CHARACTERIZATION OF MESENCHYMAL STEM CELLS IN MUSCULOSKELETAL INJURY

A Dissertation

by

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DOCTOR OF PHILOSOPHY

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ABSTRACT

The main goal of regenerative medicine is to enhance the innate healing response to more closely mimic tissue development. This may include cell-based, trophic, or small molecule therapies. In cell-based therapies, the initial premise was one of tissue replacement. Mesenchymal stem cells (MSCs) are adult-derived stem cells present throughout the body and easily harvested from individual patients. After over a decade of veterinary and human medical use of MSCs and medical research, the function of MSCs after therapeutic use in injury remains unclear.

Elucidating engraftment location and longevity of MSCs post injection may provide insight to their mechanism of action. We developed a protocol for labeling MSCs with a fluorocarbon nanoparticle that allows for non-invasive longitudinal tracking and evaluated cellular viability, proliferation, and morphology. A dose dependent cell association of the nanoparticle was seen in our study but it was not repeatable between individuals. Prior to use of this technique to track MSCs, further study is needed to elucidate where the failure occurred: uptake of label by MSCs, maintenance of label within the cell cytoplasm or loss of conjugation of the fluorophore.

Despite the use of MSCs as a therapeutic in horses for many years, there is little information on the best techniques for cryopreserving these cells for immediate use post thaw. We tested several freezing mediums for the short-term cryopreservation of equine MSCs. We found that 95% autologous serum and 5% DMSO did not negatively affect post thaw viability, growth kinetics, or morphology.

Bisphosphonates were approved for use in horses in the United States in 2014. Since, they have become wildly popular due to improvements in lameness in treated horses. We suspected that the efficacy in lameness reduction could be due to an off target effect such as an effect on the MSC because the approved dose is so low compared to the anti-resorptive dose of the same drugs in people. We investigated the impact of bisphosphonates on bone remodeling and bone cells including MSCs. We observed reduction in lameness but no changes in bone turnover or MSC characteristics.

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CONTRIBUTORS AND FUNDING SOURCES

CONTRIBUTORS

This work was supervised by a dissertation committee consisting of Dr. Ashlee Watts, advisor of the Department of Large Animal Clinical Sciences, Dr. Dana Gaddy of the Department of Veterinary Integrative Biosciences, Dr. Akhilesh Gaharwar of the Department of Biomedical Engineering, Dr. Friedhelm Schroeder of the Department of Veterinary Physiology & Pharmacology, and Dr. Mark Lenox of the Department of Biomedical Engineering.

The data flow cytometry data in Chapters 2, 3 and 4 were analyzed by Dr. Gus Wright of the Department of Veterinary Pathobiology. Clinical evaluations of lameness in Chapter 4 were scored by Dr. Sarah Sampson of the Department of Large Animal Clinical Sciences.

All other work conducted for the dissertation was completed by the student independently.

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CHAPTER I

INTRODUCTION

Regenerative medicine is a new branch of medicine and since the 90s has become commonplace in human and veterinary medicine.[1, 2] As a field, regenerative medicine focuses on promoting the body's innate healing response.[3] Traditional treatments like surgical procedures and drugs focus on the modulation of symptoms while regenerative medicine works to return tissues to normal function.[2] This novel field of medicine encompasses a variety of strategies including cell-based therapies, tissue engineering, and gene therapy.[1, 3]

Cell-based therapies are a large focus of regenerative medicine with stem cells and progenitor cells being the dominant cell type.[1, 4] Stem cells are defined by their ability to self-renew and proliferate indefinitely while maintaining the ability to differentiate into multiple cell types.[5, 6] Asymmetrical division permits stem cells to self-renew while generating a daughter cell that will terminally differentiate.[7, 8] Stem cells are classified based on their differentiation potential or potency. Stem cells with the ability to give rise to all 3 germ layers and extra-embryonic tissues are referred to as totipotent. Pluripotent stem cells, often referred to as embryonic stem cells, can only give rise to the 3 germ layers but not placental.[8, 9] Multipotent stem cells, or adult stem cells, give rise to one germ layer and are present in tissues throughout our bodies and therefore can be isolated from many tissue types.[7, 10] Multipotent stem cells give rise to progenitor cells that can be multipotent or unipotent but lack the ability to self-renew.[10] Cells and tissues

at the local environment are maintained by both progenitor cells and multipotent stem cells in different regions of the body.[7, 9]

Mesenchymal stem cells (MSCs) are multipotent stem cells that give rise to mesoderm tissues.[11] First described by Friedenstein in 1976 as fibroblast precursor cells, human MSCs are now defined by the international society for cellular therapy as plastic adherent, express CD73, CD90, and CD105, lack expression for CD45, CD34, CD14, CD79, and HLA-DR, and have trilineage differentiation potential for osteoblast, adipocytes, and chondrocyte.[12, 13] MSCs can be isolated from a variety of tissues including bone marrow, fat, and umbilical cord blood.[11, 14-16] MSCs have become popular in regenerative medicine due to their ease of isolation and their availability for autologous use without safety concerns. How MSCs improve healing is still unclear. Initially, isolated MSCs used for regenerative medicine were thought to engraft at a lesion site and differentiate into the needed cell type.[17] More recent evidence suggests that MSCs work as medicinal signaling cells to improve healing through functions other than tissue differentiation which may include, growth factor production, inflammation, immunomodulation, and other unknown factors.[18-20]

A classic example of MSCs as a signaling cell is their role supporting hematopoiesis; a specific example being MSC production of rank ligand (RANK-L) and macrophage colony stimulating factor (MCSF) to stimulate osteoclast differentiation of hematopoietic lineage cells.[21, 22] MSCs as immunomodulators secrete a variety of molecules. MSCs have demonstrated release of prostaglandin E2 after interactions with

toll-like receptors, inhibition of T cell activity via cytokines like human growth factor, and proliferation of lymphocytes via interleukin-7.[19, 20, 23, 24]

Another feature of MSCs is they might be able to be used in allogenic applications without being immunophenotyped because that they are considered immune privileged due to their lack of major histocompatibility complex II (MHC-II) expression, T cell suppression, and decreased MHC-I expression.[19, 20] Recently, increased inflammatory reaction and MHC specific antibody reactions following allogenic transplant of MSCs has been demonstrated in horse, murine and non-human primate models. [25-29]

REGENERATIVE MEDICINE: CLINICAL APPLICATIONS

The number of registered clinical trials and MSC therapy trials is steadily increasing (Figure 1.1). Since 2005, MSC therapies have become a popular area of study in regenerative medicine with applications in a variety of fields including cancers, digestive system diseases, heart and blood diseases, and gland and hormone related diseases. Muscle, bone, and cartilage studies comprise approximately one third of MSC therapy clinical trials (Figure 1.2).

When “Mesenchymal Stem Cell Human Clinical Trial” was searched on PubMed using the filters “humans”, “clinical trials”, and “01/01 to 12/31” for each year there was an increasing trend in the number of publications (Figure 1.3).

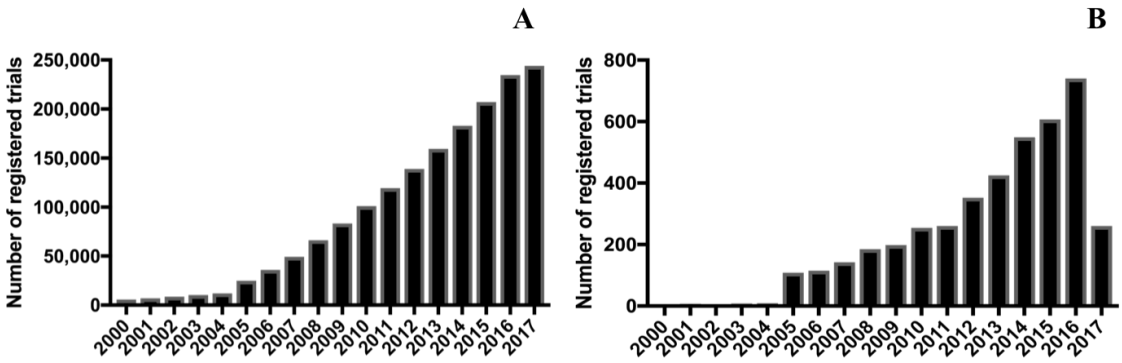


Figure 1.1 Registered Clinical Trials From 2000-2017. A) Registered clinical trials in all phases are increasing across all areas with B) MSC therapeutic trials increasing proportionally.

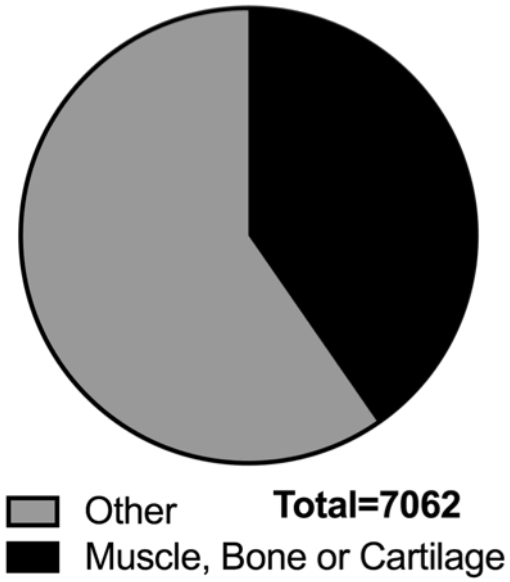


Figure 1.2 Registered MSC Therapeutic Clinical Trials. One third of registered MSC therapeutic clinical trials are in musculoskeletal injuries including muscle, bone or cartilage.

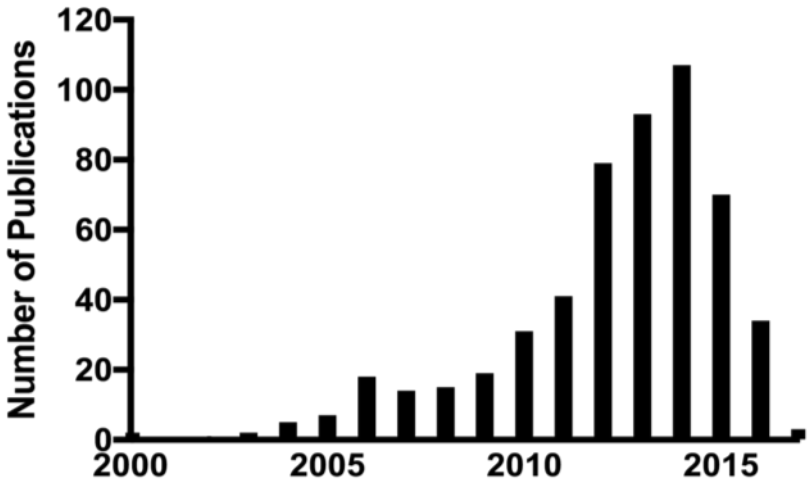


Figure 1.3 Publications of MSC Clinical Trials in Humans as Referenced on PubMed. This graph represents the human MSC clinical trials published in peer-reviewed journal during the reported year with the number of publications increasing by year.

There is a variety of ways to utilize MSCs during therapeutic applications. MSCs can be given systemically or locally. In most treatments MSCs are given systemically at a dose of 1-2 x 10^6 cells/ kg of body weight.[30] In the equine model, it is not feasible to treat systemically due to the size of the horse. In this case they are treated locally usually with a dose of 10 x 10^6 cells. Cryopreserved MSCs are used in approximately 35% of published MSC clinical trials while the rest use fresh cells.[30] In veterinary medicine most practices use fresh cells. A fresh prepped cell does not mean that the cells have not previously been frozen, just that prior to the implantation the cells are in culture. Identification of a cryopreservation medium that allows for immediate clinical use of MSCs post-thaw would be beneficial to streamline laboratory and clinical procedures

and reduce associated costs. MSCs are used in a variety of ways because we do not understand what they are doing post injection. With some many diverse ways of utilizing MSCs the use of a pre-clinical model is important for determining the most effective ways to use MSCs to help generate a standardized protocol.

REGENERATIVE MEDICINE: THE HORSE MODEL

One of the benefits of studying stem cell therapies in the horse is that the horse population, like that of man, is not homogenous in genotype or phenotype as most laboratory species are. In addition to genotype and phenotype differences, individual variation in MSC characteristics, especially in species with diversity, has been reported.[31, 32] It is important to assess MSCs in models that more accurately reflect the inherent variability among human MSC preparations.

The equine athlete is a well-accepted model for stem cell therapies in musculoskeletal injury suffering from similar injuries to those in humans.[33, 34] The horse ages similarly and experiences similar musculoskeletal injuries to human including osteoarthritis, tendonitis, and stress fractures.[33] MSCs as a treatment for tendon injuries have been vigorously studied in the horse. In studies of naturally occurring tendon lesions, horses treated with MSCs had a lower percentage of re-injury rates compared to those receiving traditional treatments.[35, 36] The horse is an important preclinical model for musculoskeletal disease and will help elucidate the best way to utilize the MSC.

REGENERATIVE MEDICINE: FUTURE APPLICATIONS

The purpose of this dissertation is to better define the best ways to utilize MSCs. Stem cells have the potential to be a powerful form of regenerative medicine if they could be harnessed by clinicians to heal tissues without scar tissue and restore function.[37] There are many diverse ways of using MSCs; however without knowing the best way to use them clinicians cannot employ their full potential.

In order to optimize the potential of MSCs as a treatment we must fully understand their role in the body after therapeutic application. To understand their function we need to know their engraftment location and effects in the local environment. Chapter 2 investigates a way to longitudinally evaluate cell engraftment, engraftment duration, and quantify cell engraftment. The environment MSCs are injected to and the state in they are in when they are injected could affect their function. Chapter 3 investigates a clinically acceptable cryopreservation formula for MSCs without xenogens and an almost completely autologous product. It is possible MSCs are modulating the environment around them. Chapter 4 investigates the impact of bisphosphonates, a musculoskeletal therapy, on bone remodeling and bone cells like MSCs.

CHAPTER II

LONGITUDINAL EVALUATION OF FLUOROCARBON LABELED MSCS

INTRODUCTION

MSCs are an increasingly popular choice when treating musculoskeletal injuries clinically in the horse due to their potential to be disease modifying osteoarthritic drugs (DMOAD) based on MSC regenerative potential.[38] MSC therapy has shown beneficial effects in musculoskeletal injuries like osteoarthritis, cartilage defects, and tendonitis in human and various animal models including caprine, porcine, and equine models.[38-41] Despite the use of MSCs to treat musculoskeletal diseases in equine clinical practice, the mechanism of action of these MSCs post injection is largely unknown.

MSCs have the potential to differentiation into multiple cell lineages.[21] It is possible MSCs act through direct cell differentiation based on their differentiation potential and has been demonstrated *in vivo*.[42] Reports have also demonstrated MSC mechanism through immune-modulation or acting as medicinal signaling cells post injection. MSCs have demonstrated modulation of cytokines via down regulation of activated T-cells in diseased environments allowing for normal tissue healing without scar tissue formation.[43] Despite the promise of a potential DMOAD, MSCs cannot be utilized fully without understanding if they act through cell differentiation, signaling, or both. Identifying engraftment location of MSCs post injection, quantifying the number of cells that engraft and the duration of engraftment could elucidate MSC function post injection.

Tracking MSCs post injection provides the opportunity to identify cell engraftment to help elucidate mechanism of action. Many forms of cell tracking after implantation exist including transgenic mice, fluorescent labeling and Y chromosome tracking.[44] Unfortunately, most methods of tracking are not optimal for two main reasons. First, these methods require tissue collection or biopsy for identification of labeled cells. Tissue collection is invasive, affects tissue healing and is not always possible depending on the location of cell injection. Second, it is impossible to evaluate the cells longitudinally or to assess tissue healing when the tissues are constantly being disrupted. Bioluminescence allows for *in vivo* longitudinal tracking without invasive techniques however, this application can only be used in the murine model.[45] A tracking method that will allow longitudinal tracking in large animal models is required.

Iron oxide nanoparticles are a non-invasive form of *in vivo* cell tracking that has been utilized over the last 10 years for longitudinal tracking. Theses nanoparticles exhibit high magnetism. High magnetism causes a disruption in signal when using magnetic imaging, which allows localization of labeled cells. Thus iron oxide labeled cells can be imaged repeatedly over time using MRI and are seen as a loss of signal or artifact on routine magnetic resonance imaging. Repeated MRI is an excellent modality for longitudinal imaging because it does not cause tissue disruption or other changes to healing.

Iron oxide nanoparticles exhibit small particle size allowing cellular uptake by endocytosis that does not affect cell viability, proliferation, differentiation, or migration.[46, 47] Coatings like lipids and surfactants are needed to stabilize iron oxide

nanoparticles for proper dispersion of the particles.[48] Cell death can cause exocytosis of the iron oxide nanoparticle into the extracellular matrix where endocytosis by another cell type is possible. These nanoparticles have been reported in ovine, mice, rats, swine and have been traced using human MSCs in various mouse models.[49-53]

Limitations to iron oxide nanoparticles as a tracking modality are twofold. First tissue healing cannot be assessed in areas iron oxide labeled MSCs are present. The presence of the iron oxide labeled cells is seen through an acquisition artifact of the MRI due to the nanoparticles magnetism, obscuring anatomical imaging and preventing assessment of tissue healing. Second, the number of iron oxide labeled MSCS present post injection cannot be quantified on MRI due to the production of signal voids larger than the cell containing them.[54] Only the presence or absence of label can be assessed. Thus we wanted to utilize a label that allows for both longitudinal tracking of cells and longitudinal assessment of tissue healing. If this were possible we would be able to assess the effects of the cells on healing while also assessing their presence in the tissues.

Perfluorocarbon nanoparticles (19F) are a new technology that have emerged in the past 5 years and provide a new technique for *in vivo* cell tracking. Hydrogen and fluorine resonate at different frequencies on magnetic imaging, allowing for evaluation for the presence of hydrogen containing anatomy and fluorine containing label using different sequences collected separately during an MRI exam. The overlay of H imaging and F imaging can then be used to demonstrate both tissue characteristics and presence or absence of fluorine label. An additional benefit is that the amount of fluorine signal

can be quantified, allowing not only confirmation of cell engraftment, but also quantification of labeled cells. Like iron oxide, 19F label is imaged by MRI and therefore can be imaged repeatedly in the same patient without invasive procedures.

Composed of 4- isotope19 fluorine molecules connected to a single carbon molecule; 19F are small particles (160nm) stable in extreme environment.[55] Endocytosis of 19F nanoparticles has been demonstrated in human MSCs and neural stem cells (NSC) cells without affecting cell viability, growth, or morphology *in vitro*. [42] Various coatings including surfactants, transfecting agents, and targeting antibodies are used to increase loading of the nanoparticles into the cell.[56] Electroporation to open cell membrane channels have been reported in non-phagocytic cells with difficulty endocytosing 19F labels.[56]

Human neural stem cells successfully endocytosed 19F nanoparticle incubated for 24 hours at 5 mg/mL before suspension in extracellular matrix and intracerebral injection.[42] Labeled hNSCs produced signal on MRI and distribution of cells in the lesion cavity believed to form new tissue though it was unclear if formed tissue was functional.[42] In another study human MSCs were labeled with 19F nanoparticles in culture for 24 hours at 2.5mg/mL for cellular uptake before being injected intramuscularly into the hindlimbs of healthy mice.[57] Labeled hMSCs produced signal on MRI with a strong relationship between MRI quantification and number or real cells *in vitro*.[57]

We wanted to develop a protocol to use 19F in horse. To allow a quick and inexpensive validation of labeling in protocol development we needed a visual

assessment of labeling. Fluorescent labels can be utilized in a variety of ways including, conjugation to antibodies that communicate with cell surface receptors and labeling cells cytoplasm and organelles.[58] This method is often used in conjugation with nanoparticle tracking to validate MRI findings in research studies where tissues samples can be collected.[59] We used a perfluorocarbon emulsion conjugated with a Texas red (19F-TR) or FITC fluorophore with an excitation/emission of 590/620 nm or 495/519 nm, respectively (Celsense, INC., Pittsburgh, PA).

