were injected IA six weeks following meniscectomy (167). In a sheep model, improved cartilage healing was observed in defects treated with chondrogenically-differentiated MSC compared to other lesions within created within the same or contralateral joint (168). In laboratory animal models, MSC have been detected in periarticular tissues indicating MSC were retained within the articular tissues, functionality of these cells has yet to be determined (169, 170).

**Safety of MSC in vivo**

Although no therapeutic dose of MSC has been established in any species, human clinical trials and lab animal studies often administer MSC in the range of 1x10^6 MSC/kg for systemic administration (104, 171). Culture of such large doses of MSC for systemic administration faces several logistical challenges using current cell culture techniques. Currently horses are often treated with local or regional MSC therapy with ~10 x 10^6 MSC total per treatment (6, 50, 172). Intravenous, intradermal, and intraarticular MSC injection has been evaluated for safety in the horse. No adverse reactions were noted following either repeated intravenous injection of relatively small allogeneic MSC numbers (2x10^5) in 291 horses, or in large (10^7) MSC numbers in 13 horses (173, 174). Additionally, intradermal allogeneic equine MSC injection did not result in inflammatory reactions, hypersensitivity, or systemic abnormalities (172). Allogeneic MSC preparations have also been reported to have no negative effects when used as intra-lesion or
intra-thecal treatment for tendonitis in two small case series (175, 176). IA MSC injection using autologous and allogeneic MSC have been evaluated using 7.5 million autologous or allogeneic (177) and 15 million autologous, allogeneic or xenogeneic MSC (178). In these studies, mild to moderate lameness, increased joint circumference, and limb edema were observed in MSC-treated limbs, which resolved without treatment. These studies indicate that although MSC injection is relatively safe, there is a 9% risk of a joint flare in the hours and days following MSC injection (6). Despite the self-limiting nature of most MSC associated joint flares, horse owners should be made aware of potential complications prior to injection of MSC suspensions.

These studies of allogeneic MSC preparations suggests that MSC may not stimulate a similar immune reaction as occurs with other allogeneic cell populations. Although allogeneic MSC injection is generally well-tolerated, allogeneic MSC does stimulate an immune response. In the horse, intradermal injection of allogeneic MSC has resulted in ex vivo humoral and cell mediated responses (137, 179). Although these findings are supported by work in other species (180-184), the significance of these responses however have not been confirmed in vivo leaving concerns over immune mediated allogeneic MSC destruction unanswered. Decreased antibody response has been observed when equine leukocyte antigen haplotypes are matched between allogeneic donors and recipients (179). This however is not commonly performed in research or clinical settings due to the necessary expertise and added expense. These observations together suggest there may be potential for developing frozen allogeneic MSC products that could be available for treatment of the equine athlete at the time of diagnosis of injury. A significant advantage of allogeneic MSC therapy is that time permits selection and screening of cell cultures for desired therapeutic properties, e.g. anti-inflammatory properties, differentiation towards chondrocytes for cartilage repair, or even in vitro pre-differentiation towards the cell type of interest prior to transplantation.
Logistical Challenges for MSC Therapy in Veterinary Medicine

*MSC Transport medium*

Currently MSC for use in clinical veterinary medicine are either prepared onsite or shipped chilled or frozen by overnight carrier. As the majority of veterinary facilities do not have the equipment or expertise to culture MSC, optimizing transport conditions is an important consideration. Ideally MSC suspensions could be administered in an excipient manner in order to reduce on site handling at the time of treatment and decrease the risk of contaminating MSC products. Equine MSC are transported chilled (2-8°C) commonly suspended in balanced electrolyte solutions, autologous bone marrow supernatant, or in medium containing autologous allogeneic, or xenogeneic serum (185, 186). When in transit for more than 24 h, viability decreases significantly in these commonly used media (185). Additionally, allogeneic or xenogeneic serum-containing medium is not considered ideal as foreign proteins may initiate immune reactions in the recipient animal (185). Frozen MSC suspensions are associated with variable cell loss and cell viability post-thaw dependent on the cryomedium used (187).

One strategy used for maintaining cell viability during transport is to mimic intracellular conditions in the transport medium in order to reduce stresses associated with osmotic and concentration gradients across the cell membrane. Balanced electrolyte solutions are designed to replicate similar electrolyte concentrations the extracellular fluid. Since blood, serum, plasma, and bone marrow aspirate all originate from the extracellular fluid compartment they all encourage water and electrolyte flow across the cell membrane due to osmotic and concentration gradients. Hypothermosol (HT) is a cell transport medium that contains electrolyte concentrations more similar to the intracellular rather than extracellular environment (Appendix I). It also
contains various energy sources for maintaining cell viability and free radical scavenger (FRS) Trilox (vitamin E analog) have been added to aid in preserving cell viability (188).

HypoThermosol®-FRS (HT-FRS) is a proprietary, serum and protein free, current good manufacturing practice (cGMP) compliant, solution optimized for preserving mammalian cell viability at 2-8°C that is manufactured by Biolife Solutions, Bothell, WA. Hypothermic preservation of numerous cell types, tissues, and organs have been reported with HT-FRS outperforming all other media tested (187-192). HT-FRS has been evaluated as a cryopreservation medium for preservation of various cell cultures. Maximal viability following cryopreservation has been reported when cell cultures are suspended in HT with the addition of dimethylsulfoxide (DMSO) (192, 193). In fact viabilities reported approach those reported for a DMSO containing cryopreservation solution “based on HypoThermosol® technology” marketed under the name CryoStor® (194). Although not marketed as such, considering the evidence presented in previous studies, it appears likely that the product marketed as CryoStor® contains relatively similar constituents as HT-FRS with the exception of 2, 5, or 10% DMSO as specified (192, 193).

Human BM-MSC viability is better preserved—compared to other commonly used media—following simulated chilled transport or cryopreservation in HT-FRS® or CryoStor® containing 10% DMSO (CS-10), respectively (187). Similar results have also been achieved in equine CB-MSC when comparing HT-FRS and CS-10 to bone marrow supernatant, balanced electrolyte solutions serum containing media for simulated transport of chilled and frozen MSC, respectively (personal communication TG Koch, January 2015).

Although not readily available from the manufacturer, the constituents of HT-FRS are published in early reports of its use (Appendix I) (195). The high [K+] and [Mg²⁺] concentrations indicate IV HT-FRS injection rates should be less than 11.7mL/kg/h to remain below the
threshold for safe \([K^+]\) administration (0.5meq/kg/h). Considering the small volumes MSC would be suspended in for an excipient MSC injection, HT-FRS administration in regenerative medicine should not raise any safety concerns.

**Additional considerations for MSC therapy in veterinary medicine**

In order to improve accessibility to MSC therapy, a commercially available MSC product would ideally be stored at veterinary clinics and ready to be administered once prescribed. Liquid nitrogen storage of biological products such as semen is common practice in veterinary medicine. MSC cultures are commonly stored long-term in liquid nitrogen as well. Study of the effect of transportation on fresh or frozen MSC preparations indicate transportation of frozen MSC suspensions is ideal if any delay in shipping occurs (185). However, currently the functionality of MSC immediately following thawing is unknown.

Another practical consideration is the MSC aspiration and injection process, which should not adversely affect viability in order to achieve maximal therapeutic benefit. The Effect of aspiration has not been studied on MSC however negative pressure associated with vacuum assisted wound closure has been observed to decrease MSC viability. Injection of human and rat MSC suspensions suggests there are no negative effects associated with the injection process (196, 197). Recently, one publication and two abstracts presented at veterinary conferences suggest that equine MSC are adversely affected by the injection process (185, 198, 199). Differences in methodology and concerns regarding interpretation of data (see Appendix III) leaves this question largely unanswered.

**Challenges for Equine Cell-Based Synovitis Therapies**
One potential drawback of current IA MSC or SVF therapy using autologous MSC is that cell isolation occurs following injury. The time needed to isolate and culture expand the MSC prior to injection delay treatment by 10-21 days. This in turn excludes treatment of acute injuries at the time of diagnosis and allows significant inflammation to occur. This results in altered chondrocyte metabolism and catabolic damage to articular cartilage prior to treatment (8, 177). This period of time between injury and cell-based therapy is often reported as intentional in order to allow inflammation associated with injury to subside (6, 65, 75, 163). These recommendations are based on the assumption that inflammatory conditions may reduce the effectiveness of MSC and observations of transformation of BM-MSC to a pro-inflammatory phenotype in the presence of inflammatory cytokines in vitro if the MSC and responding cells are MHC-II miss-matched (144). Others have recently reported that less differentiated MSC do not induce MHC-II surface marker up-regulation (145, 147) nor do they increase recognition of MSC by NK cells or in the presence of inflammatory cytokines (145-147). These findings indicate that optimal timing of treatment may depend on the tissue source of the MSC. The MSC could potentially be assessed or manipulated for functionality prior to use during this expansion phase in order to enhance treatment efficacy. SVF therapy has the advantage of reducing this treatment delay to hours or a few days depending on whether the SVF is generated in-house or by a third-party, but characterization or culture-specific manipulation of the cell fraction is not feasible.

A potential solution to the delay associated with cultured MSC includes harvest and storage of autologous stem cells in preparation for future injuries of an individual patient or the preparation of allogeneic MSC for clinical use on an as needed basis (107, 177). Prior to clinical use of MSC preparations, effective dose, timing of treatment following injury, and reliable repeatable methods of detecting differences in treatment groups need to be established. These
parameters are needed prior to large, randomized, blinded, placebo-controlled clinical trials in order to evaluate autologous or allogeneic MSC as a future therapy in equine sports medicine.
Rationale, Hypothesis, and Objectives

Rationale

Joint disease, usually starting as synovitis, is responsible for the greatest economic loss in the equine industry. Current synovitis therapies achieve a short-term decrease in inflammation but serve only as palliative care if inflammatory stimuli persist. IA MSC therapy may have potential to modulate inflammation as treatment of synovitis in the horse. Current practice of IA injection of MSC or SVF in the horse is preceding clinical evidence of the safety and efficacy profiles of such therapies. Determination of dose, optimum timing of treatment, optimum cell selection/culture methods for specific injuries, case selection, and strategies to maximize MSC viability require further evaluation. Research is required to establish the primary benefits, and provide a clinical basis for use.

The majority of research on MSC therapy in veterinary medicine has utilized autologous cell suspensions established after the onset of injury. This practice minimizes risk associated with allograft reactions, but prevents selection of MSC cultures for anti-inflammatory properties or differentiation into specific cell types due to the time required to isolate and culture MSC. The several weeks required to culture MSC also excludes treatment of acute injuries. Establishment of allogeneic MSC as a treatment option would allow MSC therapy to be initiated at the veterinarian’s discretion rather than be dictated by logistical constraints associated with collection, culture, and transportation. Such allogeneic MSC products may be further potentiated by pre-selection or pretreatment(s) for desired functions.

Additional logistical hurdles associated with collection, culture, transportation, and injection of cell based products need to be addressed prior to establishment of MSC therapy as a viable treatment option. In order to maximize any potential benefits attributed to treatment with
MSC it is necessary to simultaneously develop strategies to maximize viability and retain/select for desirable properties in MSC cell suspensions.

Ultimately, development of a MSC product that could be maintained in stock, in a similar manner to conventional therapeutic agents, would allow the equine industry to maximize any potential benefit that may result from MSC therapy. As with conventional therapeutic agents, safety, efficacy, transportation, handling, and storage requirements need to be established prior to off-the-shelf MSC therapy becomes reality.
Hypothesis

Equine allogeneic CB-MSC are safe and effective for treatment of synovitis.