Our objective was to develop a protocol for labeling equine MSCs that 19F labeled MSCs would allow for longitudinal evaluation of cell engraftment, quantification, and duration in the same patient. Specifically we wanted to develop a protocol that would not compromise viability, proliferation, or morphology *in vitro* and quantify the number of 19F labeled equine MSCs in an *ex vivo* model using our 3Tesla MRI.

MATERIALS AND METHODS

MSC Characterization

Cryopreserved MSCs isolated from sternal bone marrow were evaluated from 10 horses ranging in age from 2-16 years (median 11). Cryovials of MSCs were thawed in a 35ºC water bath until there was no longer an ice ball present. After thawing, 1mL of DPBS (Lonza, Walkersville, MD) was added to the cell suspension and allowed to sit for 5 minutes. Following 5 minutes, the cell suspension was added in a drop wise manner to 20mL of DPBS. A 100 µL aliquot of the cell suspension was used to

determine post thaw viability and a total cell count using fluorescein diacetate and propidium iodide as previously described.[60] The cell suspension was centrifuged (300G, 4ºC, 7 brake) for 5 minutes. Cells were seeded at 10,000 cells/cm^2 in culture media (Dulbecco's modified Eagle's medium 1g/L glucose (Corning, Corning, NY) supplemented with 10% FBS (HyClone Inc, Logan, UT), 2.5% HEPES buffer (Corning, Corning, NY), and 10,000units/mL penicillin, 10,000 ug/mL streptomycin, 25microg/mL amphotericin B (Life Technologies, Grand Island, NY)) and allowed to recover for 24 hours.

Three of the 9 horses underwent trilineage differentiation and immunophenotyping as previously reported.[60] Briefly, MSCs were immunophenotyped for CD90, CD45RB, and MHCII, CD 44, CD 29 using dilutions of 1:400 1:10, and 1:100, and respectively. Multipotency was assessed by trilineage differentiation of osteocytes, adipocytes, and chondrocytes. Osteogenic cultures were induced for 21 days before staining with Alizarin red, adipogenic cultures for 6 days before staining with oil red O and chondrogenic pellets for 21 days before sectioning and staining with toluidine blue.

MSC Labeling & Visualization

MSC cultures were labeled with perfluorocarbon emulsion when cultures reached 70% confluence. Culture media was exchanged and 19F emulsion with either texas red (19F-TR) fluorophore or FITC flourophore conjugate (19F-FITC) was added directly to the cultures at concentrations of 0, 2.5, 5, 7.5, and 10 mg/mL of culture media and

incubated for 4, 8, 18, or 24 hours. At the end of incubation, cells were visualized by microscopy and fluorescently photographed (Olympus, Center Valley, PA) using commercially available software (cellSens, Olympus, Center Valley, PA). After imaging, cultures were rinsed 3 times with HBSS (Lonza, Walkersville, MD), 1x trypsin was added and incubation occurred at 37C for 5 minutes followed by serum neutralization with 10% equine serum in HBSS. Cells were collected and centrifuged (300G, 4ºC, 7 brake) for 5 minutes before being resuspended in culture media. A 100 μL sample was taken and total cell number was determined by live dead assay. One thousand of each set of labeled cells were seeded onto 10 cm plates for colony forming unit (CFU-F) assays. CFU-F plates were maintained for 10 days with medium exchanged every 3 days followed by staining with 3% crystal violet (Sigma Aldrich, St. Louis, MO) and quantification of colony number.

Remaining cells were reseeded at 10,000 cells/cm^2 to a 6 well plates, T75 flask, or chambered slide (Corning, Corning, NY). Twenty-four hours after seeding cultures were visualized by fluorescent imaging or taken for flow cytometric analysis of fluorescent labeling. Forty-eight hours after reseeding, cultures were lifted and counted as described above and population-doubling times were assessed. Washed pellets were resuspended in 95% equine serum and 5% DMSO, transferred to cryovials (Thermo Fisher, Waltham, MA) and were placed in a freeze container (Thermo Fisher, Waltham, MA) and the container was placed into the -80 for 24 hours before being transferred to liquid nitrogen.

Nuclear Magnetic Resonance

Cryopreserved MSCs labeled with fluorocarbon emulsion were thawed and counted as previously described before being lysed by 125 μL of Triton-X 100. The lysed cell suspension was transferred to a 5mm Class A 500 MHz nuclear magnetic resonance (NMR) glass tube (Wilmad,Vineland, NJ) with 125 μL of D_2O (Merck, Kirkland, QC) and a fluorine control of 250 μL trifluoro acetic acid (TFA; AMRESCO, Solon, OH). Tubes were capped and samples fluorine NMR was performed.

RESULTS

Characterized MSCs were able to differentiate into osteocytes, adipocytes, and chondrocytes, expressed CD90 and CD29 and lacked expression for MHCII, CD45RB, and CD44 (Figure 2.1; Table 2.1). One of three horses characterized expressed CD44. Reports have shown mixed populations of CD44 in equine MSCs.

CFU assays of 19F-TR labeled MSCs were variable between individuals ($p=0.0001$; Table 2.2). A time dependent trend was noted with longer incubation periods producing CFUs with increased colony numbers ($p=0.0007$; Figure 2.2).

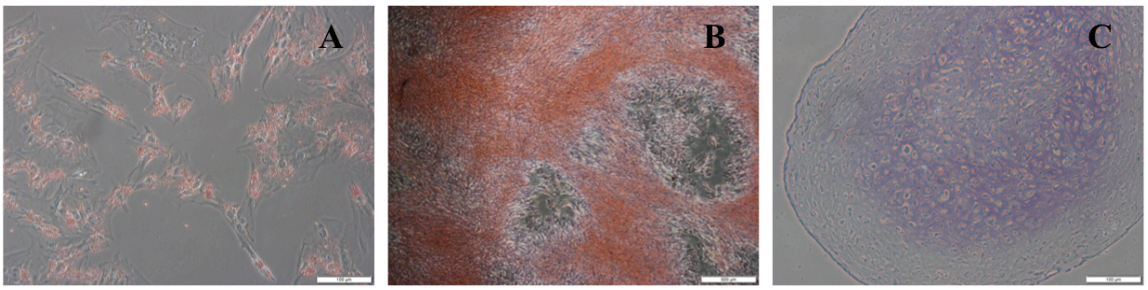


Figure 2.1 Trilineage Differentiation of Bone Marrow Derived MSCs. MSCs isolated from bone marrow were able to undergo trilineage differentiation. A) Adipogenic differentiated cells stained with Oil Red O; original magnification 20x scale bar 100um, B) osteogenic differentiated cells stained with Alizarin Red; original magnefication 4x scale bar 500um, C) chondrogenic differentiated cells stained with Toludine Blue; original magnification 20x scale bar 100um.

	MHCII	CD90	CD45	CD29	CD44
Horse 8	2.63	72.4	2.19	100	36.2
Horse 9	3.43	71.7	1.62	100	53.9
Horse 10	6.64	75.5	1.74	100	14.1

Table 2.1 Cell Surface Marker Expression of Bone Marrow Derived MSCs. MSCs isolated from bone marrow were immunophenotyped for known MSC related cell surface markers.

		0 mg/ mL	25. mg/ mL	5 mg/ mL	7.5 mg/ mL	10 mg/ mL
4 hours	Median	31.0	75.0	96.0	102.0	98.0
	SD	197	192.9	54.4	153.3	61.5
	IQR	95.0	45.0	45.0	193.0	80.0
8 hours	Median	93.0	45.0	59.0	92.0	98.0
	SD	90.6	90.1	106.6	60.8	92.6
	IQR	58.0	38.0	88.0	53.0	110.0
18 hours	Median	14.5	16.0	15.5	14.5	22.0
	SD	2.1	2.8	3.5	2.1	21.2
	IQR	1.5	2.0	2.5	1.5	15.0
24 hours	Median	162.5	113.5	101.0	110.5	108.5
	SD	165.7	69.7	77.7	91.5	136.0
	IQR	142.3	74.5	100.5	135.5	98.0

Table 2.2 Colony Forming Units Were Variable Between Individuals. Colony forming units of cells incubated for 4, 8, 18 and 24 hours at 0, 2.5, 5, 7.5 and 10 mg/mL produced high variability with large interquartile ranges (IQR).

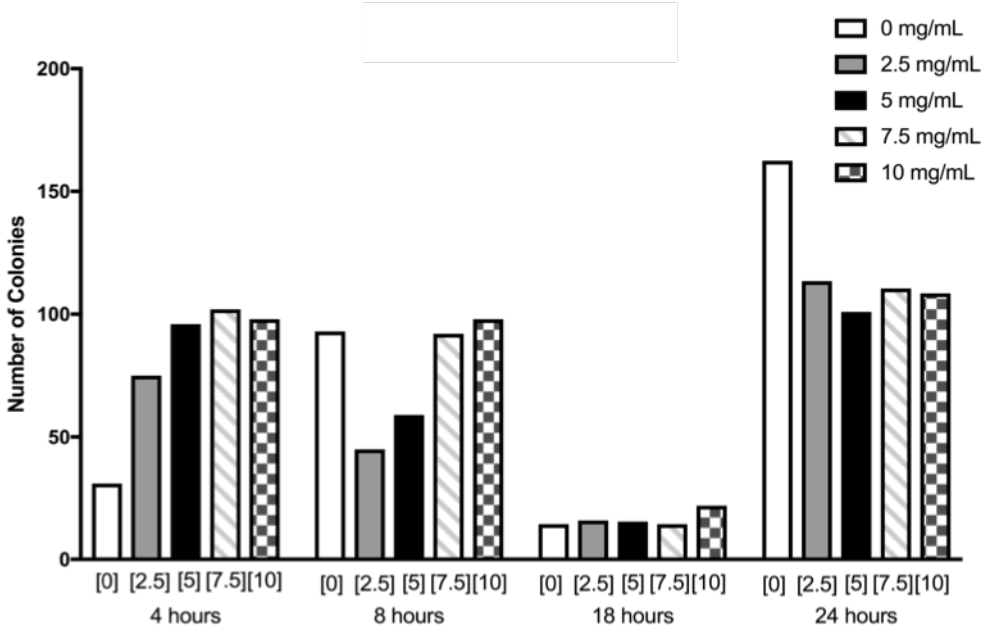


Figure 2.2 Time Dependent Response of Colony Forming Units Between Individuals. Colony forming units of cells incubated for 4, 8, 18 and 24 hours at 0, 2.5, 5, 7.5 and 10 mg/mL produced high variability with an increase in number of colonies formed as incubation time increased.

Population doubling time was calculated based on incubation periods and concentrations with shorter incubations of 4 hours and lower concentrations of 2.5mg/mL producing the shortest doubling times (Table 2.3).

Horse 1 19F-TR labeled MSCs were detectable using fluorescent imaging, indicating cell association of the nanoparticle in a dose dependent manner (Figure 2.3). Subsequent horses labeled with 19F-TR either did not produce signal under fluorescent imaging or produced signal that appeared to be aggregated nanoparticle extracellular (Figure 2.4). To test whether the lack of signal was due to the sensitivity of our

microscope, we used a microscope with better sensitivity and resolution. As the new microscope lacked brightfield imaging, DAPI a nuclear stain was used to locate MSCs and the presence of 19F-TR was assessed. Fluorescent signal of 19F-TR was not detected using the new equipment (data not shown).

Population Doubling Times

Incubation Period		Labeling Concentration	
4 hour	0.73	0 mg/mL	-5.45
8 hour	0.87	2.5 mg/mL	2.05
18 hour	-1.60	5 mg/mL	9.82
24 hour	1.16	7.5 mg/mL	5.02
		10 mg/mL	-4.66

Table 2.3 Population Doubling Times of 19F Labeled MSCs. Populations doubling time in days of 19F labeled MSCs were variable between individuals.

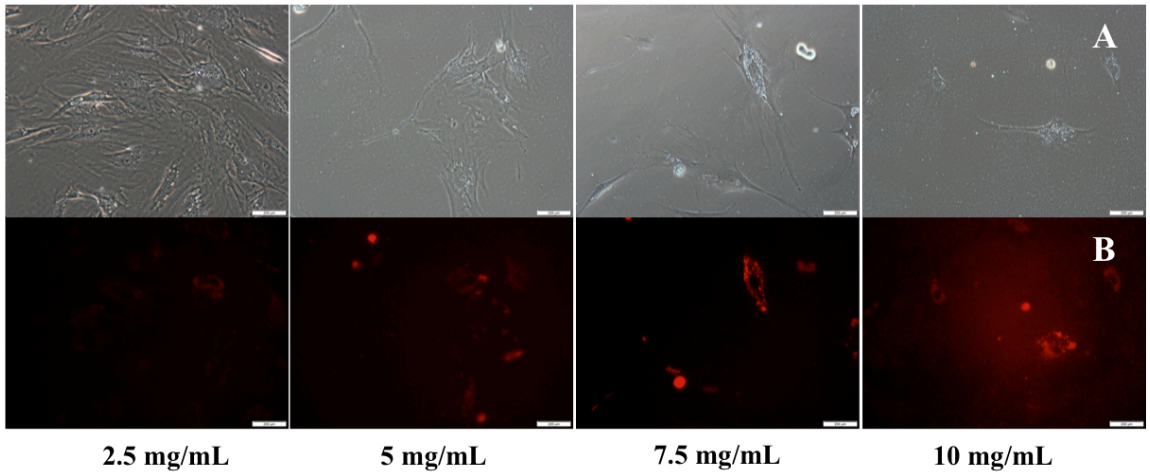


Figure 2.3 Dose Dependent Relationship of 19F Labeled MSCs on Brightfield and Fluorescent Imaging. MSCs of Horse 1 were labeled with 19F for 18 hours and evaluated on A) brightfield and B) fluorescent imgaging; original magnification 10x scale bar 200 um.

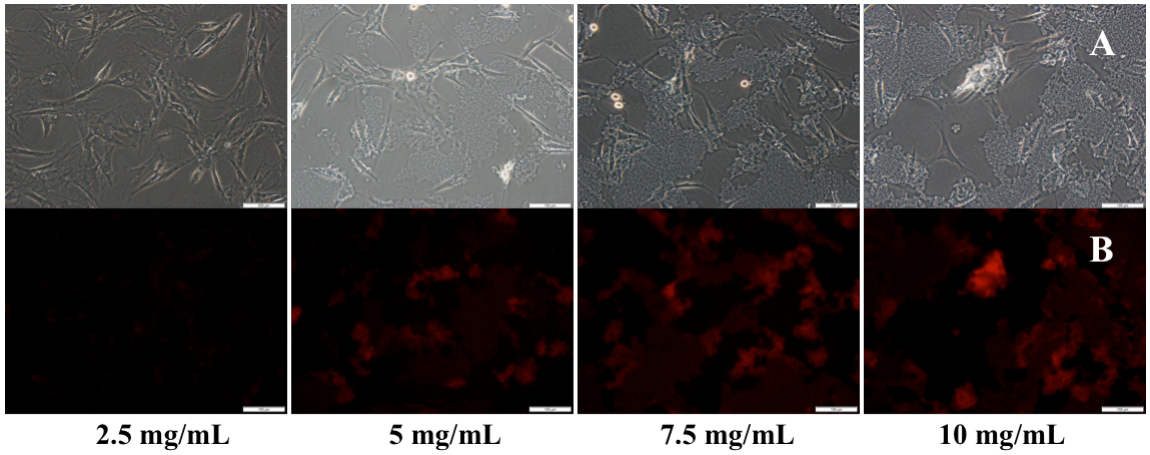


Figure 2.4 Aggregation of 19F Nanoparticle Complicates MSC Labeling. Representative image of Horse 9 labeled with 19F for 24 hours and evaluated on A) brightfield and B) fluorescent imaging; original magnification 20x scale bar 100 um.

Two horses were labeled with 19F-FITC to allow for flow cytometry analysis of fluoroscent signal. No singal was detected under fluorescent imaging of 19F-FITC lableled cells. A dose dependent cell association was seen in 19F-FITC labeled cells using flow cytometric analysis in the first horse (Figure 2.5). However the dose dependent relationship was not repeated in the second horse indicating that individual differences between donors exist (Figure 2.6). Either the labeling process was affecting the fluorophore or the nanoparticle was being exocytosed by the cells.

We thought it was possible that the poor label uptake indicated by appearance (or lack thereof) of the fluorophore conjugate was due to loss of the conjugate. If this was happening, it was possible that there was adequate 19F label in the cells. Fluorine NMR was used to assess the 19F nanoparticle independent of the fluorophore (TR or FITC). Fluorine was present in 8 hour incubations at higher concentrations and in all concentrations of 24 hour incubated MSCs indicating the nanoparticle was associated with the cells in a dose and time dependent manner (Figure 2.7).

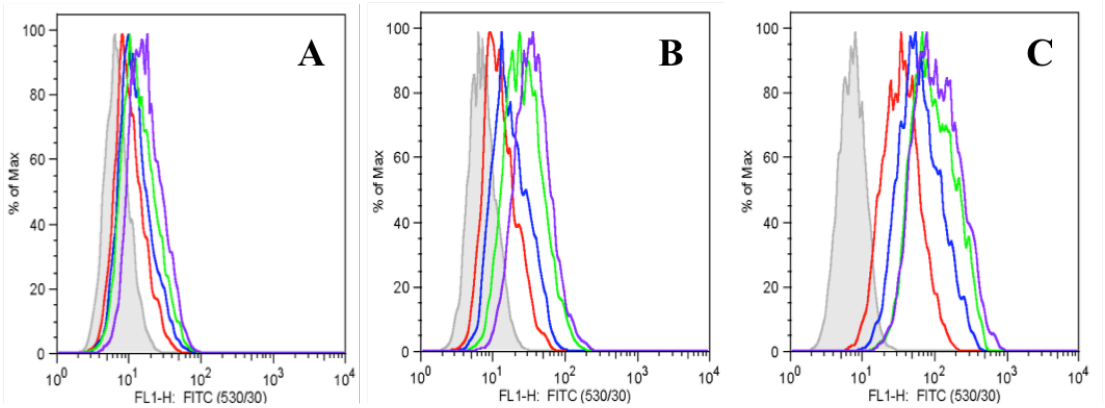


Figure 2.5 Dose Dependent Relationship of 19F Labeled MSCs Via Flow Cytometry. Horse 5 labled MSCs with 19F for A) 4 hours B) 8 hours and C) 24 hours demonstrated a dose and time dependent relationship. Gray peaks represent unstained cells while red, blue, green and purple peaks represent 2.5, 5, 7.5 and 10 mg/mL, respectively.

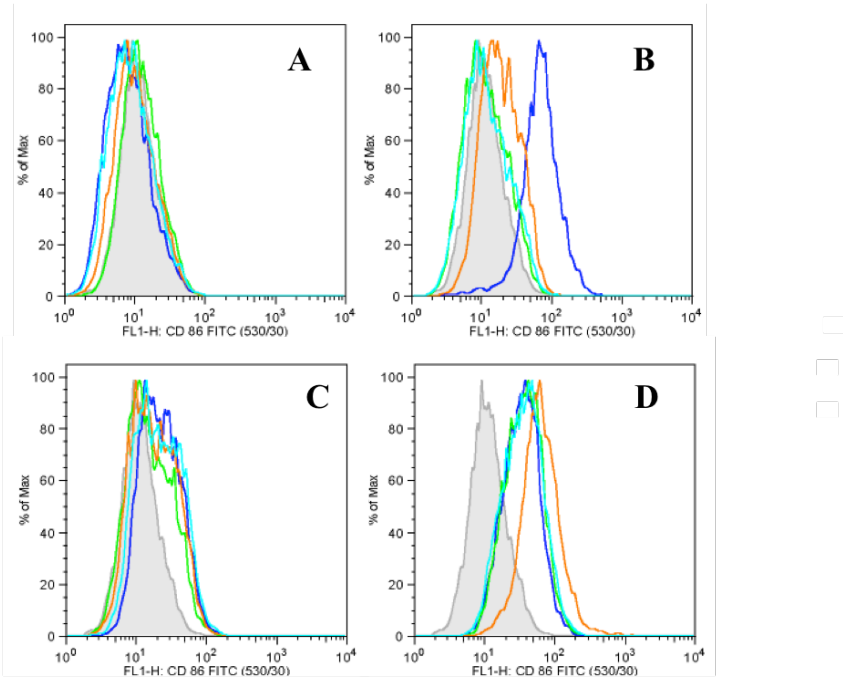


Figure 2.6 No Dose Dependent Cell Association of 19F. Horse 7 labeled with 19F for A) 4 hours B) 8 hours C) 18 hours and D) 24 hours did not demonstrate a dose or time dependent cell association. Gray peaks represent unstained cells while navy, green, orange and light blue peaks represent 2.5, 5, 7.5 and 10 mg/mL, respectively.

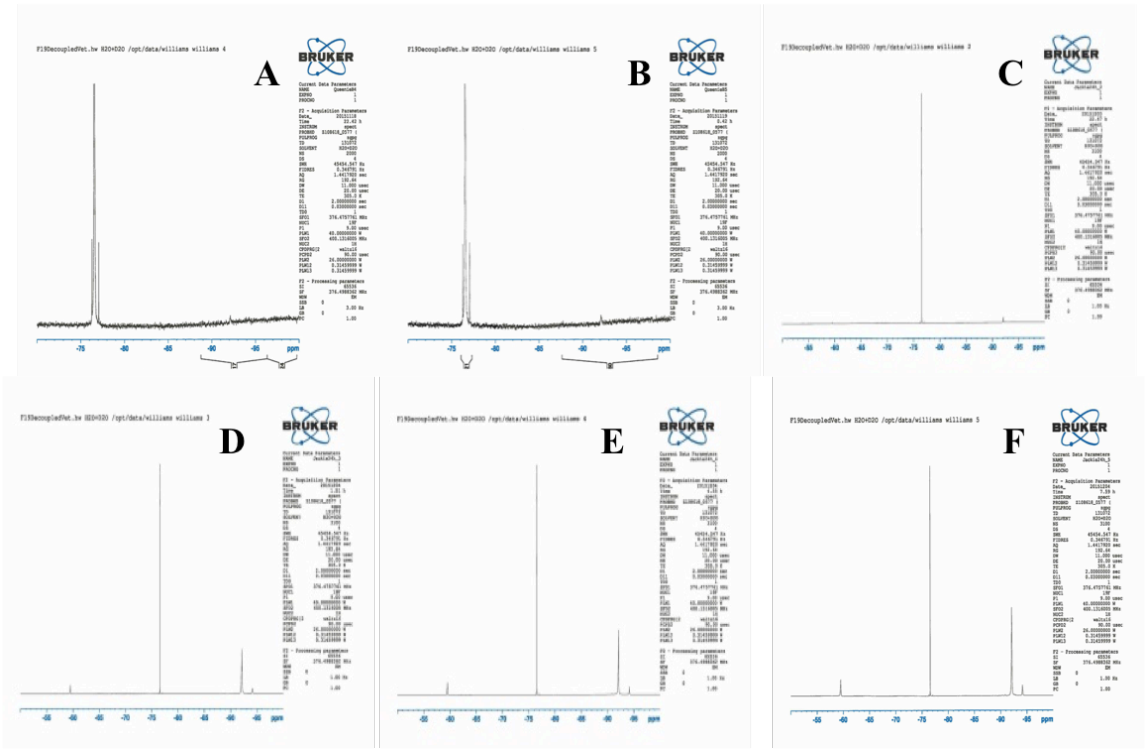


Figure 2.7 Dose Dependent Cell Association of 19F Via Nuclear Magnetic Resonance. Fluorine from 19F nanoparticles produced a signal at -92 ppm only for MSCs labeled for A) 8 hours, 7.5 mg/mL B) 8 hours, 10mg/mL C) 24 hours, 2.5 mg/mL D) 24 hours 5 mg/mL E) 24 hours, 7.5 mg/mL F) 24 hours 10 mg/mL. Fluorine control, Trifluoric Acid, produced a signal at -76 ppm.

DISCUSSION

Our objective was to develop a protocol for labeling equine MSCs with 19F that would not compromise viability, proliferation, or morphology and allow for quantification of 19F labeled MSCs in an *ex vivo* model using our 3Tesla MRI. Inability to reliably detect the nanoparticle between individuals inhibited quantification of labeled MSCs *ex vivo*.

Although we sometimes demonstrated a dose dependent amount of fluorescent label it was inconsistent between horses, which is not what we expected. This may be due to difficultly in labeling non-phagocytic cells like stem cells, which has been demonstrated in hNSCs.[42, 56, 57] The difference to non-phagocytic cells is because phagocytic cells, like macrophages, have specialized receptors like mannose and complement that activate cytoskeleton rearrangements by actin filaments leading to internalization of products.[61] In contrast to phagocytic cells, non-phagocytic cells like MSCs do not have specialized receptors to enhance phagocytosis and do not readily endocytose products. Endocytosis of nanoparticles by MSCs endocytosis of nanoparticles has been aided through clathrin-mediated endocytosis where products are packed into clathrin-coasted vesicles.[62] It is possible insufficient label uptake was due to lack of activation of clathrin recruitment to the plasma membrane by the 19F label coating. Another possibility is that MSCs from specific horses did not adequately take up the nanoparticle because of continuous exhaustion, which has been demonstrated in MSCs loaded with nanoparticles leading to cell cycle arrest and inability to continue uptake of nanoparticle.[63]

We used NMR to quantify 19F association with the cells independent of fluorophore signal. A dose dependent cell association of fluorine was seen in 19F labeled cells from all tested horses. Therefore we thought it was possible that lack of signal on fluorescent imaging was due to loss or changes of the conjugate rather than insufficient uptake of the nanoparticle. However, the possibility still exists that, NMR detected nanoparticle attached to MSCs extracellularly.

Exocytosis of the nanoparticle is another possible reason for failure when developing labeling methods with internalized labels. MSCs have demonstrated relatively quick endocytosis and exocytosis process with material found in the extracellular matrix as soon as 24 to 48 hours after uptake.[63] Varying rates of exocytosis between individuals could explain the dose dependent relationship seen by in some assays that was not repeatable between individuals. This is similar to reports demonstrating lack of specificity of tracking with 19F nanoparticles because of exocytosis or cellular death of 19F labeled cells *in vivo* resulting in 19F release to the extracellular environment. In one report of a stroke model in rats, nineteen percent of cells containing the 19F nanoparticle were host cells and not the transplanted 19F labeled hNSCs.[42] Another study reported 19F signal was identified in macrophages rather than 19F labeled MSCs when evaluated under fluorescent imaging.[57] Identification of engraftment location or duration of engrafted cells may not be accurate *in vivo* if 19F is exocytosed and endocytosed by a macrophage or other cell type.

A limitation to our study was the inability to expand cultures to a number that would allow for characterization of 19F labeled MSCs. However, previous reports have demonstrated 19F does not have an effect of MSC ability to undergo differentiation.[57]

CONCLUSIONS

Cell tracking with a flurocarbon nanoparticle has the potential to allow for longitudinal identification of cell engraftment location and duration without disruption to tissue healing or disrupted identification of tissue healing. However further research and

development of 109F is required prior to use for tracking of equine bmMSCs. Improved reliability of label uptake and/or maintenance of label within cells to be tracked is required. A mechanism to improve endocytosis could be coating activating clathrin recruitment to the plasma membrane. A mechanism to to prevent exocytosis of the label would prevent non-specific tracking of resident phagocytic cells. Identification of engraftment, quantity and duration of MSCs post injection will increase the understanding of the cells mechanism of injection in the lesion.

CHAPTER III

CRYOPRESERVATION OF EQUINE MESENCHYMAL STEM CELLS IN 95% AUTOLOGOUS SERUM AND 5% DMSO DOES NOT ALTER POST – THAW GROWTH OR MORPHOLOGY *IN VITRO* COMPARED TO FETAL BOVINE SERUM OR ALLOGENEIC SERUM AT 20 OR 95% AND DMSO AT 10 OR 5%*

INTRODUCTION

The equine athlete is a well-accepted model for stem cell therapies in musculoskeletal injury.[33] This is because the horse suffers from naturally occurring superficial digital flexor tendon injury that is similar to humans and culture derived and expanded MSCs are being used to treat these injuries.[34] Use of clinical practices in equine cellular therapies that are acceptable in human medicine would be beneficial to help ascertain the value of stem cell therapy for tendon injury in this naturally occurring large animal model.

The ideal stem cell preparation, whether frozen or fresh, is an ongoing debate in medicine.[64-67] Cryopreserved MSCs are used in approximately 35% of published MSC

* This work has been adapted from the original article " Cryopreservation of equine mesenchymal stem cells in 95% autologous serum and 5% DMSO does not alter post – thaw growth or morphology *in vitro* compared to fetal bovine serum or allogeneic serum at 20 or 95% and DMSO at 10 or 5%" by Mitchell A, Atwell K, Smith R, Watts AE. Stem Cell Research and Therapy 2015, 6:231 (doi:10.1186/s13287-015-0230-y; https://stemcellres.biomedcentral.com/articles/10.1186/s13287-015-0230-y). The original article is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.[60]

clinical trials.[30] However in veterinary medicine the majority of laboratories preparing MSCs for horses throughout the world do so with fresh cells.[68] This is not to state that MSCs have not been previously frozen, but that immediately prior to implantation in the patient, the MSCs are in monolayer culture and prepared for injection immediately prior to clinical use with transport to the animal site in cooled media. Identification of a cryopreservation medium that allows for immediate clinical use of MSCs post-thaw would be beneficial to streamline laboratory and clinical procedures and reduce associated costs. It is also possible that the cryopreservation process itself induces cell selection of 'stronger' MSCs or induces greater MSC activity and expansion potential, which could translate to improved stem cell efficacy.[69]

Because of the potential benefits of using cryopreserved MSCs, and the use of cryopreserved MSCs in human clinical trials, cryopreserved MSCs should be investigated in the treatment of naturally occurring tendon injury in horses. The first step in using cryopreserved MSCs in equine veterinary patient is to identify the ideal medium for cryopreservation. To do this, the effect on short-term viability and growth of MSCs post-thaw must be understood.[70] Our objective was to determine if a clinically acceptable formulation and serum source for short-term cryopreservation of equine bone marrow derived mesenchymal stem cells would preserve normal viability, morphology and normal growth kinetics post-thaw. Six different freezing solutions were tested with differing serum supplementation sources and concentrations of dimethyl sulfoxide (DMSO). Different DMSO formulations were tested to determine if a low concentration of DMSO was sufficient to preserve viability and growth of MSCs frozen in a slow-

freezing method. Different serum sources were tested to determine if an autologous serum source was sufficient to preserve viability and growth. We hypothesized there would be no differences in the post-thaw viability, morphology, cell growth kinetics in MSCs cryopreserved in autologous, allogeneic or xenogeneic mediums or with different concentrations of DMSO.

MATERIALS AND METHODS

Bone Marrow Derived MSC Isolation, Expansion, and Cryopreservation

All animal procedures were approved by the institution's animal care and use committee (IACUC 2012-079). No horses were euthanized for this study. Bone marrow derived MSCs were isolated from 9 healthy horses ranging in age from 5 to 16 as previously described. [71] Briefly, bone marrow was collected from mildly sedated horses into heparinized syringes for a final concentration of 5,000 units of heparin per 30ml of marrow. Red blood cell lysis was performed with ammonium chloride (7.7mg/mL NH4CL; 2.06mg/mL Hydroxymethane-aminomethane; pH 7.2). [71] The remaining nucleated cellular portion of the marrow was plated at 175microL of original raw marrow volume per cm^2 (Corning, Corning, NY) and maintained at 37ºC, 5% CO^2 in humidified air. Culture medium (Dulbecco's modified Eagle's medium 1g/L glucose (Mediatech, Manassas, VA) supplemented with 10% FBS (HyClone Inc, Logan, UT), 2.5% HEPES buffer (Corning, Corning, NY), and 10,000units/mL penicillin, 10,000 ug/mL streptomycin, 25ug/mL amphotericin B (Life Technologies, Grand Island, NY)) was exchanged 3 times per week. Once colonies or monolayers reached 70% of

confluence, cultures were passaged until there was a minimum number of MSCs available for the experiment of $3x10^6$ MSCs and for other experiments not outlined in this manuscript.

During each passage, cells were detached from culture flasks by incubation with 5mL per 175cm^2 of 0.25% TrypsinEDTA (Corning, Corning, NY) for 5 minutes followed by collection, serum neutralization of trypsin with 5mL per 175cm^2 of 10% equine serum in HBSS and centrifuged. Mesenchymal stem cells were re-suspended in culture medium and re-seeded to new tissue culture flasks at 5,000 MSCs/cm^2. Once the minimum total MSC number was achieved, MSCs were collected, rinsed by centrifugation 3 times and a portion was frozen in each cryopreservation solution at $10x10^6$MSCs/mL. Freezing mediums were a formulation of 20% serum, 10% DMSO and 70% MEM (20/10/70) or a formulation of 95% serum and 5% DMSO (95/5). Serum sources were fetal bovine serum (FBS), commercially available pooled equine serum (Allo; Thermo Scientific, Waltham, MA) or autologous serum (Auto). The same lot was used throughout the project for both FBS and pooled equine serum. After dropwise addition to the freezing solution, the cell suspension was transferred to cryovials and vials (Thermo Scientific, Waltham, MA) were placed immediately in a room temperature isopropyl alcohol freeze container (Thermo Scientific, Waltham, MA) and the container was placed in a -80^{o}C freezer for 24 hours. After 24 hours, vials were transferred to liquid nitrogen storage in the liquid phase.[72]

Immunophenotyping

Mesenchymal stem cells grown for each horse underwent immunophenotyping for expression of MHCII (Bio-Rad, Raleigh, NC), CD44 (Bio-Rad, Raleigh, NC), CD29 (Beckman Coulter, Brea, CA), CD90 (VMRD Inc, Pullman, WA), and CD45RB (VMRD Inc, Pullman, WA) using flow cytometry. Antibodies were chosen based on previously published work and dilution of 1:400 for CD90, 1:10 for CD45RB and 1:100 for CD 44, Cd29, and MHCII. [32, 73] MSCs were aliquoted at 1 million cells per Eppendorf tubes in 50 microL of staining buffer.

Mesenchymal stem cells stained with primary antibodies (MHCII, CD29, and CD44) had antibody dilution added and was incubated for 45 minutes at 4ºC. Pellets were centrifuged (400G, 7 brake) for 5 minutes. Pellets were washed with 200 uL of DPBS and centrifuged again before being resuspended in DPBS for analysis.

Mesenchymal stem cells stained with secondary antibodies (CD90, CD45RB) had antibody dilution added and cells were incubated on ice for 15 minutes before being centrifuged (2000rpm, 4ºC) for 3 minutes. Cells were washed with 100 uL of DPBS and centrifuged 2 times. One hundred uL of secondary antibody dilution (1:100) was added and cells were incubated on ice in the dark for 15 minutes before being centrifuged (400G) for 5 minutes. Cells were washed with 200 uL DPBS and centrifuged again before being resuspended in 500 uL of DPBS for analysis. Both primary and secondary antibodies had 5 uL of 7-AAD (Biolegend, San Diego, CA) added immediately prior to analysis for assessment of viability.

Trilineage Differentiation

Multipotency of MSCs used in the experiment was assessed by inducing trilineage differentiation on MSCs from 8 of 9 horses using techniques previously described. [74-76] For chondrogenic differentiation, 3 aliquots of 500,000 MSCs were centrifuged (300G, 5 minutes, 4ºC, 7 brake) in polypropylene conical tubes to form pellets. Supernatant was aspirated gently not to disturb the pellet and 1mL of chondrogenic media was added. Chondrogenic media containing Dulbecco's modified Eagle's medium 4.5g/L glucose supplemented with 1% FBS, 2.5% hepes buffer, 10,000 units/mL penicillin, 10,000 ug/mL streptomycin, 25 ug/mL amphotericin B, 0.2% transforming growth factor β (Life Technologies, Grand Island, NY), 301.89 ug dexamethasone (Sigma Aldrich, St. Louis, MO), 50 ug/mL L-ascorbic acid (Sigma Aldrich, St. Louis, MO), 40 ug/mL proline (Sigma Aldrich, St. Louis, MO) and 1% ITS premix (VWR, Radnor, PA) was exchanged 3 times per week for 21 days. Pellets were fixed in 4% PFA (Sigma Aldrich, St. Louis, MO) for 10 minutes followed by routine embedding, sectioning and staining with toluidine blue (Sigma Aldrich, St. Louis, MO).

For adipogenic differentiation, MSCs were seeded to 10cm plated at 1,000 MSCs per cm^2. Once the plates reached 70% confluence, media was exchanged to adipogenic induction media for 3 days (Dulbecco's modified Eagle's medium F12 (VWR, Radnor, PA) supplemented with 3% FBS, 10,000 units/mL penicillin, 10,000 ug/mL streptomycin, 25 ug/mL amphotericin b, 5% rabbit serum (Life Technologies, Grand Island, NY), 33 uM/L biotin (Sigma Aldrich, St. Louis, MO), 17 uM/L pantothenate (Sigma Aldrich, St. Louis, MO), 1 uM/L insulin (Sigma Aldrich, St. Louis, MO), 1

uM/L dexamethasone, 225 uL isobutylmethylxanthine (Sigma Aldrich, St. Louis, MO), 89 uL rosiglitazone (Sigma Aldrich, St. Louis, MO). Media was then exchanged to adipogenic maintenance medium for an additional 3 days (adipogenic induction media without isobutylmethylxanthine and rosiglitazone). Induced and control plates were stained with oil red O (Sigma Aldrich, St. Louis, MO).

For osteogenic differentiation, MSCs were seeded to 10cm plates at 1,000 MSCs per cm^2. After reaching 70% confluence media was exchanged for osteogenic induction medium and maintained for 14 and 21 days (Dulbecco's modified Eagle's medium F12 supplemented with 10% FBS, 10,000 units/mL penicillin, 10,000 ug/mL streptomycin, 25 ug/mL amphotericin B, 10 uM/L b-Glycerophosphate (Sigma Aldrich, St. Louis, MO), 20nM/L dexamethasone, and 50 ug/mL L-ascorbic acid). Plates were stained with 2% alizarin red (Sigma Aldrich, St. Louis, MO).

Thawing

After storage in liquid nitrogen for 2-5 days, vials were thawed with gentle agitation at 35ºC in a water bath until an ice ball was no longer present. Immediately post thaw, an equal volume of DPBS was added to the cell suspension. Five minutes later, the cell suspension was collected and added drop-wise to 20mL of DPBS (Lonza, Walkersville, MD). This thawing method was defined in a pilot project to this experiment where we determined the importance of avoiding osmotic shock in the 95/5 formulations (Figure 3.1). A 100 uL aliquot of thawed cell suspension in DPBS was used for a total cell count and viability using fluorescein diacetate (67.57 mg/mL) and

propidium iodide (1.35mg/mL) in DPBS. Counting cell suspensions were plated on a Nebauer hemocytometer and visualized by fluorescence microscopy (Olympus, Center Valley, PA). The live (green) and dead (red) cells were counted. A total of 10 squares at 10x magnification were counted per sample. The cell suspension was carefully mixed by pipetting and then centrifuged to pellet the cells for removal of freezing solutions (300G, 5 minutes, 4ºC, 7 brake).