Sub-Hypotheses

1) Equine CB-MSC will retain lymphocyte suppressive properties regardless of post-thaw culture period
2) Equine CB-MSC and will maintain anti-proliferative properties in vivo when assessed in an IA LPS induced synovitis model.
3) Equine CB-MSC will not be affected by aspiration or injection through hypodermic needles,
4) Equine CB-MSC will not cause adverse reactions injected as a pooled allogeneic suspension in HT-FRS or CS-10,

Objectives:

To test this hypothesis, the objectives listed below will be assessed:

Objective 1: To determine the effects of the post-thaw culture period on CB-MSC lymphocyte suppression

Objective 2: To evaluate the effect of IA injection of lymphocyte suppressive CB-MSC as treatment for LPS induced synovitis.

Objective 3: To determine the effect of aspiration and injection on BM and CB-MSC viability in vitro.

Objective 4: To evaluate the safety of IV injection of HT-FRS, CS-10, and pooled allogeneic CB-MSC
Chapter 2: Post-thaw non-cultured and post-thaw cultured equine cord blood mesenchymal stromal cells equally suppress lymphocyte proliferation \textit{in vitro}.

This chapter is a modified version of Williams \textit{et al.} (2014) (151)
Abstract

Multipotent mesenchymal stromal cells (MSC) are receiving increased attention for their non-progenitor immunomodulatory potential. Cryopreservation is commonly used for long-term storage of MSC. Post-thaw MSC proliferation is associated with a lag-phase \textit{in vitro}. How this lag-phase affect MSC immunomodulatory properties is unknown.

We hypothesized that \textit{in vitro} there is no difference in lymphocyte suppression potential between quick-thawed cryopreserved equine cord blood (CB) MSC immediately included in mixed lymphocyte reaction (MLR) and same MSC allowed post-thaw culture time prior to inclusion in MLR. Cryopreserved CB-MSC from five unrelated foals were compared using two-way MLR. For each of the five unrelated MSC cultures, paired MLR assays of MSC allowed five days of post-thaw culture and MSC included in MLR assay immediately post-thawing were evaluated.

We report no difference in the suppression of lymphocyte proliferation by CB-MSC that had undergone post-thaw culture and MSC not cultured post-thaw (p < 0.0001). Also, there was no inter-donor variability between the lymphocyte suppressive properties of MSC harvested from the five different donors (p = 0.13). These findings suggest that cryopreserved CB-MSC may have clinical utility immediately upon thawing. One implication hereof is the possibility of using cryopreserved CB-MSC at third party locations without the need for cell culture equipment or competencies.
Introduction

Multipotent mesenchymal stromal cells (MSC) are receiving significant attention as a treatment option for various conditions. Currently MSC are thought to have either progenitor or non-progenitor cellular functions. Although studied extensively, progenitor cell integration into recipient tissues has only been observed in very small numbers challenging the importance of the MSC progenitor paradigm (116, 118, 200). Di Nicola et al (106) observed MSC-mediated lymphocyte suppression occurred in a MSC-dose dependent, time independent, and reversible manner that did not require cell-to-cell contact in vitro. These findings resulted in a paradigm shift—from progenitor to non-progenitor functions—as a main mechanism by which undifferentiated MSC exerts therapeutic effect. Non-progenitor MSC actions that have been investigated include cell-to-cell fusion (119, 123, 124), organelle transfer (125, 126), reactive oxygen scavenge (129-132), and suppression of lymphocyte proliferation (105, 106, 152).

MSC-mediated lymphocyte suppression has been observed in equine in vitro studies. Expected equine lymphocyte proliferation following stimulation with either allogeneic lymphocytes or plant-based mitogens was suppressed by equine MSC derived from bone marrow, adipose tissue and umbilical cord blood (CB) in vitro (109). Work in our lab confirmed the lymphocyte suppressive properties of equine CB-MSC (201). One potential advantage of CB-MSC compared to other MSC sources is that they can be isolated and characterized prior to the donor sustaining and injury in case of autologous use. Allogeneic use of equine CB-MSC have also recently been reported in clinical cases without observed adverse reactions (175). These reports suggest that allogeneic CB-MSC may have clinical utility as immune-modulatory agents. Equine MSC lymphocyte suppression studies to-date have explored MSC that were maintained in culture prior to inclusion in so-called two-way mixed lymphocyte reactions (MLR) in vitro. Cryopreserved and passaged umbilical cord derived endothelial cells exhibit a proliferative lag-
phase upon thawing and sub-culturing (202). The lag phase however, was observed to be 36h longer for cryopreserved endothelial cells than non-cryopreserved cells. No difference was noted between fresh and cryopreserved/thawed human umbilical vein endothelial cells with regard to anti-inflammatory and anti-coagulant activity in vitro. The thawed cells, however, were allowed to overcome their proliferative lag phase following cryopreservation prior to inclusion in these in vitro assays (202). Whether cryopreserved/thawed MSC exhibit an equivalent ‘functional lag-phase’ with regard to lymphocyte suppression is undetermined.

We hypothesized that in vitro there is no difference in lymphocyte suppression potential between post-thaw non-cultured (PTNC) equine CB-MSC immediately included in mixed lymphocyte reaction (MLR) and same MSC allowed post-thaw culture (PTC) prior to inclusion in MLR.

**Materials and methods**

**Ethics statement**

This study was specifically approved by the University of Guelph Animal Care Committee with regard to the procedures of collection of equine peripheral blood lymphocytes and equine umbilical cord blood (animal use protocols 1756 and 1570). Additional research conducted using specimens of this kind does not require review by the Animal Care Committee (falls under CCAC Category of Invasiveness A) and therefore the mixed lymphocyte reactions can be considered to have been conducted in accordance with the institutional ethics guidelines. Collection of peripheral blood and cord blood was add-on procedures to the routine care of the horses. No animals were sacrificed during the study. Equine umbilical cord blood was collected
on two privately owned commercial farms in Southern Ontario. Four of five samples were collected on one farm from Thoroughbred foals. One sample was collected on another farm from a Warmblood foal. Informed consent was obtained in writing from the horse owners/agents prior to sampling. The broodmares on the foaling farms are housed in large foaling boxes. Both farms are staffed 24/7 and mares are under constant video surveillance and carrying foaling alarms to allow for observed foaling and assisted delivery if needed. Umbilical cord blood was collected by farm staff as instructed. Instruction included video-review of cord blood collection. Cord blood was collected from an isolated segment of the umbilical cord after the umbilical cord had been clamped and detached from the foal. Peripheral venous blood was obtained from the Equine Research Herd owned by the Ontario Ministry of Agriculture and Food (OMAF). Once investigators have an approved animal care protocol from the University of Guelph Animal Care Committee access to these research horses are granted. In this study peripheral blood was collected from 5 adult mixed-bred horses. The adult horses on the research farm are housed in smaller groups with run-in sheds throughout the year. The horses are on pasture during the summer and during the winter they have access to large paddocks with gravel surface. Collection of peripheral blood was collected under mild sedation (Xylazine HCl, 0.35 – 0.40 mg/kg bwt IV; Bayer, Toronto, ON) from the jugular vein following which manual pressure was applied for several minutes to aid hemostasis.

*Lymphocyte collection*

Equine peripheral blood lymphocytes (PBL) were isolated from equine whole blood obtained from five unrelated adult horses. 450mL of whole blood was collected into a commercially available blood collection bag containing anticoagulant (Na citrate) and processed within 2 hours of collection by Ficoll (GE Healthcare, Mississauga, ON) density gradient
separation. 35mL of whole blood was layered over 15mL of Ficoll-Hypaque- plus (density 1.077g/L) within a 50mL conical tube. Samples were centrifuged at 500g for 30 minutes at room temperature (RT) with no brake. The interphase was collected and washed twice with phosphate buffered saline (PBS) following centrifugation at 500g for 10 minutes at RT. Washed PBL were suspended in 10 mL of MLR culture medium (MLR-CM) which consisted of Roswell Park Memorial Institute (RPMI 1640, Invitrogen, Burlington, ON) culture medium, 10% heat inactivated horse serum (Invitrogen, Burlington, ON), 1% penicillin/streptomycin (Invitrogen, Burlington, ON), and 1% L-glutamine (Lonza/Cambrex, Walkersville MD). Cells were counted using an automated fluorescent based cell counter (Nucleocounter NC-100, Mandel Scientific Company, Guelph, ON), re-suspended freshly prepared cryomedium (10% DMSO in MLR-CM) and frozen in 1.8mL cryovials at a concentration of 6x10^6 cells/mL. Cells were slowly frozen at a rate of -1°C/min to -80°C (Mr. Frosty, Nalgene, Mississauga, ON) before transfer for long-term storage in liquid nitrogen. At the time of use, the PBL were thawed in a 37°C water-bath, followed by centrifugation at 500g for 5 minutes at RT. The PBL were suspended in 5 ml MLR-CM, counted and adjusted to a concentration of 2x10^6 PBL/mL.

**MSC collection, culture, and cryopreservation**

Cryopreserved CB-MSC cultures from five unrelated foals (N=5) were included. The CB-MSC cultures were established as previously described (107, 203). These primary cell cultures were cryopreserved at passages ranging from P1-P3 and preserved at a concentration of 1x10^6/mL. Same-batch MSC vials from each of the five foals were designated to one of two treatment groups—PTC and PTNC. Previously four of the five CB-MSC cultures evaluated in this study had consistently expressed CD29, CD44, CD90, and not expressed MHC-I, MHC-II, CD4, CD8, CD11a/18, and CD73.
MSC-PTC

Five days prior to MLR set-up, one CB-MSC cryovial from each foal was thawed in a 37°C water bath, slowly diluted into 5mL of MSC culture medium (MSC-CM) consisting of Dulbecco’s modified eagle medium containing 30% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine. MSC suspensions were centrifuged at 300g for 5 minutes, supernatant removed and suspended in MSC-CM. An automated cell count was performed using an automated fluorescent-based cell counter (Nucleocounter NC-100, Mandel Scientific Company, Guelph ON). Thawed MSC were seeded in polystyrene culture flasks at a density of 5,000 MSC/cm² and incubated at 38°C, 5% CO₂, in a humidified atmosphere for 5 days. MSC-CM was changed on day two and four of the incubation period. On the fifth day MSC were detached from the culture flasks using trypsin-EDTA and suspended in MSC-CM, and counted. Following centrifugation the MSC were suspended in MLR-CM at a concentration of 2x10⁵/mL.

MSC-PTNC

On day five, the day of MLR set-up, the second same-batch cryovial from each foal was thawed. The MSC were suspended in MSC-CM as described above. MSC were counted and suspended in MLR-CM at a concentration of 2x10⁵/mL.

MLR

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Cryopreserved PBL were thawed in a 37°C water bath, slowly diluted into 5mL of MLR culture media (MLR-CM). PBL suspensions were centrifuged at 300g for 5 minutes, the supernatant was removed, and cell pellet was suspended in MSC-CM. An automated cell count was performed using an automated fluorescent-based cell counter (Nucleocounter NC-100, Mandel Scientific Company, Guelph ON). PBL were subdivided into tubes for use as stimulator PBL and responder PBL, respectively. Stimulator PBL and CB-MSC were mitotically inactivated with 20Gy γ-radiation (Theratron 780C Cobalt 60, MDS Nordion, Ottawa ON). Irradiated stimulator PBL (PBLx) from all five horses was pooled in equal proportions at a concentration of 2x10^5PBLx/mL.