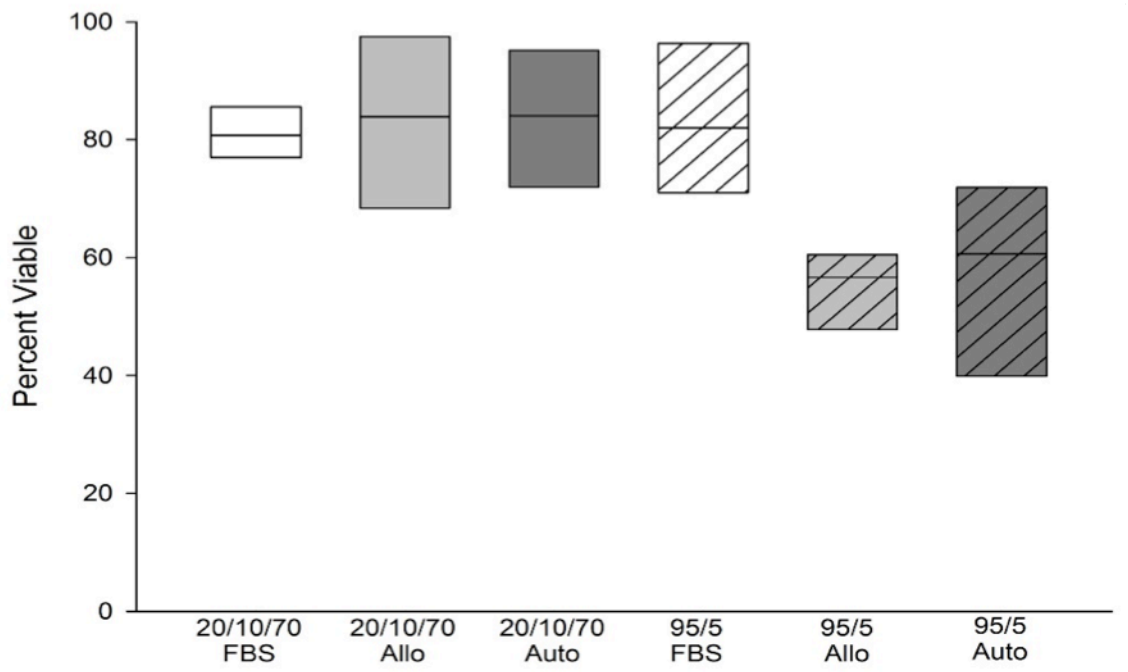


Figure 3.1 Pilot Project Percentage of Viable Cells Post Thaw. Percentage of viable cells post-thaw of MSCs from 9 horses cryopreserved in 6 different solutions from our pilot project (median, quartile). In the pilot project, there was a minor variation in the thawing process. The viable MSCs were significantly lower when MSCs were frozen in 95/5 Allo and 95/5 Auto solutions.

Post Thaw Cell Staining with CellTrace™Label

Cell Trace™ violet dye was used to determine the speed of cellular proliferation. CellTrace™ violet dye binds to intracellular amines without interfering with cellular activity. As the cell divides, the dye is distributed equally between the 2 daughter cells, resulting in dye dilution that reflects the number of cell divisions that have occurred since labeling with CellTrace™.[77, 78] The amount of dye dilution in each cell and thus the number of cell divisions that have occurred, or the generation of that cell, is determined using flow cytometry. A cell that has dye dilution reflecting 2 cellular divisions is a second-generation cell and so on. Post centrifugation, the supernatant was removed and pelleted MSCs were resuspended in 1 mL DPBS to be labeled with CellTrace™ violet dye, as per manufacturer instructions. Briefly, cells were labeled in suspension by adding 1microL of staining solution to 1mL of DPBS containing $\leq 10x10^6$ cells. CellTrace™ violet dye and MSCs were incubated for 20 minutes at room temperature in the dark with gentle agitation every 3 minutes. Complete media was added at 5 times the staining volume and incubated at room temperature in the dark for an additional 5 minutes. The suspension was centrifuged, resuspended in complete media at the same volume and incubated for another 10 minutes at room temperature in the dark, with agitation every 3 minutes. Stained MSCs were then treated as described below for post-thaw monolayer culture of MSCs.

Post-Thaw Monolayer Culture of MSCs

Following labeling, MSCs from each condition were seeded at 10,000 viable MSCs/cm^2 to tissue culture flasks (Corning, Corning, NY) to evaluate growth kinetics, viability and morphology and 1,000 viable MSCs were seeded to a 10 cm plate for colony-forming-unit assay. Cultures were maintained as outlined above prior to cryopreservation.

Monolayer cultures of MSCs were visualized by microscopy and photographed (Olympus, Center Valley, PA) using commercially available software (cellSens, Olympus, Center Valley, PA). Each monolayer culture was given a morphology score of excellent (cells were spindle shaped), good (cells were wider or more star shaped), fair (cells were flattened and/or contained large vacuoles) or poor (cells were flattened, vacuolar and foamy in appearance) and a debris score of none (<5 floating cells per 40x field), mild (<20 floating cells per 40x field floating cells), moderate (<40floating cells per 40x field), or severe (≥40 floating cells per 40x field) by an investigator blinded to treatment group assignment (Table 3.1).

One week after seeding the 10 cm plates, colonies were stained with 3% crystal violet (Sigma Aldrich, St. Louis, MO) and colonies were manually counted without magnification. The evaluator was masked to treatment group assignment.

	Morphology	Debris
Excellent/None	cells were spindle shaped	<5 floating cells per 40x field
Good/Mild	cells were wider or more star shaped	<20 floating cells per 40x field
Fair/Moderate	cells were flattened and/or contained large vacuoles	<40 floating cells per 40x field
Poor/Severe	cells were flattened, vacuolar and foamy in appearance	≥40 floating cells per 40x field

Table 3.1 Morphology and Debris Scoring Rubric for MSC Cultures. MSC cultures were scored as excellent, good, fair, poor or none, mild, moderate, severe for morphology and debris, respectively.

Cell Generation Assay

After 24 and 72 hours of monolayer culture post-thaw, cells were detached and collected by addition of trypsinEDTA. Total cell number was determined. Cells were co-labeled with propidium iodide and flow cytometry was used to assess the concentration of remaining CellTrace™ cytoplasmic dye. Approximately 12,000 to 35,000 events were collected per condition. The cell generation with the greatest concentration of cytoplasmic dye at 24 hours post-thaw was defined as the parent generation. ModFit LT software program was used to determine division rates of the

MSCs (Verity Software House, Topsham, ME). Results were reported as the current cell generation and the proportion of cells in that generation.

Statistical Analysis

Raw data were imported to a commercial statistical software program (Statistix 9, Analytical Software, Tallahassee, FL). Differences between the conditions for continuous data were evaluated by 1-way-ANOVA with Tukey's post hoc tests and by Kruskal-Wallis ANOVA with pairwise comparisons as appropriate for data structure. Differences in paired data within a group were evaluated by Wilcoxon-signed rank test. Differences were considered significant when $p \leq 0.05$.

RESULTS

No differences were found in the post thaw viability, morphology and growth kinetics of previously frozen MSCs with each of the tested solutions. Bone marrow was collected from 9 mixed breed mares aged 5-16 years. Passage number of MSCs ranged from P3 to P6 (Table 3.2). Differences in passage number were due to the need for different total numbers of MSCs from each horse for other experiments not outlined in this manuscript.

	Age	Passage	Total Cell Number
Horse 1	8	4	$67.8x10^6$
Horse 2	5	6	$77.4x10^6$
Horse 3	16	4	$104.8x10^6$
Horse 4	12	4	$12.7x10^6$
Horse 5	11	3	$32.6x10^6$
Horse 6	7	4	$21.2x10^6$
Horse 7	13	6	$32x10^6$
Horse 8	12	3	$49.4x10^6$
Horse 9	14	6	$60.4x10^6$

Table 3.2 MSC Culture Characteristics. MSCs were isolated from horses ranging in age from 5 to 16 years. Passage numbers of MSC cultures ranged from P3 to P6.

All assay time points were met for each donor and formulation except for horse 6 in 95/5 FBS, due to a laboratory error immediately after CellTrace™ labeling. This freezing medium (95/5 FBS) was repeated later and all assays were performed. For repetition of horse 6 95/5 FBS, the same passage was available but had been cryopreserved for 18 months. Statistical significance, with and without this data for horse 6 in 95/5 FBS, was unchanged.

Within each solution, there were significantly fewer viable and attached MSCs at 24 hours post thaw compared to the number of viable MSCs seeded to each flask

(20/10/70FBS, p=0.02; 20/10/70Allo, p=0.05; 20/10/70Auto, p=0.012; 95/5 FBS, p=0.05; 95/5Allo, p=0.05; 95/5Auto, p=0.02). There was no significant difference at 72 hours post thaw (Figure 3.2).

Between the solutions, there were no significant differences in any of our assays. Immediate post-thaw cell viability for each condition ranged from 81 to 88 percent (Figure 3.3).

Cell debris scores at 24 hours were generally mild to moderate debris, with 1-2 horses in each medium having severe debris and 1-2 horses in each medium with no debris (Figure 3.4).

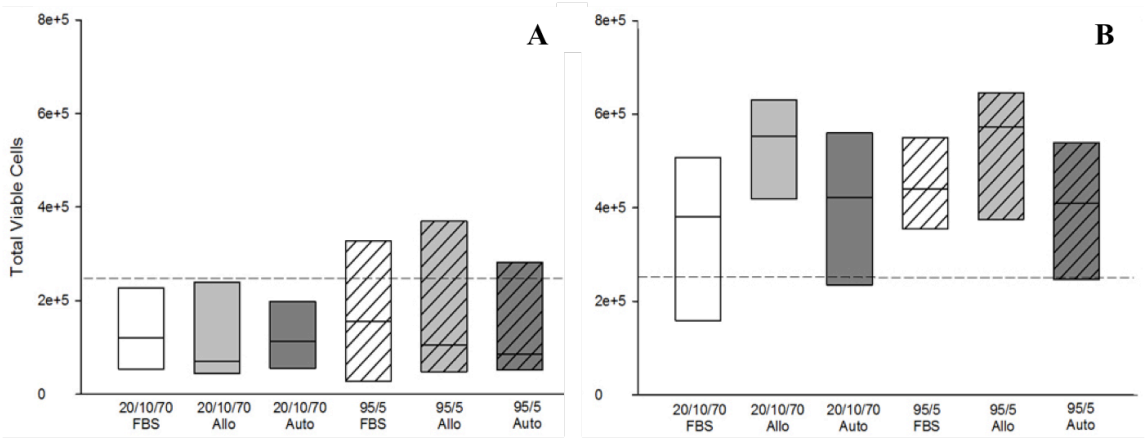


Figure 3.2 Total Viable Cell Number. Viability of MSCs from 9 horses cryopreserved in 6 different freezing solutions after A) 24 hours and B) 72 hours in monolayer culture post-thaw (median, quartiles).

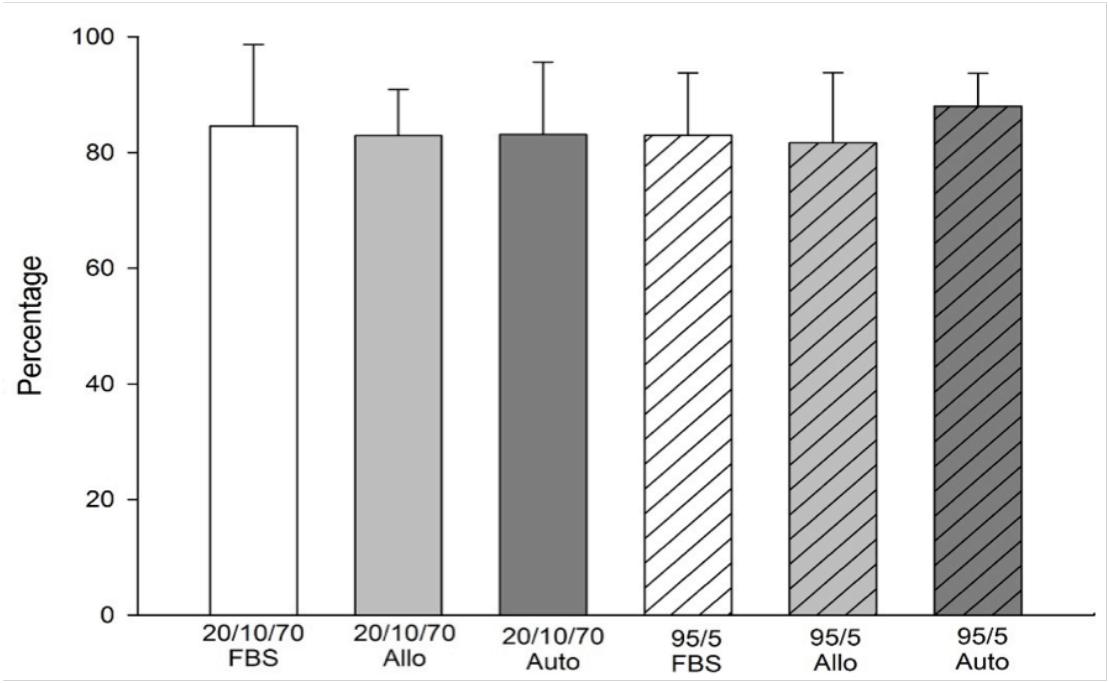


Figure 3.3 Percentage of Viable Cell Post-Thaw. Viability immediately post-thaw of MSCs from 9 horses cryopreserved in 6 different solutions.

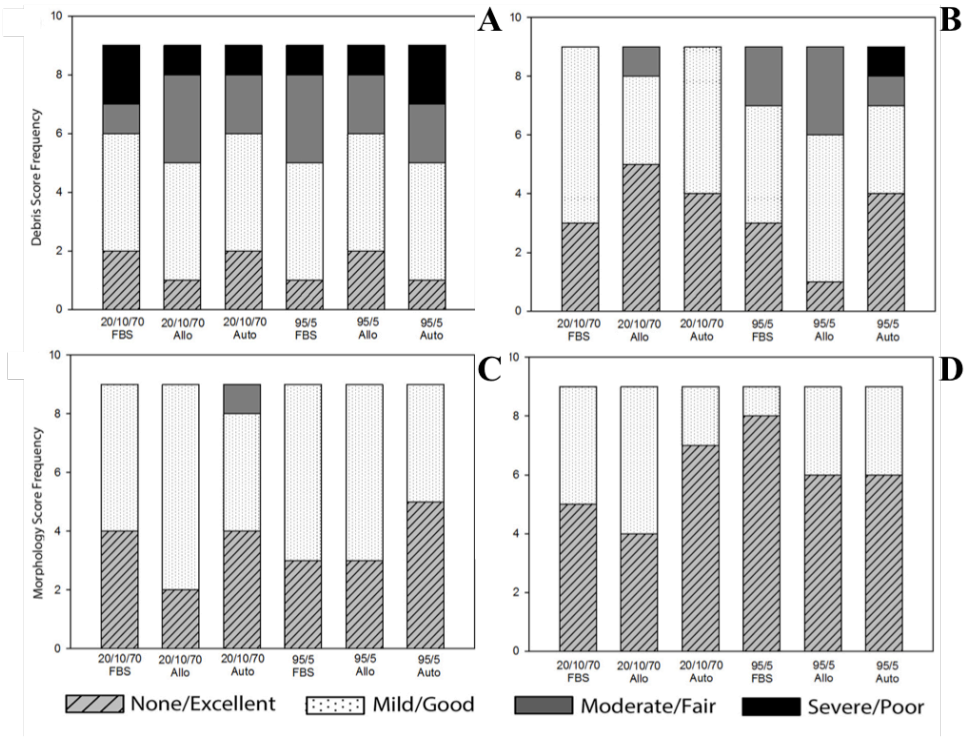


Figure 3.4 Debris and Morphology Scores. Frequency of A), B) debris and C), D) morphology scores of MSCs from 9 horses cryopreserved in 6 different solutions in monolayer cultures at A), C) 24 hours and B), D) 72 hours.

Mesenchymal stem cells receiving severe debris scores were from the same 2 individual donors. Horse 1 MSCs received a debris score of severe for all 6 freezing solutions and the total viable MSCs at 24 hours were extremely low, ranging from 25,000 to 55,000 viable MSCs. Mesenchymal stem cells from 1 other individual donor (horse 6) received severe debris scores in 2 formulations, 20/10/70FBS and 95/5Auto, and scores of mild and moderate for all other formulations. The cell counts at 24 hours were also very low for these formulations (145,000 and 45,000). In contrast to scores at 24 hours, cell debris scores at 72 hours were generally none to mild (Figure 3.4).

Exceptions to this were the MSCs from the same 2 individuals that had received severe debris scores at 24 hours. The MSCs from these horses also received worse scores at 72 hours: horse 1 had moderate debris in all formulations except 20/10/70FBS and 20/10/70Auto and horse 6 had severe debris in 95/5Auto. Cell morphology scores at both 24 and 72 hours post thaw were generally good to excellent without differences between groups (Figure 3.5). Total viable cell counts were not different between the groups at 24 or 72 hours (Figure 3.2). Percentage of confluence at 72 hours ranged from 70-80% for all conditions. Numbers of colonies from the 10cm plates ranged from 72-115 colonies (Figure 3.6).

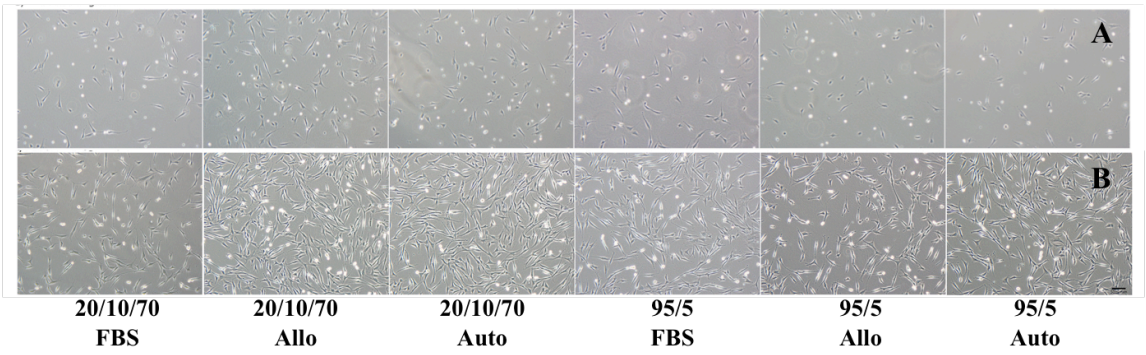


Figure 3.5 Images of Monolayer Culture. Microscopy images of MSCs from Horse 3 crypreserved in 6 different freezing solutions after A) 24 hours and B) 72 hours in monolayer culture post-thaw; original magnification 4x scale bar 500um.

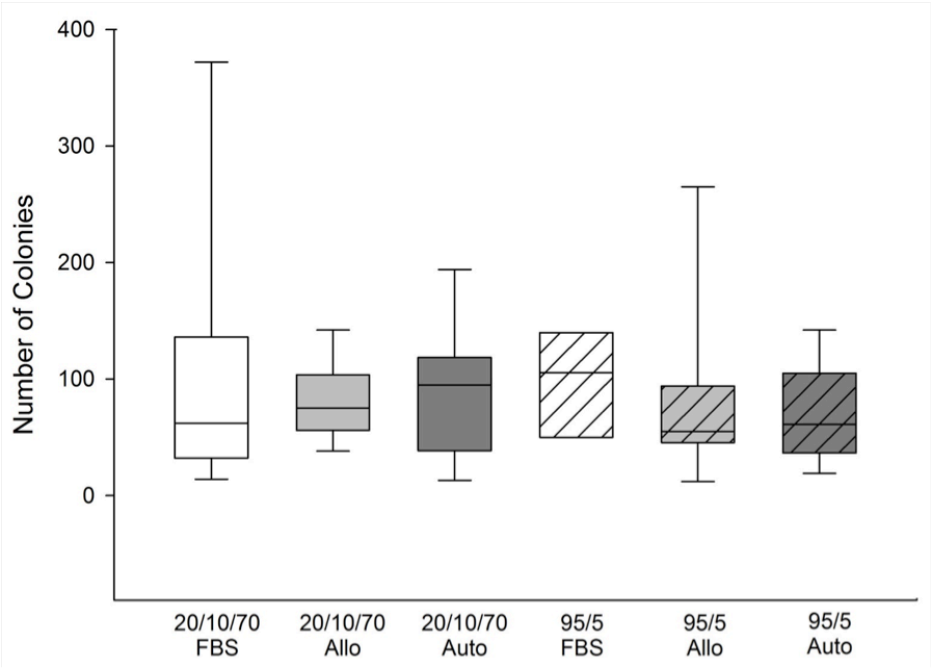


Figure 3.6 Total Colony Number. Numbers of colonies on CFU-F assay from MSCs cryopreserved in 6 different freezing solutions (median, quartile). One thousand total viable MSCs were seeded to 10cm plates. Colonies were stained and manually counted without magnification 1 week later.