Non-irradiated responder PBL (PBL), PBLx, and irradiated MSC (MSCx) were combined in a 10:1:1 ratio, respectively, in 96 well round bottom plates and cultured five days at 38°C, 5% CO₂, in a humidified atmosphere. Lymphocyte proliferation was determined by bromodeoxyuridine (Brd-U) assay, see below for details. A series of controls were prepared on each plate including: negative control (PBL + autologous PBLx), positive control (PBL + allogeneic pool of PBLx), Brd-U staining controls (PBL + FITC or 7-AAD in the absence of Brd-U), and unstained (PBL only). On the fifth day of MLR culture Brd-U was added to each well and cultured for an additional 24 hours before fixation and staining for flow cytometry.

MLR reactions were performed in triplicate reactions using responder PBL from three different horses; resulting in nine replicates of each reaction. Identical experiments were setup using MSC that had received 5 days of PTC or PTNC MSC. These paired MLR were cultured, processed, and evaluated simultaneously.

**Outcome assessment**
On day 6 of MLR culture, cells were fixed and stained for Brd-U flow cytometry using a commercially available kit (BD Biosciences, Mississauga ON,) according to the manufacturers directions. In brief, MLR-CM was removed, cells were washed, fixed, and stained using FITC anti-BrdU antibody (to detect proliferative cells) and 7-AAD antibody (viability stain). After 24 hours, cells were analyzed by flow cytometry. The gate to identify resting and stimulated lymphocytes was maintained consistent throughout the experiments.

Statistical Analysis

Raw data was imported into a statistical analysis software package (SAS, SAS institute, Cary, NC). A general linear model was used to analyze the effect of treatment (MSC cell line, positive/negative controls), lymphocyte donor, and post-thaw culture period using the PROC MIXED function. All two and three way interactions were initially evaluated and non-significant effects and interactions were removed from the model. Residual analysis was performed in order to determine if ANOVA assumptions were met, to detect potential outliers, and evaluate the need for data transformation. The residuals were formally tested for normality using the four tests offered by SAS (Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises, Anderson-Darling) and plotted against the predicted values and variables used in the model. For the purpose of determining statistical significance α was set at 0.05.

Results

CB-MSC suppressed lymphocyte proliferation compared to the positive control, Figure 1 (p<0.0001). No difference was observed between CB-MSC cultures or between CB-MSC cultures and the negative control (p=0.13). All MLR that included CB-MSC, no matter the post-
thaw status, proliferated significantly less than their associated positive control, Figures 2 and 3. Positive controls were always significantly different than negative controls (p<0.0001). The odds ratio of the positive control staining for Brd-U was 4.99 (95% CI 3.21-7.76) times the odds of any other treatment staining Brd-U positive.

**Discussion**

Cryopreserved equine CB-MSC appear to constitutively suppress lymphocyte proliferation *in vitro* independent of post-thaw culture period. The noted homogeneity in the lymphocyte suppressive effects among CB-MSC suggests that although minor inter-donor variation did exist, screening of individual CB-MSC cultures prior to *in vivo* therapy may not be warranted.

Clinical use of cryopreserved CB-MSC immediately upon thawing would allow the attending veterinarian to determine treatment time independent of MSC procurement. Following injury, time is a critical factor as inflammatory mediators and cells quickly localize to the area of injury facilitating an inflammatory response (8, 21). Despite the importance of inflammatory events in tissue healing, acute excessive inflammatory events or chronic inflammation is often associated with impeded tissue healing or further tissue damage (8, 204, 205). This occurs as a result of activation of the host immune responses including elevated cytokine levels, reactive oxygen species, the release of host matrix metalloproteinases, and other collagenolytic enzymes (204).

Development of an “off the shelf” cryopreserved CB-MSC therapy appears feasible using an allogeneic strategy. Although lameness was not associated with IA MSC injection into healthy joints, mild inflammatory responses to IA injection of autologous and allogeneic MSC has been
reported in the horse (118, 177). Equine allogeneic MSC from CB and adipose tissue were not associated with adverse reactions when used as treatment for tendonitis in two small case series (175, 176). The possible immune-privileged nature or immune evasive properties of MSC indicates potential for immediate MSC treatment using a cryopreserved allogeneic MSC product. One caveat of the study is that PTNC CB-MSC were added into the reaction wells five days before evaluation of lymphocyte proliferation. We can therefore only conclude that any recovery period required by MSC prior to being lymphocyte suppressive is less than five days. In the initial report of lymphocyte suppression in MLR (106), the effect of human bone marrow-derived MSC was reported to occur independent of the time that MSC were added. Work with cryopreserved human MSC suggests recovery of normal cellular function occurs relatively quickly post-thaw (206, 207). These studies investigated the effect of cold and osmotic shock associated with cryopreservation on human BM-MSC and concluded that the metabolic disturbances observed in thawed MSC ceased to exist after 24h (206) and could be minimized using proper technique (207). We speculate that the recovery period is short, and clinically irrelevant, as no difference in lymphocyte proliferation was observed. This notion is supported by the mean response we observed within CB-MSC cultures. In three of the five CB-MSC cultures evaluated, lymphocyte proliferation was less in the wells treated with CB-MSC that were from the PTNC group. In fact, of the two CB-MSC cultures where trends towards significant differences were observed within a sample pair, the sample with the lower mean had been treated with CB-MSC from the PTNC group.

In conclusion, our findings suggest that cryopreserved equine CB-MSC may have clinical utility immediately upon thawing. One implication hereof is the possibility of using cryopreserved CB-MSC at third party locations without the need for cell culture equipment or competencies.
Acknowledgements:

We would like to thank Mr. William Sears for his expertise in performing the statistical analysis and Ms. Kim Stewart for her help with irradiation of cells for the MLR assays.
Figures:

Figure 1: Mean FITC anti-BrdU staining following two-way MLR. Lymphocyte proliferation of un-stimulated (negative control) and allogeneic stimulated (positive control) compared to stimulated lymphocytes treated with MSC. Three biological replicates and three technical replicates were used in the negative control, positive control, and for evaluation of each of the five MSC cultures. Five thousand lymphocytes in each sample were observed and designated either FITC positive (proliferative in the previous 24h) or FITC negative (not proliferative) Umbilical cord blood MSC from five cultures were either added after a 5-day post-thaw-culture period or identical MSC vials were thawed immediately prior to the experiment. No difference was observed between post-thaw-culture and post-thaw-non-culture groups. Different letters indicate statistically significant differences between means (α=0.05). Error bars represent the 95% confidence interval.
Figure 2: FITC anti-BrdU staining of five different CB-MSC cultures. Cryopreserved CB-MSC were either cultured five days or thawed immediately prior to MLR set-up. Each bar represents the mean percentage of FITC positive cells in nine replicates (triplicate wells for each of the three unrelated responder lymphocyte donors, 5000 lymphocytes evaluated in each sample). In no instance were significant differences observed between post-thaw-culture and post-thaw-non-culture groups. Different letters indicate statistically significant differences between means ($\alpha=0.05$). Error bars represent the 95% confidence interval.
Figure 3: Cell proliferation during 2-way MLR was detected with a FITC-labelled antibody to BrdU. Dot plots represent fluorescence of lymphocytes identified by forward and side scatter after co-culture with post-thaw-cultured (PTC) and post-thaw-non-cultured (PTNC) CB-MSC. The percentage fluorescence (right quadrant) indicates the proportion of lymphocytes proliferating in autologous (negative control), allogeneic (positive control) and allogeneic plus CB-MSC reactions. Differences in lymphocyte proliferation induced by PTC and PTNC CB-MSC were not significant.
Chapter 3: Equine allogeneic umbilical-cord-blood mesenchymal stromal cells reduce synovial fluid nucleated cell count and induce mild self-limiting inflammation when evaluated in a LPS induced synovitis model

This chapter is a modified version of a manuscript that is currently in press at Equine Veterinary Journal.

Equine Veterinary Journal submission number: EVJ-GA-14-372.R2
Abstract:

Improvement has been reported following intra-articular (IA) injection of mesenchymal stromal cells (MSC) in several species. These observations have led to use of IA MSC in equine practice with little understanding of the mechanisms by which perceived improvement occurs. This study evaluates the effect of IA allogeneic umbilical-cord-blood- (CB-) derived MSC using lipopolysaccharide (LPS) induced synovitis model. We hypothesized IA CB-MSC would decrease inflammatory response associated with LPS injection.

Feasibility studies were performed to evaluate IA LPS or CB-MSC alone into the tarsocrural joint. Following this in the principle study, middle carpal joint LPS synovitis was induced bilaterally then CB-MSC were injected into one middle carpal joint. Lameness, routine synovial fluid (SF) analysis, and SF biomarkers were evaluated at 0, 8, 24, 48, and 72 hours.

LPS injection alone resulted in transient lameness and signs of inflammation. In joints untreated with LPS, injection of 30-million CB-MSC resulted in mild synovitis that resolved without treatment. Mild (grade 1-2) lameness in the CB-MSC-treated limb was observed in two horses, severe lameness (grade 4) in the third 24h post-injection. Lameness did not correlate with synovitis induced by CB-MSC injection. Simultaneous injection of LPS and CB-MSC resulted in significant reduction in SF total nucleated, neutrophil, and mononuclear cell numbers compared to contralateral LPS-only joints. No difference was detected in other parameters associated with SF analysis or in SF biomarkers. The incidence of lameness was only different from baseline at 8 h, where horses were lame in CB-MSC limbs.

We conclude allogeneic CB-MSC reduced SF cell populations and stimulated mild self-limiting inflammation in the synovitis model. Continued evaluation of the effects of IA CB-MSC therapy on synovitis in horses is needed to evaluate anti- and pro-inflammatory properties of CB-MSC. Immediate interests are dose, timing of treatment, and treatment frequency.
**Introduction:**

Joint injury is the most significant cause of wastage in the equine industry (3, 4). These injuries are commonly treated with a variety of medications that focus on controlling inflammation and serving as palliative and short-term care. These medications (67), such as non-steroidal anti-inflammatories, corticosteroids, polysulfated glycosaminoglycan, hyaluronic acid, and autologous conditioned serum are considered symptom-modifying agents and may be associated with further cartilage damage (53, 67).

Multipotent mesenchymal stromal cells (MSC)—also referred to as mesenchymal stem cells—are cells derived from mesoderm tissues that are plastic adherent under *in vitro* culture conditions and capable of at least tri-lineage differentiation towards chondrogenic, osteogenic and adipogenic cell fates. Cell surface markers—although used to define human MSC—are not widely used to define equine MSC. Despite some variability in phenotype of equine CB-MSC reported (108, 109, 201, 208), in our lab CB-MSC (including the cell culture evaluated in this study) reliably display cell surface markers CD29, CD44, CD90, and not MHC I, MHC II, CD4, CD8, CD11a/18, and CD73 (201). MSC are also generating interest for their non-progenitor functions in the organization of cellular repair (105, 106). A mechanism by which MSC may provide therapeutic effect is through anti-proliferative or anti-inflammatory influence on inflammatory cells (105). Di Nicola and colleagues (106) reported that human MSC cultured with stimulated lymphocytes, suppress lymphocyte proliferation in a dose dependent, time independent, reversible manner that does not require cell-to-cell contact. Similar results have been observed using equine MSC (8, 109, 151). These results from *in vitro* mixed lymphocyte reactions (MLR) suggest equine MSC may have immune-modulatory functions *in vivo*. Equine umbilical cord blood- (CB-) MSC immune-modulatory effects have yet to be evaluated *in vivo*. 
In synovial joints, inflammatory insults are seen as lameness, joint effusion, edema, increased synovial fluid (SF) cell count, changes in inflammatory cell populations, and increases in SF total protein. Cartilage damage results if cartilage reabsorption exceeds its low regenerative capacity (8, 22). This occurs through a cascade of inflammatory events leading to chondrocyte destruction, cartilage matrix depletion, and type II collagen breakdown (22). The resulting chain reaction initiates further cytokine signaling and propagates cartilage damage (17, 22, 23).