At 24 hours post-thaw, the majority (mean; standard deviation) of MSCs remained in their parent generation: 20/10/70 FBS (98.4%; 3.15), 20/10/70 Allo (98.2%; 2.36), 20/10/70 Auto (99.5%; 0.44), 95/5 FBS (98.8%; 1.48), 95/5 Allo (98.6%; 1.92), and 95/5 Auto (98%; 2.96; Figure 3.7). At 72 hours post-thaw, the majority (mean; standard deviation) of MSCs were in the fourth generation: 20/10/70 FBS (54.9%; 10.25), 20/10/70 Allo (55.1%; 7.94), 20/10/70 Auto (57%; 10.32), 95/5 FBS (51.5%; 15.17), 95/5 Allo (54.2%; 12.98), 95/5 Auto (59.1%; 11.78) (Figure 3.7).

When the number of MSCs contributing to the total cell number at 72 hours was calculated, based upon the mean proportion of cells in each generation at 72 hours, it was lower than the cell count at 24 hours (20/10/70 FBS, 65,372; 20/10/70 Allo, 95,865; 20/10/70 Auto, 60,675; 95/5 FBS, 80,897; 95/5 Allo, 80,033; 95/5 Auto, 64,578)

Mesenchymal stem cells for each horse were immunophenotyed (Table 3.3). The majority of horses were negative for MHCII, CD44, CD45RB and positive for CD29 and CD90. Eight of 9 horses underwent trilineage differentiation and were positive for osteogenic, chondrogenic and osteogenic differentiation (Figure 3.8).

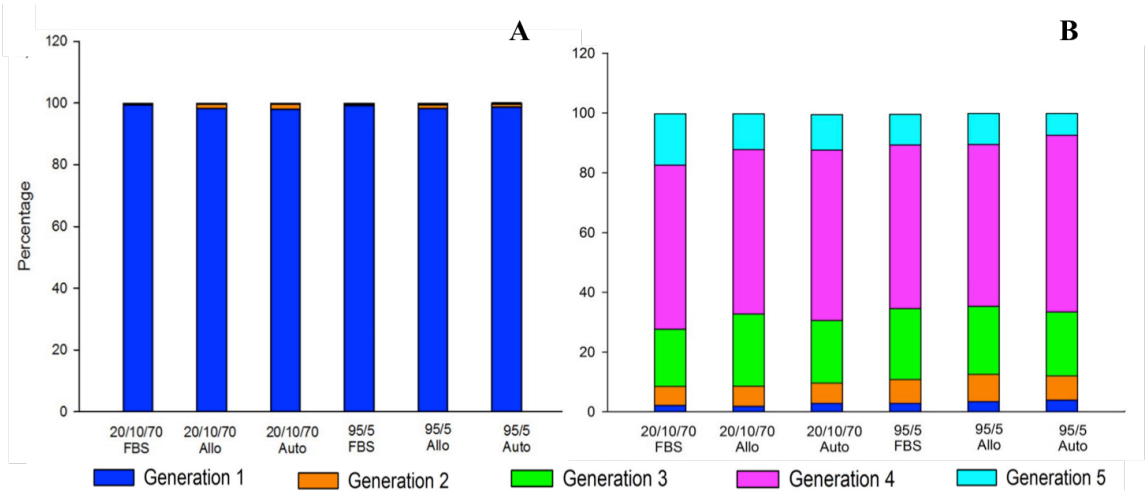


Figure 3.7 Cell Generations Post-Thaw. Percentage of MSCs cryopreserved in 6 different solutions in generations 1-5 at A) 24 hours and B) 72 hours post-thaw and monolayer culture (mean).

	MHCII	CD44	CD29	CD45	CD90
Horse 1	1.39	68.7	100	7.27	100
Horse 2	1.41	3.19	100	1.56	99.8
Horse 3	1.52	1.88	99.8	2.5	83
Horse 4	1.13	71.8	100	5.52	89.7
Horse 5	1.24	10.8	99.4	14.9	94.7
Horse 6	1.83	9.64	100	6.42	99.5
Horse 7	60.9	18.7	100	39.5	92.8
Horse 8	5.01	48.7	99.9	21.2	90.7
Horse 9	45.4	17	99.8	17.7	99.4

Table 3.3 Cell Surface Marker Profiles. MSCs cryopreserved in 6 different freezing solutions were evaluated for expression of CD90 and CD29 and lack of expression of MHCII, CD44 and CD45.

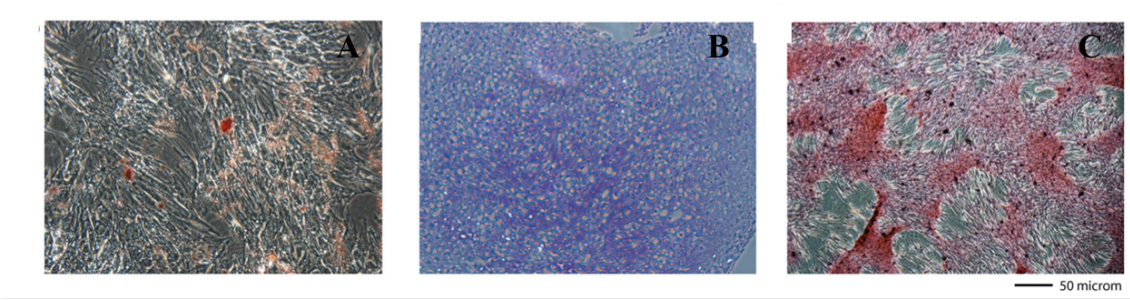


Figure 3.8 Trilineage Differentiation. Images of MSCs from a representative horse after A) adipogenic B) chondrogenic and C) osteogenic differentiation; scale bar 50um.

DISCUSSION

We sought to identify whether a clinically acceptable medium for short-term cryopreservation of equine bone marrow-derived MSCs would preserve normal post-thaw viability and growth. Mesenchymal stem cells from 9 middle-aged adult horses at a broad range of passage numbers were utilized to best mimic the clinical scenario of autologous MSC therapy where differing total numbers of MSCs might be required due to differences in tendon lesion size and severity. Varying concentrations of autologous serum, pooled equine serum or FBS; 2 concentrations of DMSO; and the presence or absence of a cell culture media were tested. Standard immediate and longer-term post-thaw viability assessments included total live and dead analysis, CFU-F assay and assessment of MSC morphology and cellular debris. A more novel analysis we used to assess growth was to stain MSC cytoplasm in a way that would not interfere with cellular activity and could be accurately measured by flow cytometry, giving us the number and frequency of cellular divisions for single cells.[79] Analysis of remaining cytoplasmic dye 24 and 72 hours after staining, allowed evaluation of growth kinetics of MSCs from each cryopreservation medium, and in combination with total cell numbers and culture scoring, enabled indirect assessment of post-thaw apoptosis induction.

One of the benefits of studying stem cell therapies in the horse is that the horse population, like that of man, is not homogenous in genotype or phenotype as most laboratory species are. In addition to genotype and phenotype differences, individual variation in MSC characteristics, especially in species with diversity, has been reported.[31, 32] It is important to assess MSCs in models that more accurately reflect the

inherent variability among human MSC preparations. Utilizing a greater number of individuals in MSC experiments better reflects responses from a diverse population. Using MSCs from 9 individual donors we found no differences between any of the freezing medium formulations in the post-thaw viability or early growth and morphology of MSCs by any of our assay methods. However, when we looked at individual horses, there were marked differences in cell expansion between the media solutions 72 hours post-thaw in a few of the horses. For example, 37% of MSCs from horse 4 frozen in 20/10/70Allo were in generation 5, while the other 5 freezing solutions were much lower, ranging from 12-31% of the MSC population in generation 5. As a contrasting example, only 0.5% of MSCs from horse 6 frozen in 20/10/70Allo had reached generation 5, while the other 5 freezing mediums had much higher percentages of MSCs in generation 5, ranging from 6-62%. Had we included one of these horses in a smaller group size, we might have erroneously identified differences between the formulations.

The media formulations we tested were either 20% serum, 10% DMSO and 70% cell culture media or 95% serum and 5% DMSO. The 20/10/70 formulation was elected as the standard cryopreservation medium formulation used in cell culture for many cell types. Within this group, our question was whether use of xenogen-free serum sources was possible. The 95/5 formulation that has been recently reported was elected to answer 2 questions.[80] Can an almost entirely autologous product (95%) and a reduced DMSO concentration be used? The lack of deleterious effects when an autologous product was used with a low concentration DMSO could move cryopreserved MSCs closer to an off-the-shelf product and would also streamline preparation of autologous MSCs.

Culture and cryopreservation of MSCs in FBS has been a standard technique for many years. Because of a desire to move toward an entirely xenogen-free product in stem cell therapies, 2 equine serum sources were tested. Based upon other work in our lab (data not shown) and that of others, we think there are individual differences in the quality of serum for the growth of MSCs.[30, 81] Because of these potential variations in serum quality between individual horses, autologous serum and a commercially available pooled equine serum were tested. If individual serum quality differences exist, it does not appear to negatively affect the post-thaw viability and growth of MSCs frozen in autologous serum at either concentration we report here. Therefore, either the commercially available equine serum or autologous serum can be used for short-term xenogen-free MSC cryopreservation. An entirely autologous product versus an allogeneic, xenogen-free product would be desirable to minimize many more risks, both known and unknown.

Despite being cytotoxic and potentially toxic to the patient who will receive the cells, 10% DMSO is the most commonly used cryoprotectant agent with or without cell washing for DMSO removal prior to cell infusion to patients.[67, 82, 83] Because of its cytotoxicity and varying reports of the effectiveness of lower DMSO concentrations in human cell cryopreservation, 5% DMSO was also tested.[84, 85] A lower DMSO concentration, if effective, might minimize toxic effects that occur prior to freezing and in the immediate post-thaw period when MSCs are in the cryopreservation medium. Based upon our results, 5% DMSO is sufficient as a cryoprotectant for short-term MSC cryopreservation. Using this lower concentration of DMSO would be especially

important if a post-thaw rinse of MSCs was delayed or avoided altogether prior to clinical application.

Lack of differences among the cryopreservation mediums we tested is in stark contrast to results of a pilot project in our lab. In the pilot project, the same 6 freezing solutions and serum sources on MSCs from 6 middle-aged horses were tested, but we utilized a very minor variation in the thawing process. The difference in the thawing method was that post-thaw MSCs were slowly transferred in a drop-wise manner to a large volume (20mL) of DPBS, as has been previously reported, rather than the step-wise introduction to DPBS over 5 minutes we report here.[68] This minor difference in methods resulted in profound deleterious effects of cryopreservation mediums consisting of 95% serum of both equine types with post-thaw viabilities of less than 60% (Figure 3.1). Susceptibility of all cell types to post-thaw osmotic shock is well known and enhanced susceptibility has been suggested in human MSCs. [69, 86] Absence of balanced isotonic solution and/or a lower concentration of cryoprotectant in our 95/5 formulation both could have led to increased susceptibility to osmotic shock. Regardless, it appeared in our pilot project, that the use of 95% FBS was somewhat protective of the enhanced susceptibility to osmotic shock in the 95/5 formulation compared to either equine serum source. In the experiment of this report, careful handling of MSCs to reduce osmotic shock resulted in no differences among the 95/5 or 20/10/70 formulations. The importance of MSC handling immediately after the thawing process should be underscored.

First reported in 2011, post-thaw growth arrest of MSCs followed by a very rapid proliferation rate of surviving MSCs was seen in our study.[69] As originally suggested, this might be selection of 'better' MSCs with a younger phenotype and faster proliferation rate while inducing apoptosis of the 'less strong' MSCs post-thaw. Our study demonstrated a lack of MSC division of the plastic adherent population in the first 24 hours with >95% of viable MSCs still in the defined parent generation, and a greater number of non-adherent cells in the first 24 hours post-thaw reflected by the higher debris scores at 24 hours and lower total cell count of adherent MSCs after 24 hours of culture than the number of MSCs seeded for all groups. These floating cells were likely apoptotic MSCs, rather than surviving but dysfunctional cells because much higher numbers and monolayer densities would have occurred at 72 hours had the floating cells recovered function after 24 hours. Additionally, the CFU-F assay colony number was lower when debris scores at 24 hours were high (Figure 3.9).

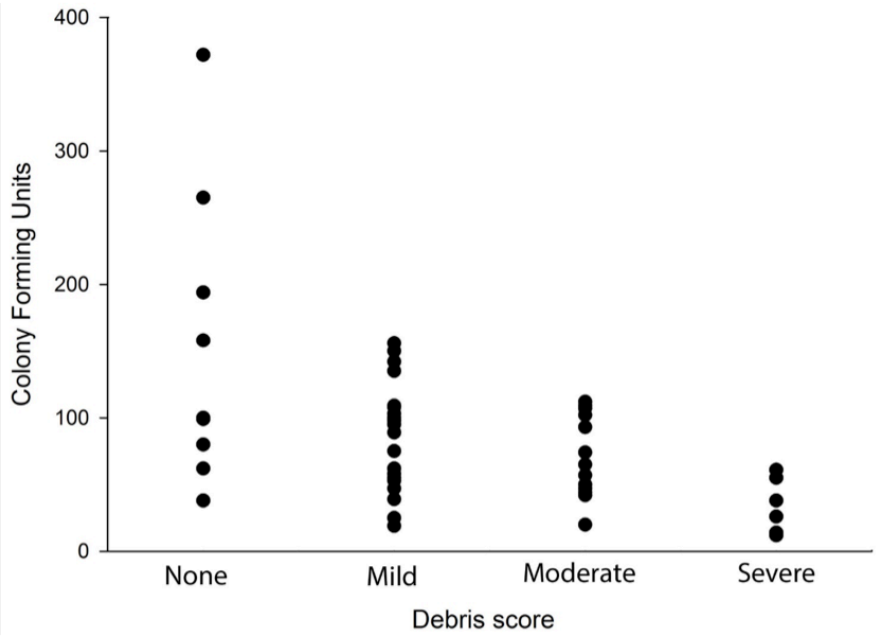


Figure 3.9 Debris Score vs Colony Forming Units. Total number of colony forming units plotted against debris scores from MSCs cryopreserved in 6 different solutions and maintained in monolayer for 24 hours post-thaw.

This growth arrest seemed to recover between 24 and 72 hours, with the majority of viable MSCs in the fourth generation, 48 hours later. However, we think there was incomplete recovery with continued apoptosis in a portion of MSCs because the total viable cell number at 72 hours was significantly lower than one would expect given our cellular generation data. An assay of apoptosis would have been helpful to prove that apoptosis occurred. Finally, although direct comparisons to growth of MSCs from the same donors that had not been frozen were not made, our impression is that the growth during the 72 hours post-thaw was much greater than we see during routine monolayer expansion of fresh MSCs. This is in contrast to a recent report where post-thaw MSC

growth was not different to suspension stored MSCs where there was a steady proliferation rate for 4 days.[68]

A limitation of our study was that cell surface markers, commonly used to characterize the phenotype of MSCs, and tri-lineage differentiation potential *in vitro*, were not assayed post thawing. These analyses were not performed for 2 main reasons. First, others have reported lack of changes in cell surface markers in fresh versus post-thaw human and porcine bone marrow-derived MSCs and that cryopreservation does not change differentiation ability. Second, others have reported lack of changes in cell surface marker profile due to serum type (autologous serum versus FBS). Therefore, we thought the minimal exposure to different mediums during freezing and thawing was unlikely to alter the cell surface marker expression or *in vitro* differentiation potential, so long as viability and growth were unchanged.

Another step that is important to note in our design, is that DMSO was removed from MSCs post-thaw with a post-thaw wash by centrifugation. In the clinical setting, if one were to use any of our tested conditions immediately post-thaw, a post-thaw wash & centrifugation step would be required if removal of DMSO was desired. This washing step would require laboratory involvement in the clinical procedure, somewhat limiting their off-the-shelf availability to the treating clinician.

CONCLUSIONS

In conclusion, we evaluated the short-term cryopreservation of equine bone marrow-derived MSCs in solutions consisting of differing concentration and types of serum and concentrations of DMSO. A low tech, commercially available freezing system that would be affordable in veterinary services was used. In this system, equine MSCs did not have differences in post-thaw viability and growth, regardless of the cryopreservation formulation or serum source used. The importance of minimizing osmotic shock of MSCs immediately post-thaw and the potential increased risk of osmotic shock with different mediums for cryopreservation as found in our pilot project should be noted. Additionally, immediately post-thaw, there was an apparent lag phase of MSCs with little cellular division and assumed apoptosis in the first 24 hours post-thaw, followed by rapid MSC growth over the next 48 hours. If a xenogen-free product with lower concentration of cryoprotectant is clinically desirable to streamline clinical and laboratory procedures by use of cryopreserved MSCs, the use of 95% autologous serum and 5% DMSO for the short-term cryopreservation of equine bone marrow-derived MSCs is recommended.

CHAPTER IV

INVESTIGATING THE IMPACT OF BISPHOSPHONATES, A MUSCULOSKELETAL THERAPY, ON BONE REMODELING AND BONE CELLS

INTRODUCTION

Bone is a dynamic tissue primarily composed of Type I collagen mineralized with hydroxyapatite that must constantly adapt to various amounts of load.[87] Osteoblasts and osteoclasts are the cellular mechanisms that maintain bone homeostasis. Osteoblasts lay down bone matrix containing mineral and non-collagenous protein before mineralization, while osteoclasts resorb bone. Both osteoblastic and osteoclastic actions are important for the remodeling, formation, and maintenance of healthy bone.

MSCs play an important role in bone homeostasis as both osteoblasts progenitors and osteoclasts support cells. Although MSCs as osteoblast progenitors have recently been questioned, osteoblasts are thought to be descendants of MSCs while osteoclasts are differentiated from the hematopoietic lineage.[88, 89] An additional role of MSCs is they are known to support hematopoiesis; specifically in osteoclastogenesis by producing RANK-L and MCSF stimulate osteoclast differentiation of hematopoietic lineage cells.[21, 22] When an imbalance in bone remodeling occurs a pathogenic state can form. Osteoporosis is a chronic condition in which both osteoclast and osteoblast activity is elevated.[90] While osteoclast can resorb bone in a few weeks, it takes osteoblast months to lay down new bone. This imbalance causes a net loss of bone density.[90] Different

treatments have been developed to manage bone turnover disorders including the use of bisphosphonate drugs.