Currently, improved clinical outcomes have been reported following intra-articular (IA) MSC injection in the horse (6), dog (165, 166, 209, 210), and human (211, 212). As a result, IA MSC injection is used commonly in equine practice without a full understanding of the mechanisms by which improvement occurs.

We hypothesize that IA injection of lymphocyte suppressive (determined by in vitro MLR) (151) equine CB-MSC, will decrease inflammation in acutely inflamed equine middle carpal joints.

Materials and Methods

Collection, isolation, and culture of CB-MSC

Cryopreserved CB-MSC cultures were procured as previously described (107, 203) from CB that had produced a consistent phenotype (201). CB-MSC cultures from five unrelated donor foals were found to have anti-proliferative/anti-inflammatory properties using two-way allogeneic stimulated MLR (151). One CB-MSC culture, at passage 3, cryopreserved once, and cultured for a total of 25 days was used in these in vivo studies. The CB-MSC culture evaluated had been previously shown to continuously express CD29, CD44, CD90, and not express MHC-
I, MHC-II, CD4, CD8, CD11a/18, CD-73 from passages 2-5 and following cryopreservation (213). Prior to injection CB-MSC were cultured in serum free media for 24h, detached from culture flasks and washed once in PBS.

*Research animals*

Six mature healthy Standardbred horses (5 female, 1 male; mean age 9.2 years, range 4-11 years) that had not received any medications for at least two months and were free of significant lameness, as determined by two board-certified equine surgeons through lameness examination, were selected. All procedures complied with institutional animal care committee protocols approved for this study (Koenig, University of Guelph Animal Care Protocol # 1879).

*Clinical lameness evaluations*

Prior to injection of any substance and each sample collection, each horse received a general physical exam and was evaluated at a walk and trot by two board-certified equine surgeons who were blinded to treatment. The leg appearing most lame and the associated lameness grade were recorded using the American association of equine practitioners (AAEP) grading scale (214).

*Feasibility studies*

Two feasibility studies were performed initially to ensure LPS consistently induced acute synovitis and to ensure that allogeneic administration of CB-MSC did not induce significant adverse reactions. Horses from the above group were randomly assigned to either the LPS (n=3) or CB-MSC (n=3) groups using a random number generator. Each horse was sedated (detomidine hydrochloride 0.01 mg/kg, butorphanol tartarate 0.01 mg/kg—i.v.), and hock circumference was 59
measured with the aid of a series of marks clipped into the hair around the circumference of the joint to standardize subsequent measurements. Both hocks were clipped and prepared for aseptic injection into the tarsocrural (TC) joint. Treatment and control joints were randomly assigned by coin flip. Arthrocentesis was performed using a 20 Ga x 1” hypodermic needle, and 2 mL of SF was collected. Either 0.5 ng of LPS diluted to 2 mL total volume (in lactated Ringer’s solution—LRS) or 30 million CB-MSC (in 2 mL saline) were injected. The contralateral TC joint received 2 mL of the diluent solution (either LRS or saline) that had been used in the original limb. General physical and lameness examinations were performed at 0, 8, 24, 48, and 72 hours post injection. 2 mL of SF was collected at each time-point (see below for details on SF handling and analysis).

Evaluation of CB-MSC and LPS co-administration

Following a washout period of at least four weeks all six horses used in the above feasibility studies were used to determine if CB-MSC reduced the inflammatory response following LPS injection. Due to the transient inflammatory event associated with LPS induced synovitis and a lack of evidence as to optimal treatment time, we chose to inject CB-MSC at the same time as LPS in order to maximize the possibility of detecting differences between treatment and control joint pairs. For this study the middle carpal joint was utilized to minimize the chance of any residual effect from the prior feasibility studies. CB-MSC dose was subsequently adjusted to attempt equal CB-MSC/synovial volume as in the feasibility study. Each horse was sedated, the carpi measured, and arthrocentesis with SF collection was performed as described above. LPS (0.5 ng) in 2 mL LRS was administered IA followed by 10-million CB-MSC suspended in 2 mL of saline. Synovitis was induced in the contralateral middle carpal joint in a similar manner,
except 2 mL saline was injected alone instead of CB-MSC suspension. Lameness examination and SF sampling occurred as described for the feasibility studies.

**SF collection and handling**

Each SF sample was divided into glass EDTA and anti-coagulant free vials and immediately transported to the laboratory at room temperature. Each EDTA vial was submitted to our university’s veterinary diagnostic laboratory for routine cytological analysis, which included total nucleated cell count (TNCC) (Coulter Z2 nucleated cell counter, Beckman Coulter, Hialeah FL), total protein (TP) by refractometry, and differential cell count (blinded board-certified clinical pathologist). From these numbers, absolute numbers of neutrophils and mononuclear cells were calculated. Published reference ranges were used for interpretation of results (21). The remaining vial of SF was spun at 13,793g for 10 minutes at 4°C and the supernatant removed. The SF supernatant was divided and stored at -80°C for future evaluation. From these aliquots in the CB-MSC feasibility study and the LPS/CB-MSC co-administration study glycosaminoglycan (GAG) (43, 52, 59) release was determined using the dimethyl methylene blue assay. Prostaglandin E2 (PGE2) (48), C-propeptide of type II collagen (CPII) (17, 52), type II collagen cleavage neopeptide (C2C) (17, 43), and chondroitin sulfate CS-846 epitope of aggrecan synthesis (43, 52) were evaluated using commercially available ELISA kits (CPII, C2C, CS-846: Ibex Technologies Inc. Montreal QC; PGE2: RnD Systems, Minneapolis, MN).

**Statistical analysis**

Raw data from joint circumference and SF analysis, biomarker was imported into statistical software (SAS 9.2, SAS institute, Carey NC). Data were analyzed using a general linear mixed model using the PROC MIXED function. Residual analysis was used to determine if
ANOVA assumptions were met, to detect outliers, and to evaluate the need for data transformation. When comparisons were made between simple effects, the simple effect ANOVA tables were used to determine statistical significance. For the purpose of determining statistical significance, $\alpha$ was set at 0.05.

The feasibility studies’ lameness examinations and TC joint circumference data was reported as mean lameness grades and mean differences in joint circumference from pre-injection measurements.

Clinical lameness examinations, from LPS/CB-MSC co-administration study, were evaluated by calculating kappa statistic to describe intra-observer agreement on the lameness leg and grade. Lameness grades were transformed from the AAEP numerical grading scale to a binary grading system by adding the lameness scores and assigning as either lame (sum≥2) or not lame (sum=0 or 1) for calculation of the kappa statistic. Odds ratios were calculated using Fischer’s exact test to compare lameness at post injection time points to baseline.

Power calculations were not performed in this study, as the expected variance was not known. Had differences not been detected, the observed variance would have been used to calculate the necessary sample size to observe differences. If the calculated sample size were feasible, a second round of sampling would have been initiated.

Results

LPS feasibility study

Eight hours following LPS injection into the TC joint, a moderate increase in hind-limb lameness (mean lameness grade 2.7, range 2-3) was observed. At all subsequent time points,
lameness had returned to baseline levels (all horses grade 1) as noted in table 1. Peak joint circumference was observed 8h post LPS injection (table 1).

SF cytology following LPS injection into the TC joint revealed most significant elevations in TNCC, TP, and the proportion of neutrophils in the SF 8 h post-injection (p<0.001, each). A mean (95%CI) TNCC of 73x10^9/L (46.5-99.5x10^9), TP of 42.3g/L (38.1-46.6g/L) and 89% neutrophils (83-95%) were observed at 8h, which decreased in subsequent time points (Appendix II, Figures A-E).

CB-MSC feasibility study

Following injection of 30-million CB-MSC into the TC joint there was a moderate increase in lameness (mean lameness grade 2.6, range 2-4). Lameness persisted for 24 h, but had resolved by 48 h as observed in table 1. One horse was lame at the walk (grade 4) and was treated with epidural anesthesia (morphine 0.1 mg/kg, Q8h, 2 doses). Lameness resolved without further treatment by 48 h. Peak joint circumference was observed at 48h post injection.

Compared to control joints, injection of 30-million CB-MSC into TC joints resulted in an elevated TNCC at 8 h (p=0.01), figure 1A. In joints receiving CB-MSC injection a mean (95% CI) TNCC of 13.1x10^9/L (7.7-18.5 x10^9), TP of 33.6 g/L (17.3-65.6 g/L) and %neutrophils of 73% (41.9-104.2%) was observed at 8h. Elevated TP in CB-MSC-treated joints was observed at 48 and 72 h (p<0.001 and p=0.01, respectively), figure 1B. CB-MSC injection was not associated with altered SF differential cell count (%neutrophil) at any time. Calculated mononuclear cell numbers were increased at 8 h post-CB-MSC injection (p=0.02), figure 1D.

PGE_2 concentrations were higher in MSC-treated joints at 24 and 72 h (p<0.001, p=0.01, respectively), figure 2A. CP-II concentrations were higher in MSC treated joints compared at 24,
48, and 72 h (p=0.04, p<0.001, p=0.004 respectively), figure 2B. CS-846 concentrations were higher in CB-MSC treated joints at 48 and 72 h (p<0.001 and p=0.003, respectively), figure 2C.

No significant differences were detected in GAG or C2C concentration, or in the percentage of neutrophils in the differential cell count between CB-MSC-treated and control joints at any time (Appendix II, Figures F-H).

*Evaluation of CB-MSC and LPS co-administration*

Substantial agreement was observed between observers (kappa=0.62) when adjusted for horses considered not lame or bilaterally lame. The presence of lameness was higher in legs treated with LPS/CB-MSC than those treated with LPS alone 8 hours post injection (OR 41.6, p=0.02). At all other time points, the incidence of lameness was not different from baseline lameness values.

LPS/CB-MSC treated carpi had significantly increased circumference at 0 and 48 h (p=0.02 and p=0.01, respectively). Differences from pre-injection measurements are summarized in table 2. A highly significant increase in circumference was observed at all time points in LPS/CB-MSC treated joints, while joints treated with LPS alone were not or were marginally larger at 48 and 72h.

LPS/CB-MSC-treated joints had reduced TNCC at 8h when compared to control joints (p=0.002). Elevated TNCC was observed in LPS/CB-MSC treated limbs at 72 h relative to control joints (p=0.005), figure 3A.

Calculated absolute numbers of neutrophils (TNCC x % neutrophils) and mononuclear cells (TNCC x (1-% neutrophils)) were reduced in joints treated with LPS/CB-MSC at the 8 h time point (p=0.01, p=0.01), figures 3C & 3D respectively). The proportion of neutrophils in the SF was not different between treatment and control paired joints at any time-point.
LPS/CB-MSC treated joints had significantly elevated TP concentration at 48 and 72 h when compared to control joints (p=0.003, p=0.002), figure 3B.

Concentrations of GAG (p>0.2), PGE₂ (p>0.1), CP-II (p>0.3), C2C (p>0.3), and CS-846 (p>0.05) were not different between treatment and control joints at any time (Appendix II, Figures I-N).

Discussion

In this study, we report three main findings. First, a moderate inflammatory reaction and lameness was observed following injection of CB-MSC into TC joints untreated with LPS. Second, LPS/CB-MSC treatment of the middle carpal joint resulted in a >50% reduction in TNCC. Finally, CB-MSC injection resulted in mild, self-limiting inflammation following the initial inflammatory reaction in both the feasibility and LPS/CB-MSC co-injection studies.

Confidence in the LPS synovitis model was obtained through the LPS feasibility study, which yielded results that were consistent with those previously reported (93).