Bisphosphonates were first reported in 1968 as a derivative of the pyrophosphate family. Bisphosphonates differ from pyrophosphates as they contain a carbon ion rather than oxygen which makes them more stable and resistant to chemical and enzymatic hydrolysis.[91] The first bisphosphonates were used as water softeners to inhibit calcium carbonate precipitation.[92] In medical applications, the high affinity for calcium by bisphosphonates targets them to bone after administration. Bisphosphonates adhere to bone and can be incorporated during mineralization. They can be classified into two categories, non-nitrogen containing and nitrogen containing; with the latter having an increased binding to hydroxyapatite therefore having a more potent affect.[93] Nitrogenous bisphosphonates cause cell apoptosis by inhibiting the mevalonate pathway, which is important in many cellular processes leading to apoptosis.[94] In contrast, non-nitrogenous bisphosphonates are known to inhibit cell function by interfering with the mitochondria, eventually leading to cell apoptosis. The non-nitrogenous equine approved bisphosphonate clodronate, is metabolized into nonhydrolysable ATP analogs that accumulate in the mitochondria resulting in cytotoxic effects.[95] Osteoclasts contain a higher number of mitochondria and therefore accumulate a higher number of nonhydrolysable ATPs causing senescence preventing bone resorption followed by apoptosis.[95] Once released by the apoptotic cell, the non-hydrolysable ATP analogs have a high affinity for calcium leading to storage in the bone through formation of

complexes with hydroxyapatite crystals that can be taken up by osteoclasts at a later time.[96]

During osteoclast resorption collagenous and noncollagenous bone proteins are released into the serum and urine, which can be used as biomarkers of bone metabolism.[97, 98] Two commonly used bone biomarkers are osteocalcin and CTX-I.[99] Osteocalcin is a non-collagenous bone protein incorporated into the extracellular bone matrix by osteoblasts during bone formation; osteocalcin levels reflect the active osteoblast in bone.[100] Osteocalcin levels also reflect active osteoclast in the bone as it is liberated during resorption.[100] CTX-I is a C terminal telopeptide of type I collagen, the most abundant protein in bone. CTX-I is released during bone resorption by cathepsin K, an enzyme produced by osteoclasts during bone resorption.[97] Osteocalcin and CTX-I are commonly used to monitor skeletal development, severity of bone disease, and effects of bisphosphonates.[101, 102]

Today, bisphosphonates are potential disease modifying osteoarthritic drugs (DMOAD) primarily used to treat diseases with increased bone resorption and bone loss.[103] To date, there are no DMOADs that have shown efficacy in slowing the progression of osteoarthritis and all current treatments are simply symptom modifying. Aside from their anti-resorptive effects, bisphosphonates have demonstrated chondroprotective, anti-inflammatory and analgesic affects.[103] Bisphosphonates have commonly been utilized in humans to treat metastatic bone pain; however, the pathway in which they can relieve pain has yet to be elucidated.[104] Studies have shown pain relief properties independent of known anti-resorptive action including anti-inflammatory

effects.[103, 105-107] Tiludronate and clodronate have demonstrated inhibition of nitric oxide and pro-inflammatory cytokines in macrophages *in vitro* as well as tiludronate inhibition of vascular endothelial growth factor (VEGF) synthesis in osteoblasts.[107, 108] Down regulation of pro-inflammatory cytokines and formation of vessels by inhibition of VEGF could modulate pain independent of resorption activity.

In humans, nitrogenous bisphosphonates have been used to treat hypercalcemia and osteoporosis.[109] Long-term bisphosphonate treatment can lead to oversuppresion of bone turnover decreasing the skeleton's ability to heal properly and reducing bone's mechanical properties.[110] Rare but severe complications including include atypical fractures and osteonecrosis of jaw have been reported in patients on long-term bisphosphonate therapies due to over suppression of bone turnover.[110-112] To prevent over suppression of bone turnover and reduce chances of adverse events, patients are now taking drug holidays in which they stop bisphosphonate usage for a determined period of time.[113] Yearly exams are recommended for patients on bisphosphonate therapies evaluate bone turnover makers, like CTX-I and assess the likely hood of an adverse event occurring. Though duration and length of holidays are specific to each patient depending on bone mineral density, bone turnover markers like CTX-I and the time in which the patient has been on the drug; generally this is every 3 to 5 years. [113] Bisphosphonate therapy is continued when bone turnover levels being to elevate to levels consistent to those before treatment.[113]

In late 2014, the FDA approved the bisphosphonate drug OSPHOS®, containing the active non-nitrogenous bisphosphonate clodronate, for the treatment of navicular

syndrome in horses. Navicular syndrome is a chronic disease affecting the podotrochlear apparatus and is considered one of the most common causes of forelimb lameness in the horse.[114, 115] OSPHOS® has become a popular choice for veterinarians treating chronic lameness issues in the horse due to the off target analgesic effects. Lameness, or limping, in the horse is a result of musculoskeletal pain.[116]

Despite its clinical popularity, little research has been done to understand the effects of clodronate in the horse. Without information on the effects of clodronate on the bone after treatment at the clinical dosage we must wonder if we are putting our clients at risk. The occurrence of one rare event like atypical fracture or osteonecrosis of the jaw in the horse could be detrimental to the horse industry. CTX-I levels have been evaluated in horses treated with the non-nitrogenous bisphosphonate tiludronate. Horses treated with tiludronate intravenously once daily for 10 days were compared to horses treated with a constant rate infusion and evaluated for CTX-I levels were evaluated on days 1, 3, 6, 10, 20, 30, and 60 after treatment. A significant decrease in CTX-I levels was noted between day 0 and day 1 and continued through day 3 for both treatment groups.[96] This is similar to CTX-I levels after treatment with bisphosphonates in human reports.[117]

In a different study a force plate analysis was used to assess the effects of tiludronate in horses treated intravenously (IV) compared to those receiving a regional limb perfusion (RLP). Client owned horses with no previous history of tilduronate treatment, scored grade 2 or 3 front limb lameness by the AAEP scale, and showed clinical improvement in lameness after administration of local analgesic to the palmar

digital nerves were randomized for treatment selection for the study. After treatment with tiludronate by either RLP or IV at one dose of 1 mg/kg, lameness by force plate were evaluated on days 14, 30, 60, 120, and 200. A significant improvement in lameness was noted on day 120 and 200 in IV treated horses compared to RLP treated horses. [115] Delayed lameness improvement was contributed to pain relief from decreased osteoclast resorption.

Although tiludronate and clodronate are both non-nitrogenous bisphosphonates, there are differences between them within the non-nitrogenous group. Clodronate is a first generation bisphosphonate while tiludronate is a slightly more potent second generation.[118] Bisphosphonate potency is directly related to ability to bind to bone. Clodronate has demonstrated lower binding affinity (806 μM) to bone compared to tiludronate (173 μM) *in vitro*.[119]

Another difference between the two FDA approved equine bisphosphonates is that tiludronate is administered IV while clodronate is administered intramuscularly (IM). IV administration results in a higher peak of drug and a greater amount of drug that binds bone allowing for rapid uptake of the bisphosphonate by the skeleton while clodronate administered intramuscularly has decreased uptake by the skeleton.[103, 118]

There has been no research to date demonstrating efficacy of clodronate in osteoclast function. In the New Animal Drug application of OSPHOS, a pharmokinetic and 5x dosing study was conducted along with a field evaluation in which treated horses were evaluated for lameness on days 28 and 56. Efficacy was demonstrated by a reduction in lameness only and there was no data reported for the assessment of

osteoclast inhibition. A 75% success rate, determined by an improvement of a grade or more by the American Association of Equine Practitioners (AAEP) scale, was noted in clodronate treated horses.

Given the complications in human therapies, we believe it is critical to understand the effects of clodronate in the horse, as atypical fractures could be detrimental to the industry. Our goal was to determine if clodronate given at the clinically relevant dose could alter MSC characterization and ultimately bone remodeling. We hypothesized that there would be no charge in markers of bone remodeling and no change in osteoclast and osteoblast recruitment and there would be an improvement in lameness.

MATERIALS AND METHODS

Study Design

All animal procedures were approved by the institution's animal care and use committee (IACUC 2016-0122). No horses were euthanized for this study. Twelve university-owned horses were used for this study. Inclusion criteria include horses on the western horsemanship equestrian team that were competing during the fall 2016 semester and had not previously received bisphosphonate treatment. The head coach and the equestrian team veterinarian identified eligible horses. Horses were randomly placed into either the treatment group receiving 1.4mg/kg clodronate (OSPHOS®; n=6) or an equivalent volume of LRS as a placebo (CONT; n=6). Patient allocation was controlled in the pharmacy and remained unknown to all other parties. All horses had 12mls of

blood collected from the jugular vein weekly for 8 weeks prior to treatment and 8 weeks post treatment. Serial clinical evaluations, lameness locator data, and coach questionnaires of lameness were performed one week prior to treatment and each week for 3 weeks and at 8 weeks following treatment (see below). Bone marrow was collected from the sternum at 2 weeks prior to treatment and at 2 weeks post treatment for *ex vivo* and *in vitro* bone marrow cultures (Figure 4.1).

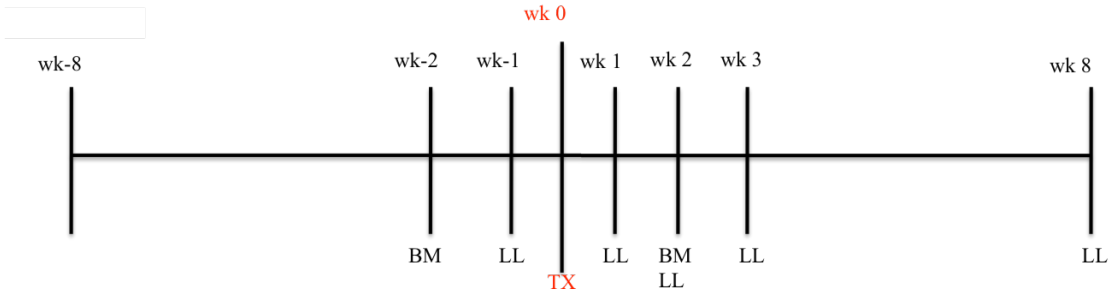


Figure 4.1 Study Design. Horses were evaluated over a 16-week period with treatment at week 0. Each week from week -8 to week 8 blood was collected from the jugular vein. Two weeks prior and 2 weeks post treatment horses bone marrow was aspirated. One week prior and at each week for 3 weeks and at week 8 post treatment lameness was evaluated.

Differentiation & Bone Resorption

Bone marrow aspirate from 2 weeks prior and 2 weeks post treatment underwent red blood cells lysis with ammonium chloride (7.7mg/mL NH4CL; 2.06mg/mL

Hydroxymethane-aminomethane; pH 7.2) before being washed and seeded into 12 well plates with or without the presence of clodronate. Osteoblast cultures were maintained for 28 days in α–MEM, 15% FBS, 1X antibiotic/antimycotic, 10nM dexamethasone, 50 uM L-Ascorbic Acid, and 10mM β-GlycerolPhosphate (basal media) followed by alizarin red staining. Pre-osteoblast cultures were grown for 10 days and stained for alkaline phosphatase positive cultures. Osteoclast cultures were maintained for 15 days in basal media and 10^{-5}M 1α, 25-Dihydroxycholecalciferol before TRAP staining. Culture media was exchanged every 5 days. Colonies from each culture were counted after the appropriate staining. Bone marrow aspirated post *in vivo* treatment was additionally seeded onto an OsteoAssay kit containing bone mineral in basal media with RANKL and MCSF, with and without $5x10^{-5}$M clodronate. Osteoassay cultures were maintained for 10 days before TRAP staining. Prior to staining, media was collected and an ELISA was performed for CTX-I levels.

MSC Characterization

Bone marrow aspirate from 2 weeks prior and 2 weeks post treatment underwent red blood cells lysis before seeding onto flask with and without the addition of clodronate. Cultures were maintained in 1g/L DMEM, 10% FBS, 2.5% hepes buffer, and 1X antibiotic/antimycotic until passage 3, or the needed cell number was reached with media exchanged every 3 days. A passage occurred when the cells are 80% confluent. At passage 3 MSCs were lifted and cryopreserved in 95% autologous serum and 5% DMSO until trilineage differentiation and immunophenotyping could be performed.

Cryopreserved MSCs were thawed with gentle agitation at 35ºC in a water bath until an ice ball was no longer present. Immediately post thaw, an equal volume of DPBS was added to the cell suspension. Five minutes later, the cell suspension was collected and added drop-wise to 20mL of DPBS (Lonza, Walkersville, MD A 100 microL aliquot of thawed cell suspension in DPBS was used for a total cell count and viability using fluorescein diacetate (67.57 mg/mL) and propidium iodide (1.35mg/mL) in DPBS. Counting cell suspensions were plated on a Nebauer hemocytometer and visualized by fluorescence microscopy (Olympus, Center Valley, PA). The live (green) and dead (red) cells were counted. A total of 10 squares were counted per sample. The cell suspension was carefully mixed by pipetting and then centrifuged to pellet the cells for removal of freezing solutions (300G, 5 minutes, 4ºC, 7 brake). Trilineage differentiation was performed by seeding MSCs into 10cm plates for adipogenic and osteogenic differentiation and maintaining MSCs in pellets for chondrogenic differentiation as previously described. [60] Immunophenotyping for CD44, CD45RB, CD29, CD90 and MHCII was performed on isolated MSCs using flow cytometry as previously described.[60]

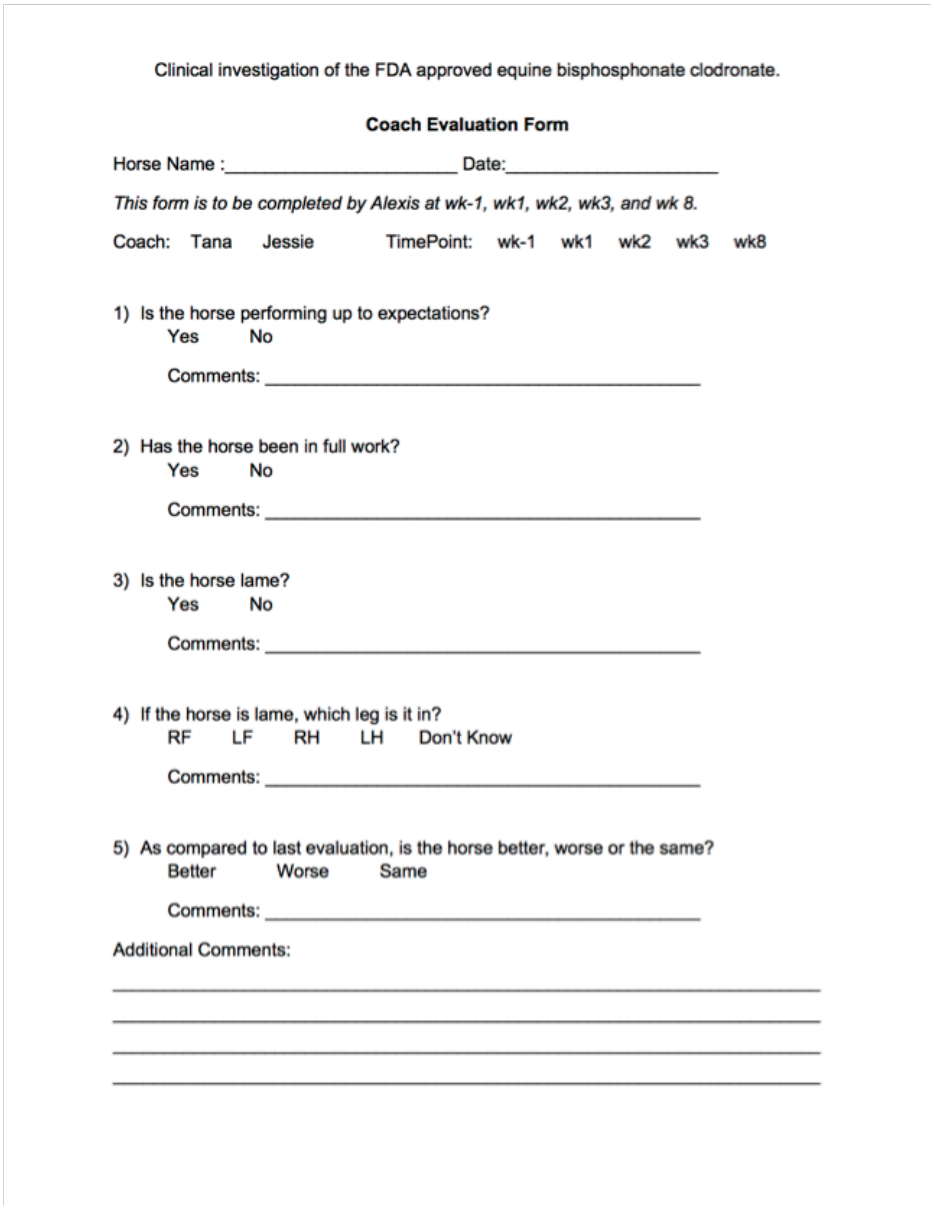
CTX-I and Osteocalcin Levels in vivo

Twelve milliliters of blood was collected from the jugular vein using a 12mL syringe and 18-gauge needle for 8 weeks prior and 8 weeks post treatment. Blood was placed in red top glass tubes and allowed to clot for a minimum of 30 minutes before centrifugation at 4000G. Serum was collected and stored in 1mL aliquots at -80°C.

Frozen aliquots were thawed and neat samples were used to assay for CTX-I and osteocalcin using commercially available ELISAs.

Lameness Effects

Lameness exams were performed one week prior to treatment and each week for 3 weeks and at 8 weeks following treatment using 3 forms of evaluation; clinical evaluations, Lameness Locator®, and coach evaluations, with all 3 forms of evaluation blinded to the others. At each lameness exam the horse was hand walked in a 10-meter straight line, hand jogged in a 20-meter straight line twice and hand jogged in two 10-meter circles to the right and left on a concrete surface. Clinical evaluations of lameness were scored while horses were jogged on concrete according to the AAEP scale with a score of 0 being no asymmetry detected under any circumstances and a score of 5 being the horse was minimally weight bearing or unable to move.[120] The Lameness Locator® is a computer based objective measure of lameness that detects asymmetry (lameness) in the horse as it moves. Marker devices were place on top of the poll, between the hips and on the right front pastern and data was collected during the 2 straight-line jogs. Lameness Locator® data was collected concurrently with the clinical evaluations. Coach evaluations were conducted after lameness exams. The coaches were asked a series of questions to evaluate the horse's work performance based on their observations of the horse in practice in a sand arena (Figure 4.2).



Clinical investigation of the FDA approved equine bisphosphonate clodronate.

Coach Evaluation Form

Horse Name :_____ Date:_____

This form is to be completed by Alexis at wk-1, wk1, wk2, wk3, and wk 8.

Coach: Tana Jessie TimePoint: wk-1 wk1 wk2 wk3 wk8

1) Is the horse performing up to expectations?
Yes No

Comments: _____

2) Has the horse been in full work?
Yes No

Comments: _____

3) Is the horse lame?
Yes No

Comments: _____

4) If the horse is lame, which leg is it in?
RF LF RH LH Don't Know

Comments: _____

5) As compared to last evaluation, is the horse better, worse or the same?
Better Worse Same

Comments: _____

Additional Comments:

_____
_____
_____
_____

Figure 4.2 Coach Evaluations. Horses performed lameness exams one week prior and each week for 3 weeks and at 8 weeks post treatment. Coaches completed a questionnaire at the time of each lameness evaluation about the horses performance in work that week.

Statistical Analysis

Raw data were imported to a commercial statistical software program (Prism, GraphPad Software, La Jolla, CA). Differences between the conditions for longitudinal data were evaluated by repeated measures ANOVA. Differences in paired data within a group were evaluated by Wilcoxon-signed rank test. Coach evaluations were evaluated using a Chi-Square test. Differences were considered significant when $p \leq 0.05$.

RESULTS

Differentiation & Bone Resorption

Bone marrow was collected 2 weeks prior and 2 weeks post treatment with clodronate. Whole marrow underwent osteoblastogenesis and osteoclastogenesis with and without the addition of clodronate. There were no significant differences in osteoblastogenesis in control, *ex vivo*, or *in vivo* treated cultures or osteoclastogenesis (Figure 4.3). However, there was a significant different in osteoblast recruitment for *ex vivo* treated OSPHOS cultures after treatment *in vivo* ($p = 0.018$; Figure 4.3).

Bone marrow collected post treatment with clodronate was seeded onto bone fragments with the addition of MCSF and RANK-L for 10 days before being fixed and TRAP stained. Media was collected from the culture period and assayed for CTX-I. There were no significant differences in CTX-I between the control, *in vivo*, and *ex vivo* treated groups indicating osteoclast activity was no inhibited by clodronate *ex vivo* at $5x10^{-5}$M (Figure 4.4).

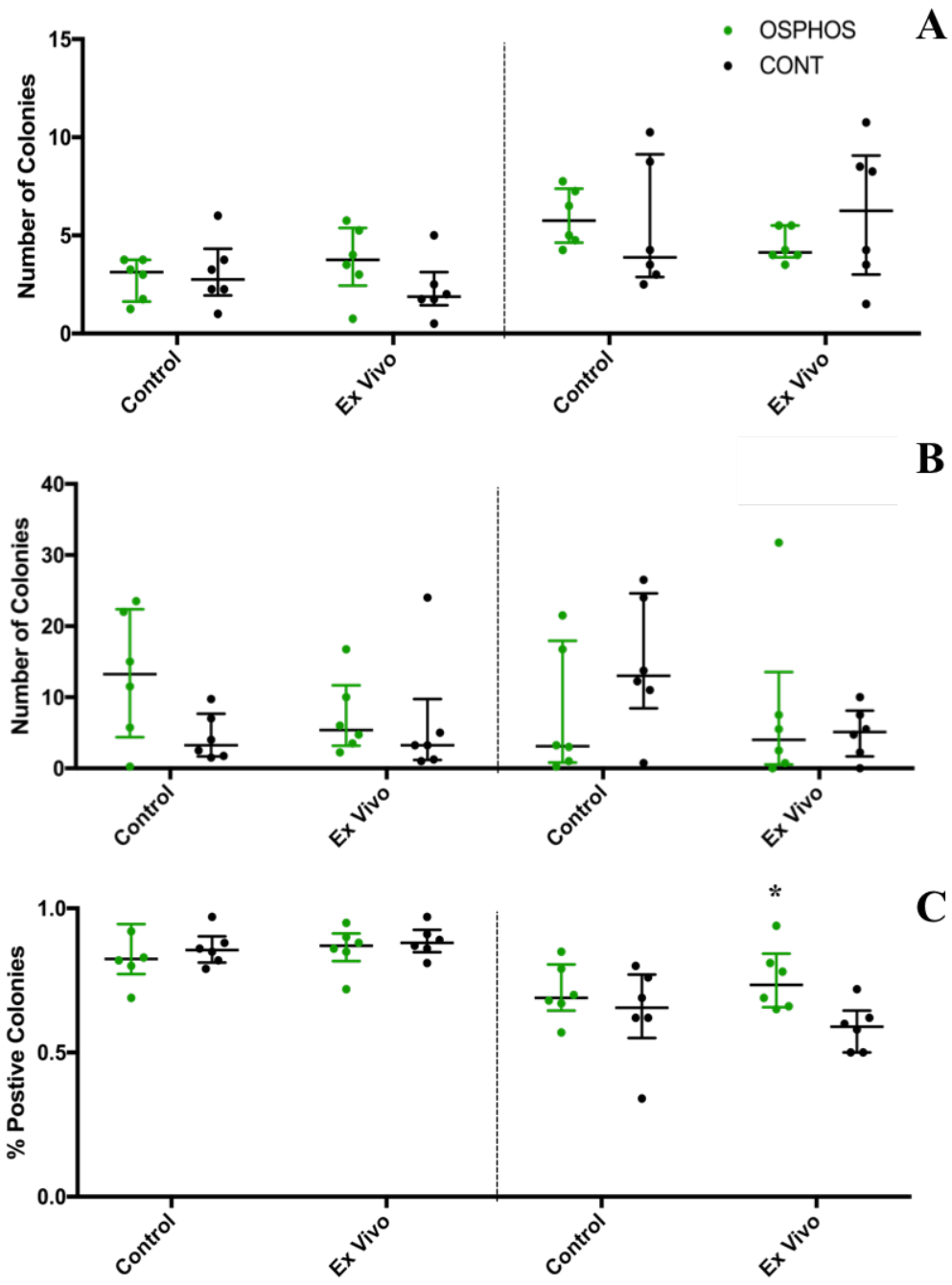


Figure 4.3 Cell Recruitment and Differentiation. Bone marrow aspirated 2 weeks prior and 2 weeks post treatment was evaluated for recruitment and differentiation capacity. There were no differences in A) osteoblast or B) osteoclast differentiation between groups. There was a significant difference in C) osteoblast recruitment after *ex vivo* treatment between the OSPHOS and CONT cultures post *in vivo* treatment (p= 0.018).

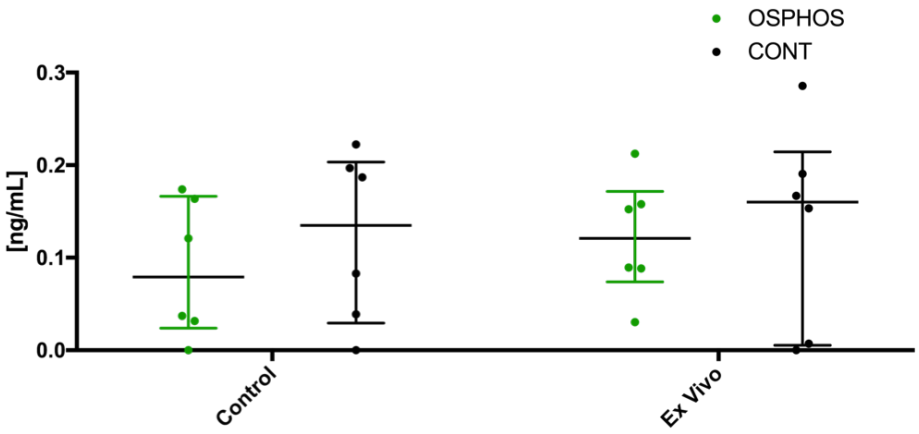


Figure 4.4 Bone Resorption Ability of Clodronate Exposed Osteoclasts. Bone marrow collected 2 weeks after *in vivo* treatment underwent osteoclast differentiation and was seeded onto bone matrix. The resulting culture media was assayed for CTX-I values representing active osteoclasts.

MSC Characterization

Bone marrow was collected 2 weeks prior and 2 weeks post treatment with clodronate. MSCs were isolated and expanded with and without the addition of clodronate for the culture period. MSCs from control, *ex vivo*, and *in vivo* treated cultures were able to undergo trilineage differentiation for osteocytes, adipocytes, and chondrocytes successfully (Figure 4.5). All horses expressed CD29, CD90, CD44 and lacked expression of MHCII and CD45 for control, *ex vivo*, and *in vivo* treated cultures (Table 4.1).

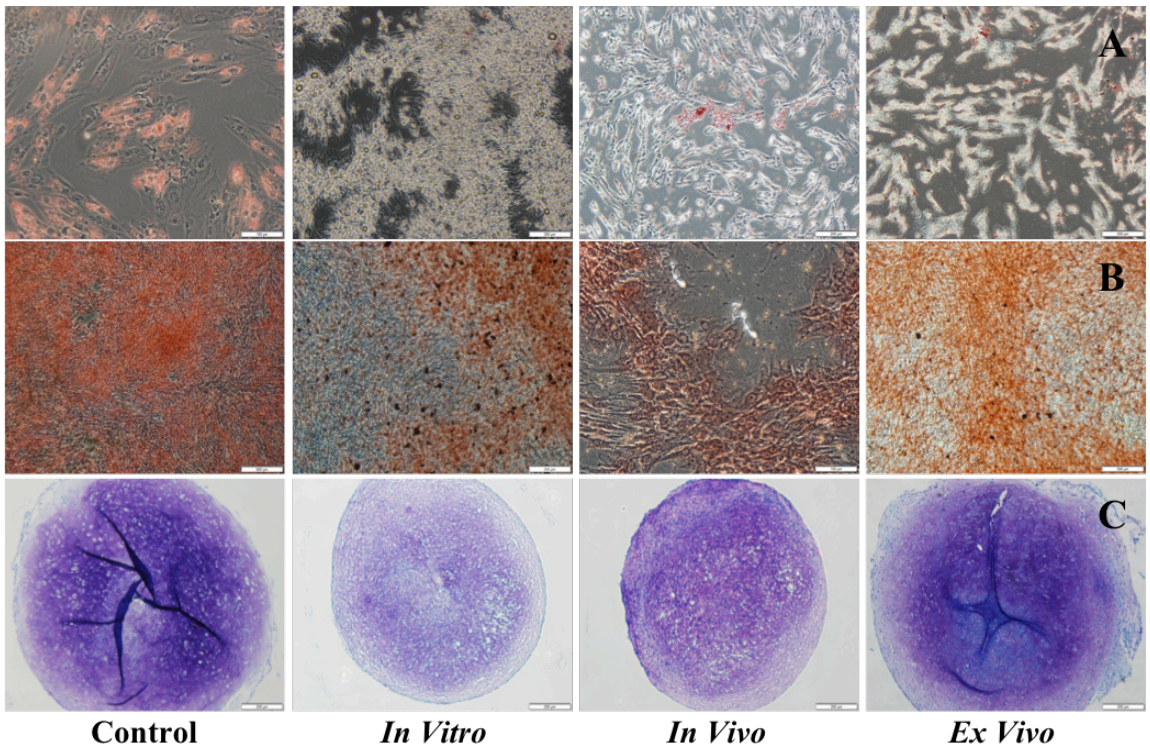


Figure 4.5 Trilineage Differentiation of MSCs Exposed To Clodronate. MSCs isolated from bone marrow aspirated 2 weeks prior and 2 weeks post treatment. Control and *In Vitro* cultures were isolated from bone marrow aspirated 2 weeks prior to treatment. *In Vivo* and *Ex Vivo* cultures were isolated from bone marrow aspirated 2 weeks after *in vivo* treatment. Representative images of A) adipocytes, 2) osteoblast and 3) chondrocytes.

		MHCII	CD44	CD29	CD45
Control	Horse 1	1.36	50	98.3	1.48
	Horse 2	1.35	25.6	99.1	2.13
	Horse 3	0.317	28.5	99.7	1.14
	Horse 4	1.06	3.04	99.1	1.87
	Horse 5	1.21	3.38	96.4	1.39
	Horse 6	0.697	96.7	99.8	0.893
	Horse 7	2.09	88.8	99.2	0.846
	Horse 9	0.0099	0	0.0396	0.128
	Horse 11	1.15	13.7	89.1	1.72
	Horse 12	1.02	23.4	98.3	2.31
Ex Vivo	Horse 1	1.79	1.92	91.3	1.44
	Horse 3	41	3.87	87.1	1.18
	Horse 4	8.62	2.94	95.2	1.99
	Horse 7	1.85	3.62	99.7	0.938
	Horse 8	47.9	3.34	49	1.6
	Horse 9	1.21	1.88	92.6	4.58
	Horse 11	1.69	12	98.6	1.42
	Horse 12	1.96	53	92.5	3.83

		MHCII	CD44	CD29	CD45
OSPHOS	Horse 1	2.95	28.6	99.3	1.47
	Horse 3	1.79	35.3	99.9	1.39
	Horse 6	1.97	26.6	98.1	2.1
	Horse 7	0.12	3.02	98.8	0.643
	Horse 8	1.55	11.2	97.8	3.89
	Horse 10	0.905	26.3	98.8	1.52
CONT	Horse 2	0.851	37.2	99.8	1
	Horse 5	0.633	11.8	96.3	1.26
	Horse 9	1.44	10.3	73.3	1.96
	Horse 11	1.05	4.17	80.2	1.98
	Horse 12	1.04	6.07	77.8	1.23
OSPHOS *Ex Vivo*	Horse 3	0.422	4.96	98.4	1.14
	Horse 6	1.13	4.1	98.7	1.63
	Horse 7	0.887	5.91	99.7	1.11
	Horse 8	0.44	5.97	87	1.16
CONT *Ex Vivo*	Horse 2	10.8	2.26	98.3	0.901
	Horse 4	1.54	5.41	93.2	1.39
	Horse 5	2.13	5.11	82.7	1.36
	Horse 9	0.867	31.3	98	1.62
	Horse 11	0.704	1.28	92	0.324
	Horse 12	0.447	12.6	99.5	0.269

Table 4.1 Immunophenotyping of MSCs Exposed To Clodronate. MSCs isolated from bone marrow aspirated 2 weeks prior and 2 weeks post treatment. Control and *Ex Vivo* cultures were isolated from bone marrow aspirated 2 weeks prior to treatment. OSPHOS and CONT cultures were isolated from bone marrow aspirated 2 weeks after *in vivo* treatment. MSCs isolated from the second bone marrow aspiration were exposed to clodronate *ex vivo*.

CTX-I and Osteocalcin Levels in vivo

Twelve milliliters of blood was collected from the jugular vein for 8 weeks prior and 8 weeks post treatment. Serum was isolated and assayed for bone turnover markers CTX-I and osteocalcin. There were no significant differences in CTX over time between

the OSPHOS and CONT groups post treatment indicating osteoclast activity was not affected at the clinical dosage (Figure 4.6).

However, clodronate treatment caused a significant increase in osteocalcin over time, specifically at weeks 1, 2, 4 and 7, indicating bone formation was occurring in clodronate treated horses (p=0.0346; Figure 4.7).

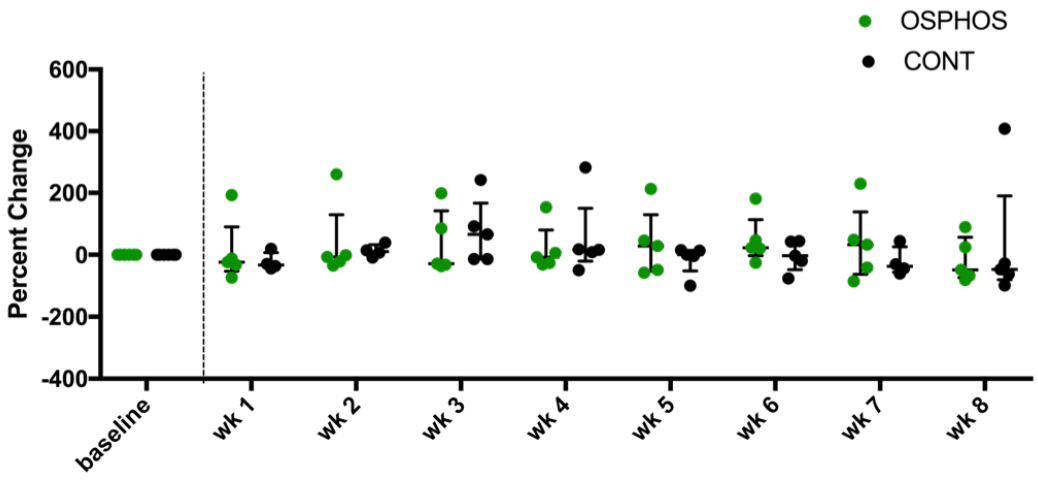


Figure 4.6 CTX-I Levels After Clodronate Treatment. Blood was collected each week for 8 weeks after treatment and evaluated for bone turnover levels. There were no significant differences between OSPHOS and CONT groups in CTX-I levels after treatment represented by the dashed line.

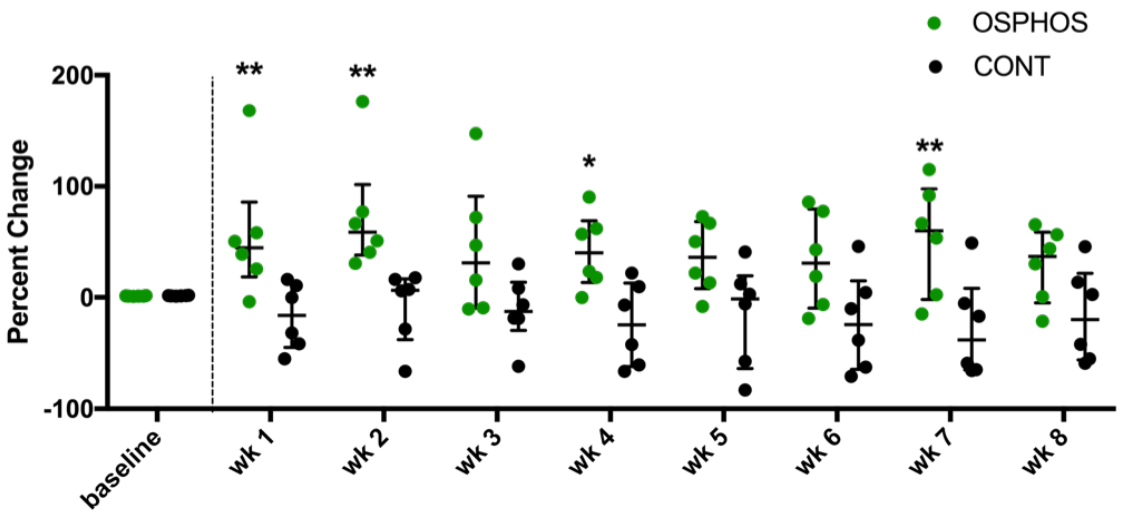


Figure 4.7 Osteocalcin Levels After Clodronate Treatment. Blood was collected each week for 8 weeks after treatment and evaluated for bone turnover levels. There was a significant differences between OSPHOS and CONT groups in CTX-I levels after treatment represented by the dashed line at week 1, 2, 4 and 7 ($p= 0.0346$).

Lameness Effects

Lameness exams were performed one week prior to treatment and each week for 3 weeks and at 8 weeks following treatment. When lameness was evaluated objectively by the Lameness Locator® no differences were found in front limb asymmetry due to treatment. However a significant difference was found in hind limb asymmetry in OSPHOS treated horses ($p= 0.0252$; Figure 4.8). When a clinician evaluated lameness, no significant differences in for either front or hind limbs over time between the OSPHOS and CONT groups when evaluated on a hard surface (Figure 4.9).

Interestingly, there was a significant difference in improvement between the OSPHOS and CONT treated groups when evaluated by the coaches (Figure 4.10). The coaches were able to successfully identify mild improvements in the horses' performance on a soft surface. This improvement in performance seen by the coaches contrasts with clinical lameness exams evaluated by a clinician or the Lameness Locator®; each horse's underlying chronic lameness was exacerbated by jogging on a hard surface possibly masking any mild improvements due to treatment with clodronate.

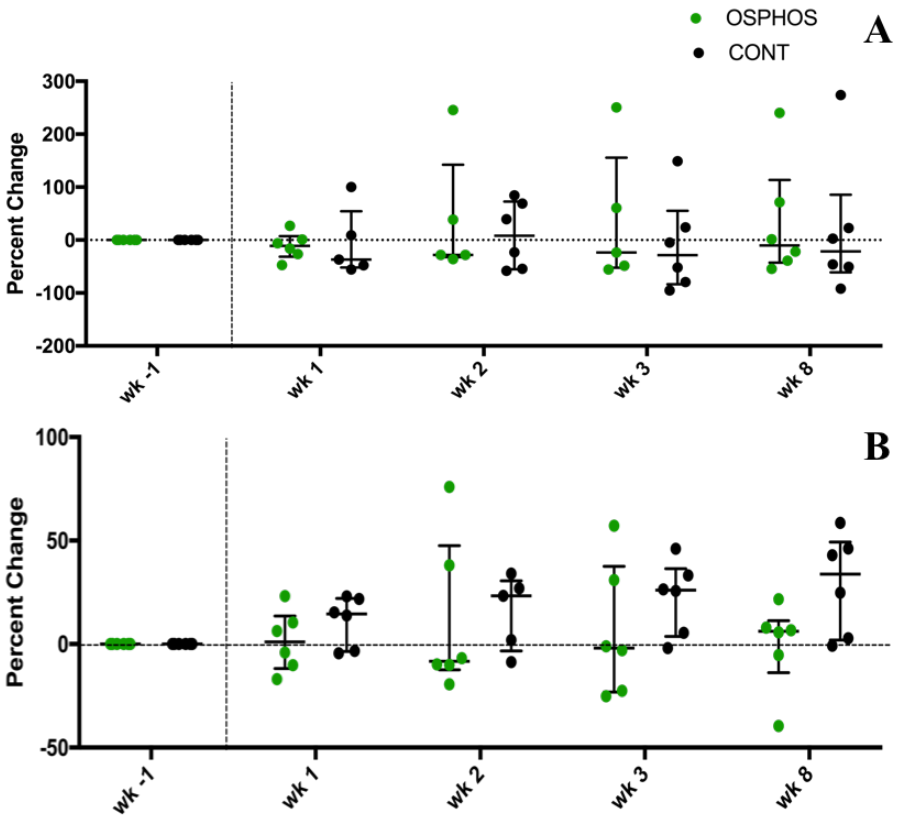


Figure 4.8 Front and Hind Limb Asymmetry. Lameness evaluations were performed using the Lameness Locator® one week prior and each week for 3 weeks and at week 8 post treatment. There were no differences in A) front limb asymmetry after treatment; represented by the dashed line. There was a significant difference in B) hind limb asymmetry between OSPHOS and CONT groups with OSPHOS treated horses having a decrease in lameness (p= 0.0252).