CB-MSC injection in joints untreated with LPS was associated with a short-term inflammatory reaction in all three horses as seen by increased TNCC, neutrophil influx, and elevations in PGE₂ at 8 and 24h. Sustained inflammation was also observed through elevated TP and PGE₂ at 72h. Two horses developed mild to moderate lameness, while grade 4 out of 5 lameness occurred in one horse. Epidural analgesia was only administered in this animal to avoid NSAID related changes in SF PGE₂ levels as observed by others (215). The lameness response of this horse is typical of acute post-injection inflammation or “joint flare”, which has been reported to have an incidence of 10% after IA injection of 13-million autologous BM-MSC (6). Autologous and allogeneic MSC have been evaluated in joints untreated with LPS using 7.5-
million (177) and 15-million (178) MSC. In these studies, mild to moderate lameness, increased joint circumference, and limb edema were observed in MSC-treated limbs, which resolved without treatment. These observations indicate a real risk of a joint flare in the hours and days following MSC injection. Despite the self-limiting nature, horse owners should be made aware of potential complications prior to injection of MSC suspensions in clinical practice.

Interestingly, peak TNCC observed in this study was less than half previously reported following autologous or allogeneic IA MSC injection into joints untreated with LPS (177, 178). This indicates that although allogeneic CB-MSC injection induced short-term inflammation, this may not occur in a dose-dependent manner. In the CB-MSC feasibility study increased lameness did not correlate with findings in the SF. The large percentage of neutrophils (73%) observed in the 8 h sample indicates a neutrophil influx when compared to the relatively uniform original mononuclear cell population. Detection of CB-MSC in the SF did not confound cell counts, as CB-MSC would likely be counted as large mononuclear cells and not neutrophils. Additionally MSC rapidly localize to tissues outside the SF (216) and the number of CB-MSC injected only accounts for 1/20th of the increase observed at 8h (217). This suggest neutrophil-based infiltration is responsible for the significant increase in TNCC observed and correlates with the subjective increase in lameness 8 and 24 h in limbs receiving CB-MSC injection in this and previous reports (177, 178).

Our use of allogeneic MSC in this feasibility study may have influenced the outcomes as Pigott et al (2013) concluded that signs of inflammation increased 25% on average with the use of allogeneic BM-MSC compared to autologous BM-MSC. However, autologous and allogeneic CB-MSC have also been compared and no difference in clinical or cytological parameters were reported (177). These findings indicate that further study is needed to evaluate the effect of MSC
dose, tissue source, and allogeneic versus autologous MSC effects to better utilize IA MSC injection as a potential therapeutic option.

LPS/CB-MSC co-administration into the equine middle carpal joint reduced the total nucleated cell count at 8 h. Although TNCC at 72 h remained elevated in the LPS/CB-MSC joints this is not considered clinically significant as both LPS and LPS/CB-MSC TNCC were within the normal range for joints previously injected with a balanced electrolyte solution (21). Calculation and analysis of neutrophil and mononuclear cell numbers indicated this suppression was a result of suppression of both neutrophil and mononuclear cells. The mechanism behind this observation is unknown, but in vitro studies from our lab and others have shown that various MSC suppress the proliferative response of stimulated lymphocytes in MLR (106, 109, 151, 201).

CB-MSC co-injection with LPS resulted in transient mild lameness with an odds ratio of 41.6 at 8 h when compared to baseline lameness scores. Two experimental studies (177, 178) and one multicenter trial using clinical cases (6) have reported a similar increase in lameness and signs of inflammation following MSC injection. Our observed differences in joint circumference following CB-MSC injection in joints untreated with LPS as well as LPS/CB-MSC injected joints further support the notion of CB-MSC-associated inflammation. Although relatively mild and self-limiting, the potential for MSC associated inflammation should be disclosed to animal owners/agents prior to consenting to experimental procedures such as IA MSC injection as there is potential for adverse reactions.

Although CB-MSC injection seems associated with an inflammatory event, the nature of the inflammation appears markedly different than that induced by LPS alone. CB-MSC co-administration with LPS reduced the total nucleated cell count observed eight hours following injection of LPS. Calculation and analysis of neutrophil and mononuclear cell numbers indicated
this suppression was a result of equal suppression of both neutrophil and mononuclear cells. We speculate that the observed differences are due to an initial CB-MSC mediated suppression of the marked inflammation that accompanies IA LPS injection followed by mild self-limiting mononuclear cell synovitis as reported by others (118). Whether the mononuclear cell synovitis is due to the CB-MSC or foreign biological substances originating from the in vitro culture process remain to be determined.

We were unable to detect changes in the biomarker levels following LPS/CB-MSC injection as hypothesized. The significant elevation in biomarker levels due to LPS injection alone and a small sample size likely prevented detection of potential differences between LPS/CB-MSC and LPS only limbs (43). IA LPS injection has been shown to stimulate a significant increase in all five biomarkers evaluated in this study with peak levels occurring at 8 or 24h post injection (43). Bilateral LPS injection in this study likewise stimulated a bilateral increase in a similar fashion. Whether the LPS injection masked CB-MSC effect on the cartilage structure, as observed in the CB-MSC feasibility study, or whether CB-MSC do not affect cartilage structure in the LPS/CB-MSC co-administration model remain to be determined. Injection of MSC into a joint with spontaneous natural OA may answer this question. In human patients with naturally occurring OA, improved clinical outcome (212) and improvement in three different methods of scoring OA (211) has been noted following IA MSC injection. Additionally, in dogs, improved lameness, as observed using force plate analysis (209, 210), and improved clinical outcomes (165, 166) have been observed in cases of naturally occurring OA. Evaluation of SF—including biomarkers of cartilage metabolism—has not, however, been performed in naturally occurring OA in the horse or other species following IA MSC injection. It is of interest that CB-MSC injected into joints untreated with LPS in the feasibility study was associated with elevations of CP-II and CS-846. These two biomarkers indicate up-regulation of anabolic
processes associated with cartilage homeostasis (17). Whether this is a result of direct stimulation of the anabolic or reparative pathways within articular cartilage by the CB-MSC or due to the inflammation induced by the CB-MSC is undetermined (43, 52).

The short duration of inflammation, 24-48 h that is induced by this LPS model of acute synovitis poses some limitations (93). Simultaneous LPS and MSC administration does not mimic all clinical situations. The findings, however, may be transferable to conditions where acute joint inflammation occurs and is rapidly treated. Such situations may include elective arthroscopic debridement of cartilage lesion, acute septic synovitis, or osteochondral fragmentation. The results may not be transferable to clinical situations of prolonged inflammation, although in vitro studies showed effects of MSC therapy independent of treatment time relative to disease onset (106). Induced reversible modeling of chronic joint inflammation by repeated low dose LPS injection might provide additional information as to the immune modulatory effects of MSC (93).

The decrease in neutrophil and mononuclear cell numbers following IA administration CB-MSC lends support to the notion that CB-MSC may have therapeutic potential for treating joint inflammation. This study also adds to the growing body of evidence that IA MSC injection induces a variable, mild, self-limiting inflammatory reaction. Continued evaluation of the effects of IA CB-MSC therapy on synovitis in horses to elucidate this apparent conundrum of anti- and pro-inflammatory properties of CB-MSC. Parameters of immediate interest are dose, timing of treatment, and treatment frequency.
Acknowledgements

We would like to thank Ms. Talia Richardson for her help with patient care and sample collection, Ms. Michelle Beaudoin-Kimble for performing synovial fluid biomarker assays and Mr. William Sears for performing statistical analysis for this study.

Funding for this study was generously provided by Equine Guelph.
Figures:

Figure 1: (A-D) Synovial fluid cytology following injection of 30-million equine CB-MSC into one tarsocrural joint of three horses with equal volume of saline injected into the contralateral joint. A) Total nucleated cell count, B) total protein, C) Neutrophil, and, D) Mononuclear Cell numbers. Error bars indicate 95% confidence interval. *p<0.05, **p<0.01, ***p<0.001
Figure 2: Synovial fluid A) prostaglandin E-2 (PGE₂), B) C-propeptide of type-II collagen (CP-II), C) chondroitin sulfate CS-846 epitope of aggrecan synthesis following injection of 30-million equine CB-MSC into one tibiotarsal joint of three horses with equal volume of saline injected into the contralateral joint. Error bars indicate 95% confidence interval.

*p<0.05, **p<0.01, ***p<0.001
Figure 3: A) Total nucleated cell count, B) total protein, C) neutrophil, and D) mononuclear cell numbers of synovial fluid following induction of transient synovitis using 0.5ng LPS injected into middle carpal joints bilaterally. One limb was treated with 10-million allogeneic umbilical cord blood derived MSC. *p<0.05, **p<0.01, ***p<0.001.
Tables:

Table 1: Lameness and Tarc circumferences after IA LPS or CB-MSC into TC joint

<table>
<thead>
<tr>
<th>Hours</th>
<th>LPS lameness grade (range)</th>
<th>TC circ.³ (LPS⁴/control⁵)</th>
<th>lameness grade (range)</th>
<th>TC circ.³ (CB-MSC⁶/control⁵)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.2 (0-2)</td>
<td>0/0</td>
<td>0.8 (0-1)</td>
<td>0/0</td>
</tr>
<tr>
<td>8</td>
<td>2.7 (2-3)</td>
<td>2.0/0.8</td>
<td>2.6 (2-4)</td>
<td>1.1/1.7</td>
</tr>
<tr>
<td>24</td>
<td>1 (1-1)</td>
<td>1.6/1.3</td>
<td>3.1 (2-4)</td>
<td>2.2/2.1</td>
</tr>
<tr>
<td>48</td>
<td>0.5 (0-1)</td>
<td>1.3/0.3</td>
<td>0.6 (0-1)</td>
<td>2.4/1.7</td>
</tr>
<tr>
<td>72</td>
<td>0.3 (0-1)</td>
<td>1.0/0.8</td>
<td>1.3 (0-2)</td>
<td>1.3/1.5</td>
</tr>
</tbody>
</table>

³ Tarsocrural Joint (n=3)
⁴ 30-million allogeneic equine umbilical cord blood derived mesenchymal stromal cells (CB-MSC)
⁵ Difference from pre-injection measurements (cm)
⁶ 0.5ng LPS suspended in 2 mL lactated Ringer’s solution
⁷ qual volume balanced electrolyte solution control
Table 2: Carpal circumference of horses (n=6) receiving IA LPS\textsuperscript{b}+CB-MSC\textsuperscript{c} or LPS\textsuperscript{bd}  

<table>
<thead>
<tr>
<th>Hours</th>
<th>LPS+CB-MSC difference (cm)</th>
<th>p-value</th>
<th>LPS+CB-MSC difference (cm)</th>
<th>p-value</th>
<th>LPS only difference (cm)</th>
<th>p-value</th>
<th>LPS only difference (cm)</th>
<th>p-value</th>
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<tbody>
<tr>
<td>8</td>
<td>0.28</td>
<td>0.03</td>
<td>0.98</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>1.25</td>
<td>&lt;0.001</td>
<td>1.05</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>1.26</td>
<td>&lt;0.001</td>
<td>0.5</td>
<td>0.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>1.28</td>
<td>0.001</td>
<td>0.8</td>
<td>0.03</td>
<td></td>
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</table>

\textsuperscript{a} Difference from baseline measurements  
\textsuperscript{b} 0.5 ng LPS suspended in 2 mL lactated Ringer’s solution  
\textsuperscript{c} 10-million allogeneic equine umbilical cord blood derived mesenchymal stromal cells (CB-MSC)  
\textsuperscript{d} Injection into contralateral middle carpal joint
Chapter 4: Aspiration, but not injection, decreases equine mesenchymal stromal cell viability

This chapter is a modified version of a manuscript submitted to BMC Veterinary Research on January 30, 2015.

BMC Veterinary Research submission number: 486578651585858
Abstract

Recently, equine multipotent mesenchymal stromal cells (MSC) have received significant attention as therapy for various conditions due to their proposed regenerative and immune-modulating capacity. MSC are commonly administered to the patient through a hypodermic needle. Currently, little information is available on the effect of such injection on equine MSC immediate and delayed viability. We hypothesize that viability of equine MSC is not correlated with needle diameter during aspiration and injection. Equine CB and BM-MSC were injected through 18-30 Ga needles and immediate viability was measured using a propidium iodide based automated cell counter. Injected MSC suspensions were cultured 24 hours and evaluated using a resazurin based assay. The experiment was then repeated with the exception of aspirating (rather than injecting) MSC suspensions through 20, 25, and 30 Ga needles. Using a 3 mL syringe, manual injection of equine cord blood (CB) or bone marrow-derived (BM) MSC with no needle and needles ranging in size from 18 to 30 Ga did not affect immediate MSC viability. Similarly, 24 h post-injection, MSC delayed viability was not different between any of the treatment groups as determined by a resazurin-based proliferation assay. Using a 3 mL syringe, aspiration of MSC through 20, 25, and 30 Ga needles resulted in significant decreases in immediate viability with no change in delayed viability when compared to aspiration without a needle. BM- and CB-MSC were observed to be similar size with a diameter±SD of 19.8±2.7 and 20.4±2.2µm, respectively. In comparison, the smallest needles, (30 Ga) have a 160 µm internal diameter. Following injection, needle diameter did not affect immediate or delayed viability of equine MSC. Following aspiration through needles sizes 20 Ga and smaller, immediate viability, but not delayed viability, decreased. As a result, an 18 Ga or larger needle should be utilized for aspiration of cell suspensions. In contrast, needle selection for MSC injection should be based on clinical preference and experience rather than concerns over decreasing MSC viability.
Introduction

Multipotent mesenchymal stromal cells (MSC)—commonly referred to as mesenchymal stem cells—are thought to have potential as a therapeutic option for orthopedic injuries. In human and veterinary medicine, promising results have been reported in vivo following treatment of joint injuries, cartilage defects, and tendon injuries using MSC therapy (6, 7, 211, 212, 218, 219). The current practice of MSC therapy requires administration of a cell-based suspension into the target tissue or structure by injection through a hypodermic needle. In order for MSC to retain their potential benefits, immediate and delayed viability must not be significantly affected by cell collection, culture, preparation, or injection. The FDA has recommended greater than 70% viability for cell based therapeutic product prior to injection (220). Currently there is little reliable evidence as to the effect of aspiration or injection of equine stem cell products through hypodermic needles.

One of the defining characteristics of MSC is their ability to adhere to tissue culture-treated polystyrene plastic. In order for MSC to be used as a therapeutic agent, they must be detached from the culture flask and suspended in liquid prior to injection. This process results in spindle-shaped adherent MSC transforming into spherical free-floating MSC. Reported diameters for suspended rat- and human-derived MSC range from 12-15 µm (196). The diameter of equine MSC in suspension is not available in the scientific literature. The effects of aspiration and injection on MSC have not been investigated separately. However, intermittent negative pressure was shown to decrease MSC viability in one human wound-healing study (221). Additionally, decreased equine MSC viability has been reported following repeated aspiration and injection of MSC suspensions (199).

While the viability of MSC obtained from rat and human bone marrow- (BM-MSC) did not appear related to needle size or injection rate (196, 222), these findings may not be applicable
to equine MSC as numerous species-dependent factors, most notably MSC size, are likely different. This being said, the internal diameter of a 30 Ga hypodermic needle is 160 µm, which is considerably larger than any cell. We therefore hypothesize that viability of suspended equine MSC following aspiration or manual injection is not correlated with needle diameter. The aims of this study were to evaluate immediate and delayed viability of MSC 24h following separate aspiration and injection through a variety of needle sizes commonly used in veterinary medicine.

**Materials and Methods:**

*Preparation of cell suspensions*

From nine unrelated horses, 5 BM-MSC and 4 CB-MSC cultures were selected from cryopreserved stock for use in this study. Standard isolation protocols were used in the collection, culture, and cryopreservation of all MSC cultures as described elsewhere (151, 203, 223). BM-MSC cultures had been cryopreserved once and CB-MSC cultures twice prior to commencement of this study. Each cryovial containing 1x10^6 MSC was thawed in a 37°C water bath and transferred to either BM-MSC culture media (DMEM low glucose, 10% fetal bovine serum (FBS), 1% L-glutamine, and 2% penicillin/streptomycin) or CB-MSC culture media (containing 30% FBS, otherwise identical) and cultured at 38°C, 5% CO₂, in a humidified atmosphere to obtain necessary MSC numbers. All MSC cultures were between passages 3-5 and 19-36 days in culture at the time of injection. MSC were detached from culture flasks using trypsin-EDTA and suspended in their respective culture media at a concentration of 5x10^6 MSC/mL. MSC suspensions were stored in polypropylene vials, at room temperature, in culture media for the
duration of the experiment. For injection and aspiration studies MSC cultures were in suspension at room temperature for 12 and 4 h respectively.

**Effect of injection on MSC viability**

Using a 3 mL Luer-lock syringe, 0.5 mL of the above cell suspension was injected over a 2 second period into 1.5 mL Eppendorf vials with the needle tip hovering at the top of each vial. One individual (LW) performed injection with the following needles attached to the syringe: no needle, 18Ga x1.5”, 20Ga x1”, 22Ga x1”, 23Ga x3/4”, 25Ga x5/8”, 27Ga x1/2”, and 30Ga x1”. Duplicate injections were performed for each cell population and needle combination. Cell viability was determined using an automated fluorescence-based cell counter (Nucleocounter NC-100, Mandel Scientific Company, Guelph, ON). Each injected cell suspension was seeded into two wells of a 96-well plate at a density of 5000/cm² and cultured for 24 h in 300 µL of their respective culture media. Subsequently, a resazurin assay, which had been previously optimized according to the manufacturer’s instructions, was used to assess delayed viability by replacing cell culture media with 300 µL of 10% resazurin (Sigma-Aldrich, Oakville ON) in phosphate buffered saline. The cells were incubated 4 h before fluorescence was read using an automated plate reader (Spectramax i3, Molecular Devices, Sunnyvale CA) at 585 nm using an excitation wavelength of 555 nm.

**Effect of aspiration on MSC viability**

Three BM-MSC and three CB-MSC cultures were selected from the above cell cultures to evaluate the effect of aspiration on MSC viability. MSC suspensions were prepared as described above with the following exception: 0.5 mL of MSC in suspension were slowly aspirated using a 3 mL syringe by one operator (LW) through the following: no needle, 20x1”, 25x5/8”, 30x1”
needles, at a rate of 0.25 mL/s. Needles were removed prior to ejection of cell suspensions from the syringe. Immediate and delayed viability were assessed as described above using duplicate samples for each cell suspension/needle combination. Fewer needles were selected for these aspiration studies since no differences were expected based on the injection experiments. In addition, needle size used for aspiration is less critical since the needle used for aspiration is commonly replaced prior to injection.

**Measurement of cell diameter in suspension**

The diameter of suspended BM- and CB-MSC was measured by capturing digital images of cell suspension using a digital inverted microscope (EVOS FL, Life Technologies, Carlsbad CA) and measuring cell diameter using software (Image J 1.48, National institutes of health, Bethesda MD, USA) calibrated to the scale bar associated with each image. The diameter of 100 cells was recorded for BM- and CB-MSC cultures with mean and standard deviation (SD) reported.

**Statistical Analysis**

Viability and fluorescence data were imported into a statistical analysis software package (SAS, SAS institute, Cary, NC). A general linear model was used to analyze the effect of MSC source (either BM or CB) and needle size using the PROC MIXED function. Residual analysis was performed in order to determine if ANOVA assumptions were met, to detect potential outliers, and evaluate the need for data transformation. The residuals were formally tested for normality using the four tests offered by SAS (Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises, Anderson-Darling) and plotted against the predicted values and variables used in the model. Cell diameters were compared using a 2 sample t-test (R 3.0.1, R foundation for statistical
Results

Effect of injection on MSC viability

Regardless of the tissue source or needle size, >89% immediate viability was achieved. Immediate viability was significantly increased in MSC injected through 25 Ga needles (p=0.01), figure 1A. There was no difference between cells originating from BM or CB, and no interaction was detected between MSC source and needle gauge. There were no differences in fluorescence from MSC cultures assessed using the resazurin assay regardless of cell source or needle gauge, and no interaction was detected between MSC source and needle gauge, figure 1B.

Effect of aspiration on MSC viability

Following aspiration, needle size had a significant effect on MSC immediate viability (p<0.0001), but not on delayed viability, figure 2. Compared to non-aspirated controls, 20, 25, and 30 Ga needles all decreased MSC viability (p=0.04, 0.0003, and <0.0001, respectively). There was no difference in MSC immediate or delayed viability between MSC from BM and CB origins, and no interaction was detected between MSC source and needle gauge.
Measurement of cell diameter in suspension

No difference was detected between BM- and CB-MSC diameters (p=0.12). Mean±SD diameters of 19.8±2.7 µm and 20.4±2.2 µm were observed for BM- and CB-MSC respectively, figure 3.

Discussion

Although equine MSC in suspension are >25% larger in diameter than those of other species (196), needle size did not decrease MSC immediate or delayed viability post-injection. Aspiration, however, significantly decreased MSC immediate viability, but did not effect-delayed viability. This finding is of significant interest for clinical administration of equine MSC as care should be taken in selecting a large diameter needle—such as 18 Ga or larger—for aspiration of cell suspensions into the syringe. After the syringe is loaded, however, needle selection can be made without concern for affecting MSC viability.

We observed significantly higher immediate viability in MSC injected through 25 Ga needles compared to all other needles and the no-needle control. The 25 Ga injections were performed sixth in the series of eight injections, which were then repeated for a second technical replicate. As such, we do not consider the experimental setup to have influenced this anomaly. Considering there are no clinical implications for increased MSC viability and that this increase was noticed using the third-smallest diameter needle evaluated, the increased viability in this group is not considered clinically significant.

The injection rate used in this study is likely slower than the rates used in clinical practice where larger gauge needles (18-22 Ga) are used. This rate was selected however, it was the maximum that could be achieved using small diameter needles (<25 Ga) and a consistent rate
was desired for this study. Significant manual force was required to achieve the desired injection rate with a 25 Ga and 27 Ga needle and the rate of 0.25 mL/s could not be achieved using a 30 Ga needle despite maximal force (to the extent of bending the syringe plunger). The use of progressively smaller needles compensated for the relatively slow injection rate used and allows us to conclude that the forces achieved by MSC injection using a 3 mL syringe are not sufficient to decrease MSC viability or proliferative ability. As smaller syringes were not evaluated, no inference can be made about any potential damage that injection through these syringes may induce on equine MSC. It is possible that a syringe with a smaller plunger may be able to create sufficient pressure and shear forces on MSC during injection to decrease immediate or delayed MSC viability. Garvin et al recently reported equine BM-MSC injected using a 2 mL syringe show increased propidium iodide and annexing V staining (necrosis and apoptosis, respectively) immediately following injection through 21 or 23 Ga needles. Differences in methodology are likely responsible for the differences observed as higher pressures, longer needles, increased injection rates, and cellular trauma associated with negative pressure (aspiration through the test needle) (221) may have influenced their results (see Appendix III). Conflicting results have been reported as to the effect of injection through various needle diameters using human and rat MSC. The most dramatic decreases in MSC viability, increases in markers of apoptosis, and decreased MSC proliferation were observed using MSC concentrations as high as 5x10^7/mL, rapid injection rates, small diameter needles and storage for more than 2h at room temperature prior to injection (197).