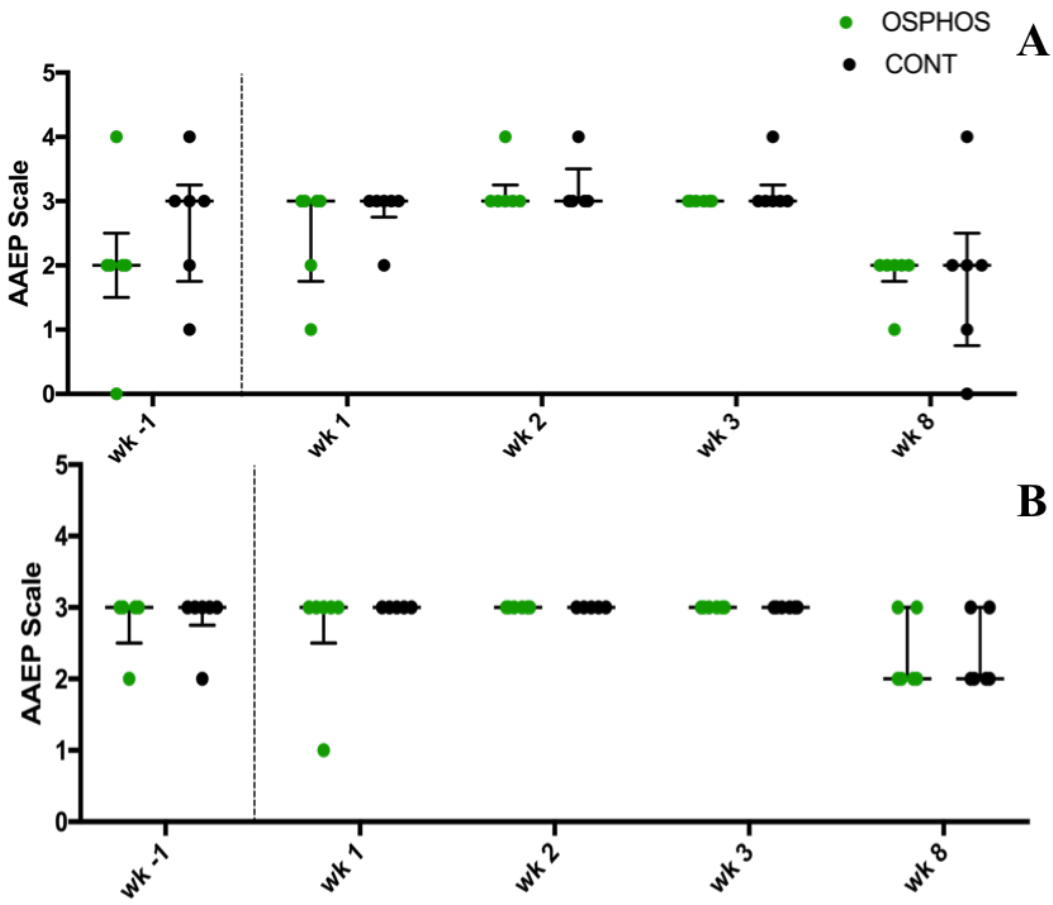


Figure 4.9 Front and Hind Limb Lameness. Lameness evaluations were performed by a clinician using the AAEP scale one week prior and each week for 3 weeks and at week 8 post treatment. There were no differences in A) front limb or B) hind limb lameness between OSPHOS and CONT groups after treatment represented by the dashed line.

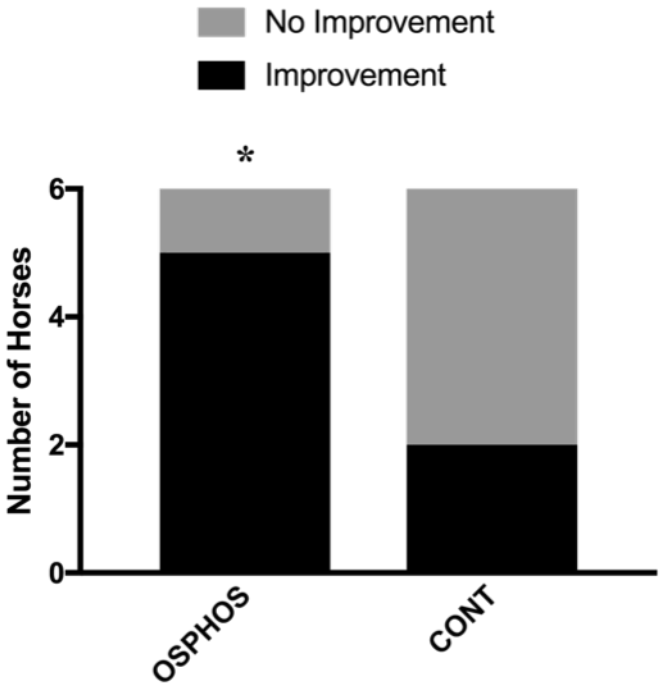


Figure 4.10 Coach Evaluations of Performance. Horse performance was evaluated by the coaches one week prior and each week for 3 weeks and at week 8 post treatment. The coaches identified improvement in 5 or 6 OSPHOS treated horses (p= 0.0395).

DISCUSSION

We sought to determine if clodronate given at the clinically relevant dose could alter MSC characterization and ultimately bone remodeling in the horse. Twelve university-owned horses ranging in age from 7 to 18 with a median age of 11 were used for this study. Weekly blood collections for 8 weeks prior to treatment and 8 weeks post treatment were used to analyze CTX-I and osteocalcin levels. Bone marrow was collected from the sternum at 2 weeks prior to treatment and at 2 weeks post treatment for *ex vivo* and *in vitro* bone marrow cultures for osteoblastogenesis, osteoclastogenesis,

bone resorption ability, and MSC characterization. Pain was evaluated by serial clinical evaluations, lameness locator data, and coach questionnaires of lameness one week prior to treatment and each week for 3 weeks and at 8 weeks following treatment.

CTX-I and osteocalcin are standard bone markers used to evaluate bone turnover *in vivo*. From treatment on there was a significant difference in osteocalcin over time between the OSPHOS and CONT groups post treatment, with the OSPHOS group having higher levels of osteocalcin at weeks 1, 2, 4 and 7 following treatment. It is possible osteoblast activity was stimulated *in vivo* by clodronate, leading to increased matrix production and increased osteocalcin levels. Clodronate stimulated osteoblast differentiation has been demonstrated *in vitro* cultures of osteoblast progenitors and MSCs. [121] Treatment with clodronate had no or an undetectable effect on CTX-I and a significant effect on osteocalcin. We believe there are 2 possible reasons for this. First, we are working with skeletally mature horses that would not be expected to have high levels of bone turnover. Therefore, there should not be large levels of CTX-I. Second, the dose administered is likely lower than that necessary to elicit a bone resorption response.

A reduction in forelimb lameness was seen in horses treated with OSPHOS in the FDA approval study.[122] Seventy five percent of OSPHOS treated horses demonstrated an improvement in lameness by one or more grade of the AAEP scale 56 days after treatment.[122] Given these marked improvements in visible forelimb lameness we expected to find similar improvements in lameness in our OSPHOS treated horses. However, we saw no change in forelimb asymmetry. In contrast, we did see a significant

improvement in hindlimb asymmetry in OSPHOS treated horses. We think the improvement in the hindlimb lameness versus lack of improvement in the forelimb lameness could be because lameness assessment was made on a hard surface. The forelimb lameness in our horses was due to foot pain, a condition known to be exacerbated by a firm surface. We think the firm surface may have decreased the likelihood of seeing a minor change in lameness. In contrast, hindlimb lameness is rarely exacerbated to the same degree by a hard surface making it more likely to be able to detect a mild improvement. No change in front or hind limb lameness was seen in horses evaluated using by a clinician. We think the lack of change in lameness was due to using the AAEP scale to score lameness. The AAEP scale defines a grade of 3 covers a broad range of lameness as defined as "consistently observable at a trot under all circumstances" covers a broad range of lameness. This means a horse with a clinically relevant change in lameness from a severe grade 3 to a mild grade 3 would appear to be unchanged using this scale.

The coach evaluations supported that a clinically relevant improvement in lameness occurred with significantly more horses receiving the evaluation of "improved" in the OSPHOS group as compared to CONT. Coaches observed the horses under work throughout the week while the clinician and the Lameness Locator® only evaluated lameness at the designated evaluation time points. It is likely the coaches are more sensitive to changes in the horses' performance and likely a better form of evaluation of lameness in the horse. The improvement in lameness seen in this study is similar to reports of the bisphosphonate tiludronate that produced a decrease in lameness

in treated horses. Interestingly, humans treated with intra-articular injections of clodronate reported a decrease in pain associated with osteoarthritis, a musculoskeletal injury.[123]

Analgesic effects of bisphosphonates have been reported in patients suffering from pain caused by metastatic breast cancer.[106] Tiludronate has demonstrated inhibition of ATPases preventing the formation of an acidic environment created during resorption.[124] It is possible prevention of these acidic environments decreases acidosis in the bone or in the body leading to decrease in perceived pain. Clodronate has demonstrated effective analgesic effects at a similar or lower dose exerting anti-resorptive affects.[125] However the lameness reduction concurrent with no change in CTX-I that we saw suggests that analgesia occurred independent of osteoclast inhibition.

Interestingly, non-nitrogenous bisphosphonates, like clodronate, have exhibited more effective analgesic effects compared to nitrogenous bisphosphonates that exhibit more potent anti-resorptive abilities.[125] In a study evaluating clodronate as a treatment for erosive osteoarthritis of the hand, intramuscular injection of 200mg of clodronate daily 10 days followed by 6 days of injection at 90 and 180 days was effective at reducing pain demonstrated by an increase in hand strength.[126] Clodronate has demonstrated a variety of analgesic effects including actions on glutamate and/or ATP-related pain transmission and reducing secretion of inflammatory cytokines and matrix metalloproteinase.[107, 126]

Osteoblastogeneis and osteoclastogenesis of control cultures and clodronate-exposed *ex vivo* and *in vivo* cultures were able to undergo differentiation as expected. *Ex*

vivo treated OSPHOS cultures after *in vivo* treatment recruited more osteoblast cells compared to *ex vivo* treated CONT cultures indicating *in vivo* and *ex vivo* treatment leads to osteoblast recruitment. Bisphosphonate drugs have a high affinity for calcium and adhere to bone. Therefore, clodronate should not have an effect of whole marrow ability to undergo differentiation. Bone marrow seeded to bone mineral and grown in MCSF and RANK-L did not have a significant difference in CTX-I availability in *ex vivo* treated cultures compared to *in vivo* treated cultures. It is possible that there were so few osteoclast in the *in vivo* treated cultures that the amount of CTX-I released was below the detection limit of the assay compared to the *ex vivo* treated cultures who had osteoclast inhibition. This is supported by microscopy which revealed more TRAP staining and resorption pits in the *in vivo* treated cultures than the *ex vivo* treated (data not shown).

MSCs were isolated from control and *in vivo* treated bone marrow were able to undergo trilineage differentiation and exhibited the expected immunophenotype. As previously mentioned bisphosphonates have a high affinity for calcium and adhere to the bone. During osteoclastic bone resorption the bisphosphonate is resorbed rendering the osteoclast inactive. MSCs isolated from whole marrow should not be affected by bisphosphonate treatment.

A limitation to our study was two horses receiving bilateral forelimb coffin joint injections with hyaluronate acid and triamcinolone and bilateral hock injections with depo-medrol. However, both horses were in the control group so any improvements in lameness from clodronate are not contributed to treatment by joint injection.

Lameness and bone turnover markers should be evaluated in horses continuing treatment at the manufactures' recommended dosing regimen of 3 months, to further elucidate the long-term effects of clodronate on bone parameters. Due to the long half-life of all the bisphosphonates, it is possible that clodronate will have a significant effect on bone turnover after re-dosing.

CONCLUSIONS

Further dosing studies should be conducted; however, the findings herein demonstrate that that clodronate improved lameness that was independent of its anti-resorptive actions. If this can be confirmed in a larger study, clodronate should be safe to use without worry of adverse skeletal effects.

CHAPTER V

SUMMARY AND CONCLUSIONS

The mechanistic action of MSCs was originally thought to be direct cell differentiation due to their differentiation potential.[42] Recent reports have indicated MSCs functioning as medicinal signaling cells modulating the environment post injection.[24] MSCs have demonstrated secretion of chemokines, growth factors, other immune modulators and signaling of endogenous progenitors to optimize tissue regeneration.[37, 43] In order to best utilize MSCs in a given circumstance, it is important to determine whether MSCs are functioning to replace damaged tissue via direct cell differentiation, act as medicinal signaling cells, or both.

Elucidating engraftment location and longevity of MSCs post injection may provide insight to their mechanism of action. In our second chapter we evaluated labeling MSCs with a fluorocarbon nanoparticle as a way to longitudinal track MSCs in the post injection environment to elucidate engraftment quantity, duration and location concurrently assessing tissue healing. However, dose dependent cell association of the nanoparticle seen in our study was not repeatable between individuals.

Possible dissociation of the fluorophore conjugate inhibited visualization of the nanoparticle and raised concerns about how the cells were labeling, intra- or extra-cellularly. It is possible that non-phagocytic cells like MSCs did not reliably endocytose the nanoparticle leading to differences observed between individuals. Varied metabolic rates between cultures, and therefore possible exocytosis of the nanoparticle, could

explain inconsistent results of labeling efficiency between individuals. Possible exocytosis of the nanoparticle raises concerns in our ability to sufficiently label the cells as well as specificity of tracking.

Modifications of the fluorocarbon nanoparticle allowing for increased endocytosis and retention of the label could improve MSC tracking. A tracking modality that would allow for longitudinal evaluation of MSCs post injection to elucidate quantity and duration of cell engraftment with concurrent assessment of tissue healing could help identify MSC mechanism of action.

If MSCs are working as medicinal signaling cells to provide a favorable environment allowing for normal tissue healing, the preparation of the cells prior to injection could affect their ability to function post injection. In our third chapter, we explored the ideal cell preparation for cryopreservation that would allow for injection of MSCs immediately post thaw while avoiding contaminating xenogens.

In our study no differences in post thaw viability, growth kinetics, or morphology were seen between 95% serum 5% DMSO and 20% serum 10% DMSO 70% complete media freezing formulations for any serum source. A post thaw lag phase was noted at 24 hours for all formulations and serum sources with possible apoptosis of MSCs indicated by our debris scores and ability to form colonies. It is possible this lag phase cause selection of "stronger" MSCs followed by marked proliferation of the cells.[69] A formulation with lower DMSO content like our 95% serum 5% DMSO formulation and an autologous serum source is recommended for clinical application to lower possible cytotoxic effects of DMSO and mitigate immune reactions from non-autologus sources.

Endogenous MSCs at the level of the bone are known signaling or "support" cells for osteoclastogenesis. Production growth factors like macrophage colony stimulating factor and RANK ligand by MSCs and osteoblast progenitors drive osteoclast differentiation of hematopoietic lineage cells.[22, 37] In our fourth chapter, we investigated the impact of bisphosphonates, a musculoskeletal therapy, on bone remodeling and bone cells like MSCs. We hypothesized that clodronate treatment at the clinically relevant dosage would provide beneficial effects on lameness (pain) without exerting an effect on osteoclastic activity.

Bone homeostasis after *in vivo* clodronate treatment was evaluated through detection of bone turnover levels, assessment of lameness, recruitment and differentiation of osteoblast and osteoclast cells, and MSC characterization. Indeed, we observed a clinical benefit, a reduction in lameness, without producing measurable changes in bone turnover suggesting clodronate exerts analgesic effects independent of anti-resorptive effects. Analgesic effects of clodronate have previously been demonstrated, specifically anti-inflammatory effects via glutamate and/or ATP related pain transmission and reduction in secretion of cytokines and chemokines by activated macrophages.[107, 126]

FUTURE STUDIES

In one study we evaluated the labeling of MSCs with a fluorocarbon nanoparticle as a way to longitudinal track MSCs in the post injection environment. It is possible endocytosis of the fluorocarbon nanoparticle was inconsistent between individual cell

cultures. Evaluating metabolic activity of cell cultures compared to their ability to uptake the nanoparticle could elucidate effects of the activity of the cells on their ability to retain the nanoparticle. If exocytosis is an issue between cultures, understanding what type of endocytosis is needed for retention of the nanoparticle could elucidate ideal labeling methods.

We investigated a clinically acceptable cryopreservation formulation of MSCs that allows for injection immediately post thaw. An initial lag phase was observed in all formulations 24 hours post thaw. Apoptosis assays should be performed to confirm if the lag phase was due to apoptosis or lack of cell attachment to the flask. Using autologous serum during cryopreservation eliminated a possible immunogenic reaction due to a xenogen (FBS). Studies have demonstrated a reduction in immune response after injection with MSCs cultured with alternative serum sources for the last 48 hours of culture.[25] Identifying culture conditions that allow for MSC isolation and expansion in xenogen free conditions should be evaluated. Platelet lysate could serve as an autologous serum source when culturing cells.

Clordronate, a non-nitrogenous bisphosphonate, given at the FDA approved dosage was studied to evaluate effects on bone turnover in *vivo* and potential off-target effects, a reduction in lameness. We demonstrated mild reduction in lameness without measureable changes in bone turnover. It is possible the dosage of clodronate needed to elicit an anti-resorptive response is higher than the FDA approved dosage of 1.8mg/kg needed to elicit an analgesic effect. *In vitro* assays of dosing response to clodronate treated osteoclastogenic cells should be evaluated to identify the concentration needed to

elicit an anti-resorptive effect. Osteoclast seeded onto bone matrix with varying concentrations of clodronate could evaluate concentrations needed to inhibit resorption. Further studies should be conducted to evaluate the effect of clodronate on lameness and bone turnover when horses are re-treated at the usual clinical regimen of 3 months.

FINAL REMARKS

Regenerative medicine is a growing field that focuses on developing methods of natural healing allowing tissues to return to normal function. Tendon, ligament, and joint related diseases are common in the horse, exhibiting similar musculoskeletal disease pathogenesis to humans.[33, 127] The prevalence of musculoskeletal injuries in the horse, and their inability to heal without scar tissue development, leads to early retirement with 61% of horses failing to return to work at their previous level.[115] Developing therapies that allow for natural healing musculoskeletal tissues without scar tissue development could enhance recovery these debilitating conditions and allow horses to return to work and more productive lives, rather than retiring.

Stem cells have the potential to be a powerful method of regenerative medicine, healing tissues without scar tissue formation, although their utility is not yet widespread due to inconsistent results in clinical trials and difficulties in writing regulation laws.[3] MSCs are an increasingly investigated therapy in regenerative medicine with the potential to work as a disease modifying treatment.[38] There are many diverse ways of using MSCs; however to maximize their full potential requires better understanding of their capacities for proliferation and differentiation within unique *in vivo*

microenvironments. The function of the MSC remains unknown as well as the original question of whether MSCs contribute by direct cell differentiation or modulation of the environment. MSC therapy can only grow and elicit answers if we continue to do research that benefits both the horse and people.

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