In previous studies aspiration through the test needle (185, 197, 198) or repeated aspiration and injection (199) likely also have a negative effect on MSC viability due to intermittent negative pressure which has been associated with apoptosis in other studies (221). Consistent with the observation of detrimental effects on MSC viability in other species.
immediate injection without aspiration of dilute cell suspensions at slow rates resulted in no change in MSC viability or apoptotic fraction using needles as small as 30 Ga (196, 222). While performing the injection studies, we observed that pre-injection MSC viability had decreased by approximately 10% towards the end of data collection. We attributed this to the time that MSC suspensions had been kept at room temperature (~12 h) and, therefore, elected to decrease the number of needles and cell suspensions evaluated for subsequent aspiration studies to reduce the time MSC were at room temperature. We considered this a reasonable strategy since in a clinical situation the needle used to aspirate a cell suspension is routinely changed prior to injection. We conclude that evaluation of 18 Ga needles should have been included in the aspiration study due to the significant decrease in viability in all needles tested. However, although statistical significance was detected between MSC aspirated through 20 Ga needles (p=0.04) and MSC aspirated without a needle, immediate viability only decreased by 0.6% on average from 97.6 to 97.0. This is most likely not of clinical significance, and viability remained well above the 70% cut-off suggested by FDA.

Our methodology assessed immediate viability by propidium iodide exclusion by the intact cellular membrane and delayed viability through metabolism of the dye resazurin. Both of these measurements provide information as to the viability and physiological state of the undifferentiated MSC, but do not assess differential potency or immunomodulatory properties of the MSC. We do not expect these functional properties of the MSC to be affected, but future work could determine if aspiration or injection affects the ability of the MSC to differentiate into adipogenic, osteogenic or chondrogenic cell fates or their lymphocyte suppressive properties in vitro.
Conclusion

Care should be taken to use large diameter needles when aspirating equine MSC. However, for injection, needle selection should be based on clinical experience, knowledge of patient behavior, and the anatomical relationship of the targeted structure without concern over MSC viability.

Acknowledgements:

This project was supported by the Danish Agency for Technology, Production and Innovation, Equine Guelph and the Department of Biomedical Sciences at the University of Guelph. None of the funding agencies were involved in design, collection, analysis, interpretation of data, writing of the manuscript, or the decision to submit this manuscript for publication. We would like to thank Mr. William Sears for his expertise in performing statistical analysis of the data included in this study.
Figure 1: Viability following injection of equine mesenchymal stromal cells; (A) Immediate and (B) delayed viability of MSC following injection through various needle diameters as assessed using a propidium iodide based automated cell counter and resazurin fluorometric assay, respectively. Error bars represent 95% confidence interval. Asterisks* (*p<0.05, **p<0.01, ***p<0.001) indicate significant difference from non-injected samples.
Figure 2: Viability following aspiration of equine mesenchymal stromal cells; (A) Immediate and (B) delayed viability of equine bone marrow- and cord blood-derived MSC using a propidium iodide based automated cell counter and resazurin fluorometric assay following aspiration through various needle diameters. Error bars represent 95% confidence interval. Asterisks* (*p<0.05, **p<0.01, ***p<0.001) indicate significant difference from non-injected samples.
Figure 3: Relative diameter of equine MSC compared to the needles evaluated
Chapter 5: Intravenous injection of HypoThermosol®-FRS, CryoStor®-10 or pooled allogeneic cord-blood-derived mesenchymal stromal cells does not result in physical exam, hematological, biochemical, or coagulation abnormalities in ponies
Abstract

Mesenchymal stromal cell (MSC) therapies in the horse often relies on third-party procurement of the cells and subsequent shipping of the cells to the treating clinician. Preservation of MSC viability and reduced risk of iatrogenic contamination of MSC suspensions prior to treatment are critical. MSC are often shipped either chilled or frozen. The cold-chain is susceptible to shipment delays whereas the functionality of frozen cells immediately following thawing is largely unknown. The safety of two commercially available carrier solutions developed for optimum preservation of mammalian cells during chilled and frozen transport and excipient use were evaluated in live horses with and without suspended allogeneic cord blood-derived (CB) MSC. We hypothesized that the carrier solutions alone or in combination with CB-MSC would not elicit measurable changes in clinical parameters, hematological including CD4+ and CD8+ counts and serum biochemical parameters.

In 9 healthy ponies a 10-mL bolus of either HypoThermosol®-FRS (HT-FRS), CryoStor10® (CS-10) or saline was injected IV (n=3/treatment). Ponies were monitored by physical examination, complete blood cell count, and biochemistry and coagulation profiles prior to, and over the next 7 days following injection. CD4 and CD8 lymphocyte populations were also evaluated in each blood sample. After one week washout period, 5x10^7 pooled (5 donor animals of 1x10^7 each) allogeneic CB-MSC were administered IV (n=9) suspended in HT-FRS following 24h simulated transport at 4°C and 10 mL acellular HT-FRS was injected IV in control animals (n=3). One week following, similar numbers of CB-MSC were administered IV immediately following thawing from frozen and 10 mL acellular CS-10 was injected IV in control animals (n=3).

In all three studies, physical exam, complete blood cell count, serum biochemistry, and coagulation panel did not deviate from established normal ranges. Proportions of CD4+ and CD8+ counts and serum biochemical parameters.
CD8+ lymphocytes increased at 168h post injection in CB-MSC treatment groups regardless of the carrier solution. Decreases in CD4+/CD8+ double positive populations were observed at 24 h and 72 h in CB-MSC treated animals. There was no difference in viability between CB-MSC suspended in HT-FRS or CS-10.

HT-FRS and CS-10 can be safely used for low volume excipient injection of MSC suspensions. HT-FRS and CS-10 both adequately maintain CB-MSC viability following hypothermic or frozen simulated transport, respectively. IV injection of 5x10^7 pooled allogeneic CB-MSC was not associated with any adverse reaction. CB-MSC injection is associated with increased CD4+ and CD8+ lymphocyte populations and decreased CD4+/CD8+ double positive lymphocytes. Although pooled allogeneic CB-MSC do not elicit clinical abnormalities, allogeneic stimulation of CD4+ and CD8+ lymphocytes may occur. The clinical implications of the altered lymphocyte counts are unknown. Further study including in vitro or in vivo evaluation of cell mediated or adaptive immunity to autologous, identical allogeneic, or MSC arising from additional unrelated individuals would help determine the nature of this response.
Introduction

Equine mesenchymal stromal cells (MSC) have been isolated from a variety of tissues including bone marrow (224-226), adipose tissue (226, 227), umbilical cord blood (CB) (107, 203), and umbilical cord tissue (226, 228). Collection of CB is a non-invasive source of CB-MSC, which are highly proliferative, capable of triliniage differentiation, and possess lymphocyte suppressive properties in mixed lymphocyte reactions (107, 151, 201, 203, 223).

Currently in veterinary medicine, MSC preparations are available following a period of in vitro culture expansion for autologous administration. Such use is limited by the several weeks needed for preparation of cultured MSC from autologous cell or tissue samples, effectively excluding immediate treatment of acute injuries. Allogeneic MSC use allows treatment of acute lesions with the luxury of time in screening and characterizing MSC cultures. In fact, the future of regenerative medicine may include cryopreserved MSC suspensions that have been screened for desirable characteristics being stocked at veterinary primary care facilities for immediate treatment at the time of diagnosis of injury.

Allogeneic MSC use has been shown to be safe for in vivo intravenous (173, 174), intraarticular (6, 178, 229), intradermal (172), intrathecal (175), and intralesional tendon injection in the horse (186, 218, 230). Although there are reports of an increased inflammatory response following intra-articular injection of allogeneic MSC (178), inflammation subsided quickly and no difference in immune response was detected between allogeneic and autologous MSC (118).

Regardless of MSC source or allogeneic/autologous use, MSC suspensions require transport from laboratory to veterinary clinic, usually by commercial overnight carrier. Decreased MSC viability has been demonstrated following transportation in various media (185, 231). One strategy reported to slow the decrease in MSC viability is to transport MSC in serum containing media. If the serum is allogeneic or xenogeneic in nature, then it is often recommended that the
MSC undergo multiple washes prior to injection to remove the majority of foreign antigens introduced by the serum. This process is inconvenient, it is associated with a decrease of total MSC numbers, and increases the risk of bacterial contamination. A cell carrier medium, which requires removal, as described prior to injection of the MSC is referred to as an ancillary medium. An excipient medium is a cell carrier solution, which provides necessary support but is otherwise unreactive and can be injected with the MSC. Excipient media allows a convenient and more standardized final product formulation since no manipulations are required at the time of treatment. HypoThermosol®-FRS (HT-FRS, Biolife Solutions, Bothell WA) and CryoStor® (CS, Biolife Solutions, Bothell WA) are cell preservation media for excipient use with chilled or frozen mammalian cells, respectively. These media are serum and protein free, commercially available, procured with adherence to current good manufacturing practices and are optimized for maintaining cell viability at 2-8°C (HT-FRS) or in a frozen state (CS-10). HT-FRS has been shown to preserve viability of cells, tissues, or organs better than cells, tissues or organs transported in any other media (187, 190-192, 232, 233). HT-FRS has also been shown to be an excellent cryopreservation media when combined with up to 15% DMSO (187, 193). CS-10 is a cryomedia with 10% DMSO.

We hypothesized that the carrier solutions alone or in combination with CB-MSC would not elicit measurable changes in clinical parameters, hematological including CD4+ and CD8+ counts and serum biochemical parameters, in healthy ponies.
Methods

Collection, isolation, and culture of CB-MSC

Cryopreserved CB-MSC cultures from our frozen stock were used. The CB-MSC were procured as previously described (107, 203). The CB-MSC display a consistent phenotype before and after cryopreservation of high expression of CD29, CD44, CD90, and none or low expression of major histocompatibility complex (MHC) class I, MHC-II, CD4, CD8, CD11a/18 and CD73 (201). CB-MSC cultures from five unrelated donor foals were expanded in culture to achieve necessary MSC numbers in DMEM containing 30% FBS, 1% Penicillin/Streptomycin, 1% L-glutamine. CB-MSC cultures suspended in HT-FRS had been cryopreserved once, were between passages 6-7, and had been cultured for a total of 42-50 days. CB-MSC cultures suspended in CS-10 had been cryopreserved once, were between passages 4-5, and had been cultured for a total of 35-40 days. Beginning 48 hours prior to injection, CB-MSC were cultured in serum free media for 24h following which they were detached from cell culture flasks using trypsin EDTA, washed once in phosphate buffered saline and stored, suspended in HT-FRS in a reusable temperature controlled shipping container (Greenbox 2-8°C thermal management system, ThermoSafe ®, Arlington Heights, IL) in the lab until just prior to the time of injection. MSC suspended in CS-10 were detached from cell culture flasks 18 days prior to injection, gradually frozen at a rate of -1°C/min to -80°C before transfer to liquid nitrogen storage. These CB-MSC were transported to the research farm on dry ice and thawed under lukewarm tap water approximately 20 minutes prior to injection. Acellular vials of HT-FRS or CS-10 (to act as controls) were handled in an identical manner to their respective CB-MSC containing vials.
Research animals

Twelve mature healthy ponies (4 female, 8 male; age 4 years) that had not received any medications for at least two months prior to the present study were used in these studies. Physical exam and baseline CBC, biochemistry and coagulation profiles indicated all horses were free from obvious disease. All procedures complied with institutional animal care committee protocols approved for this study (Koch, University of Guelph Animal Care Protocol #3247).

Although similar to horses in many ways the pony may be a better in vivo model for study of potentially immunogenic substances. Ponies express increased polymorphonucleocyte function and greater inflammatory response to inflammatory stimuli (234, 235). As a result, when evaluating a potentially immunogenic substance for safety, the pony may be a more sensitive model than the horse.

Experimental protocol

Study 1

Ponies were randomly assigned into treatment groups by lottery. Prior to injection, baseline (0 h) physical examination was performed in which temperature, pulse, respiration and demeanor were recorded and 12 mL of blood was collected from the jugular vein. A single 10-mL IV injection of one of CS-10 (n=3), HT-FRS (n=3), or physiologic saline (n=3) was administered to nine ponies. The remaining three ponies were not used in study 1. Temperature, pulse, and respiration were recorded from all treated ponies at 0, 1, 3, 6, 12, 24, 48, 72, 168 h post injection. 12 mL whole blood was collected from treated ponies at 0, 1, 24, 72, 168 h post injection.
Study 2

Ponies were randomly assigned into treatment groups as indicated above. All 12 ponies received a single IV injection of either 10 mL HT-FRS (n=3) or 5x10^7 pooled allogeneic CB-MSC (1x10^7 CB-MSC from five unrelated MSC donors) (n=9). Ponies were monitored and blood samples collected as indicated above.

Study 3

Ponies were once again randomly assigned into treatment groups as indicated above. All 12 ponies received a single IV injection of either 10 mL CS-10 (n=3) or 5x10^7 pooled allogeneic CB-MSC (1x10^7 CB-MSC from five unrelated MSC donors) suspended in CS-10 (n=9). Ponies were monitored and blood samples collected as indicated above.

**Evaluation of post transport viability**

Following injection, vials that had been used to transport CB-MSC suspensions were transported back to the laboratory at room temperature where MSC viability was calculated using the residual ~100-200uL of CB-MSC suspension in each vial using a hemocytometer counting chamber and the trypan blue exclusion assay. In total, CB-MSC suspensions had been stored under transport conditions (2-8°C in study 2, dry ice cooler in study 3) for 24 hours followed by approximately 2h at room temperature before viability measurements were obtained.

**Evaluation of blood samples**

Blood samples were submitted to our veterinary diagnostic laboratory for complete blood count (Avida 2120i, Siemens Canada, Oakville ON), serum biochemistry profile, and coagulation
profile (PT, PTT, fibrinogen). In addition CD4+ and CD8+ lymphocyte populations, which were analyzed by, flow cytometry. Briefly whole blood was prepared by combining 100 uL of whole blood with 1400 uL 1x RBC lysis buffer (9 parts dH₂O, 1 part 10x RBC lysis buffer (1.7M NH₄Cl, 0.1M KHCO₃, 1mM NH₄EDTA, pH 7.3) and incubated 5 min at room temperature. One mL flow buffer (phosphate buffered saline/1% horse serum, 15mM Na azide, 0.5mM Na₄EDTA) was added and spun at 1200 rpm for 5 min. Separate 15 minute incubations followed, with CD4 and then CD8 antibodies (catalogue # MCA108PE & MCA1078F, AbD Serotec, Raleigh, NC). The cell pellet was washed in flow buffer after each incubation. Finally the cell pellet was suspended in flow buffer for flow cytometry (BD Accuri C6, BD Biosciences, Mississauga ON). Gates to differentiate cell populations were maintained consistent throughout the experiment.

**Statistical Analysis**

Raw data from physical exams, CBC, biochemistry profiles, and coagulation profiles was imported into statistical software (SAS 9.2, SAS institute, Carey NC). Data was randomly blocked by horse and was in the form of a split plot in time design; hence there are repeated measures in time. Data was analyzed using a general linear mixed model to evaluate fixed effects (treatment, time and treatment by time interaction) and random effects (horse) using the PROC MIXED function. Various error structures, supplied by the statistical analysis software (AR, ARH, TOEP, banded TOEP 2-TOEP(t-1), TOEPH, banded TOEPH2-TOEPH t-1, unstructured, banded unstructured 2-banded unstructured t-1) were tested and one error structure was selected for each model based on the lowest Akaike Information Criterion (AIC). To improve the power of each study identical treatments were combined between studies 1-2 (HT-FRS) and studies 1-3 (CS-10) after determining there was no study effect in the linear model. Residual analysis was performed on each dataset to determine if ANOVA assumptions were met, detect potential
outliers, and evaluate the need for data transformation. The residuals were formally tested for normality using the four tests offered by SAS (Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises, Anderson-Darling) and plotted against the predicted values and explanatory variables used in the model. For the purpose of determining statistical significance α was set at 0.05.

Data resulting from post injection CB-MSC viability measurements was analyzed in a similar manner to evaluate solution effects on MSC viability.

Following statistical analysis, mean as well as upper and lower limits of the 95% confidence interval of each value (resulting from CBC, biochemistry profile, and coagulation panel) was divided by the mean prior to treatment so that all data could be plotted on the same scale.

Results

Physical exam parameters (temperature, pulse rate, respiration rate) remained within normal limits throughout the duration of all three studies, figure 1. Differences in temperature and respiratory rate were observed as indicated in figure 1. Throughout the duration of the study all animals remained bright, alert, and responsive.

CBC, biochemistry profile, and coagulation profiles did not deviate from the normal ranges provided by the diagnostic laboratory. Significant differences in parameters are indicated in figures 2 to 10.

Flow cytometry of whole blood samples to assess CD4+ and CD8+ lymphocyte populations revealed a significant decrease in the double positive (CD4+, CD8+) population 24 and 72 hours following MSC injection compared to matched HT-FRS (p=0.03, <0.0001, respectively) and CS-10 (p<0.0001, 0.04 respectively) controls. Significant elevation of CD4+
and CD8+ lymphocyte populations was observed at 168 h in animals receiving pooled allogeneic CB-MSC suspended in HT-FRS (CD4+:p=0.03, CD8+:p=0.03) and (CS-10 CD4+:p=0.02, CD8+:0.03). The CD4:8 ratio was decreased at 72h in CB-MSC in HT-FRS treated animals compared to HT-FRS treated animals alone (p=0.04). The CD4:8 ratio was also decreased at 24 and 72 hours in CB-MSC in CS-10 treated animals compared to CS-10 treated animals alone (p=0.04, 0.001, respectively), figures 11-13.

Post injection the viability of residual CB-MSC suspensions was not different between CB-MSC suspended in HT-FRS (mean-74.3, 95% CI: 69.5-78.5%) or CS-10 (mean-76.0, 95% CI: 71.4-80.0%), p=0.6, figure 14.

**Discussion**

We report no adverse clinical reaction to the IV injection of 10mL of either HT-FRS or CS-10 in healthy four-year-old ponies. Although a number of statistical differences were detected in physical exam CBC, biochemistry profile, or coagulation profile parameters, in no case were mean parameters outside of previously established normal ranges in a clinically significant manner. These significant differences were likely detected because of the small sample sizes, random variation within the herd, and as a result of chance. A similar lack of inflammatory response has been observed following IA injection of HT-FRS in horses (see Appendix IV). Likewise no adverse reactions were observed following IV injection of CB-MSC pooled from five unrelated horses. No abnormalities were detected in CBC, biochemistry, or coagulation profiles where mean values did not differ from established normal ranges. This indicates that although statistically different, physical examinations and routine blood work did not detect clinically different abnormalities between treatment groups.
We did, however, observe differences in CD4+, CD8+ and double positive (both CD4+ and CD8+ receptors) lymphocyte populations. In general, we observed an increase in CD4+ and CD8+ populations at 168h post injection and a decrease in the double positive population at the 24 and 72 h time points as well (Figure 11). Increases in the CD4+ and CD8+ populations suggest allogeneic stimulation of both T-helper and cytotoxic T-cell populations, respectively. While double positive CD4+CD8+ lymphocytes are reportedly rare in human and dog (236, 237), they represent a greater proportion of lymphocytes in swine and chickens (238-240). While the proportion of double positive lymphocytes was relatively small (less than 10%); lymphocytes originating from Thoroughbred horses and Connemara ponies have been reported to contain as high as 39% double positive lymphocytes (241). Although little is known about the function of this population they are thought to appear or disappear from CD4+ lymphocytes through the gain or loss of the CD8+ receptor (241, 242). Interestingly, in the presence of MSC CD4+/CD8+ double positive lymphocytes appear resistant to MSC-mediated lymphocyte suppression and quickly disappear during in vitro culture (241).

CB-MSC isolated under similar conditions to those used in this study maintained a consistent lymphocyte suppressive in vitro phenotype and express CD29, CD44, CD90 and low/absent expression of MHC I, MHC II, CD4, CD8, CD11a/18, and CD73 markers (201). Despite a supposed lack of both MHC I and MHC II on these CB-MSC, a significant shift of in vivo CD4+ and CD8+ lymphocyte populations indicates possible allogeneic reaction and a need to confirm the phenotype of the CB-MSC populations injected. Additional research in these animals should include Foxp3 staining to evaluate CD4+ regulatory T-lymphocytes (Treg) populations, administration of additional dose(s) of MSC with similar monitoring, or harvest of peripheral blood lymphocytes for evaluation for MSC-mediated lymphocyte suppressive properties. The immune response resulting from subsequent CB-MSC doses may reveal
significant information as to the mechanisms by which allogeneic CB-MSC elicit possible immune reaction. Numerous reports indicate allogeneic MSC administered by various routes may prime the immune system for a greater response, following future MSC injections or organ transplant in the recipient animal, as reviewed by (243). Such investigation may indicate formation of alloantibodies, increased CD8+ lymphocyte response, the duration any potential immunity persists and thereby will advance the understanding of potential ways allogeneic MSC may be used to avoid stimulation of unwanted immune reactions. Future research would be strengthened by knowledge of equine leukocyte antigen haplotype of donors and recipients so that allogeneic administration could be confirmed and clinical utility of haplo-matching assessed.

We report safe administration and excellent maintenance of CB-MSC viability following simulated transport in HT-FRS and CS-10. Although the makeup of these proprietary solutions is not readily available, a presumably similar formulation of HT-FRS has been published when “hypothermosol ” was first described (195, 244). Assuming CS-10 is similar to HT-FRS with 10% DMSO added (187, 193) the main safety concern for administration of these solutions is the high levels of K+ (42.5 meq/L) in solution. While IV administration of hyperkalemia solutions is contraindicated, excipient injection of cell suspensions in small volumes especially to large animal species eliminates risk of inducing cardiac conduction abnormalities following injection, since administration of 10mL/kg/h (approaching 0.5 meq/kg/h maximum safe administration rate of K+) is very unlikely to occur in a regenerative medicine practice.

Conclusion

HT-FRS and CS-10 can be safely used for low volume excipient injection of MSC suspensions. HT-FRS and CS-10 both adequately maintain CB-MSC viability following
hypothermic or frozen simulated transport, respectively. Clinical abnormalities were not observed in ponies injected with HT-FRS, CS-10, or allogeneic CB-MSC when evaluated by physical examination, CBC, serum biochemistry, and coagulation profile. CB-MSC injection is associated with increased CD4+ and CD8+ lymphocyte populations and decreased CD4+/CD8+ double positive lymphocyte populations. These findings indicate that although intravenous injection of pooled allogeneic CB-MSC does not elicit clinical abnormalities, allogeneic stimulation of CD4+ and CD8+ lymphocyte populations may occur. The clinical implications of the altered lymphocyte counts are unknown. Further study including in vitro or in vivo evaluation of cell mediated or adaptive immunity to autologous, identical allogeneic, or MSC arising from additional unrelated individuals would help determine the nature of this response.

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