DISSERTATION

EVALUATION OF ALLOGENEIC BONE MARROW-DERIVED MESENCHYMAL STEM CELLS FOR USE IN EQUINE JOINTS: IN VITRO TO PRECLINICAL EVALUATION

Submitted by

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ABSTRACT

EVALUATION OF ALLOGENEIC BONE MARROW-DERIVED MESENCHYMAL STEM CELLS FOR USE IN EQUINE JOINTS: IN VITRO TO PRECLINICAL EVALUATION

Joint disease is prominent in the equine population and horses provide a highly translational model for human joint disease. Mesenchymal stem cells (MSCs) have been investigated as a treatment of musculoskeletal disease in the horse with autologous MSCs showing promise as a treatment of desmitis, tendonitis and joint disease including meniscal injury and osteoarthritis. However, the culture expansion of autologous MSCs is both labor intensive and time consuming with an average expansion time of 2-4 weeks. Allogeneic MSCs would offer multiple potential advantages over autologous MSCs use including timing of treatment, potential for characterization, and selection of donors for desired stem cell characteristics. The safety of allogeneic MSCs must be established prior to clinical use. Allogeneic MSCs have been evaluated in vitro and in vivo, but rarely have allogeneic MSCs been directly compared with autologous MSCs. In addition, pre-clinical models must control for the large variability present in individual horses' reactions to joint injections as well as the variability in how different joints react to intra-articular treatments. Further, the safety of allogeneic MSCs must be examined in both the normal joint and inflammatory joint as MSCs may react to the joint environment. The goals of the research described in this dissertation were to directly compare the immune suppressive ability of autologous and allogeneic bone marrow-derived MSCs (BMDMSCs) in vitro, and directly compare both the normal and inflamed joint response to autologous and allogeneic BMDMSCs in vivo.

In the first part of this work we compared the immune suppressive properties of allogeneic and autologous BMDMSCs in vitro. No difference was detected between the ability of allogeneic versus autologous BMDMSCs to suppress lymphocytes in modified mixed lymphocyte reactions. This work also established prostaglandin E_2 as an important mediator of immune suppression used by allogeneic BMDMSCs.

Following in vitro studies, two preclinical, in vivo studies were performed. In the first study, allogeneic and autologous BMDMSCs were administered into clinically normal, contralateral, metacarpophalangeal joints. No difference was detected in the clinical or cytological response of the normal equine joint to allogeneic versus autologous BMDMSCs. After establishing the response of the equine tibiotarsal joint to recombinant IL-1β (rIL-1β), an additional in vivo study was conducted to determine the inflamed joint response to allogeneic versus autologous BMDMSCs. In this study, no difference was detected in synovial fluid parameters, subjective lameness, or joint effusion between the inflamed joint response to allogeneic versus autologous BMDMSCs. In addition, no decrease in joint inflammation was detected as a result of autologous or allogeneic BMDMSC administration.

The work described in this dissertation has improved our understanding of the equine joint response to allogeneic and autologous BMDMSCs. Further, it supports future exploration into the use of allogeneic BMDMSCs for musculoskeletal disease in the horse. Specifically, this work should be followed with a direct comparison of the efficacy of allogeneic versus autologous BMDMSCs for joint disease in the horse.

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Chapter 1: Literature Review[1]

1.1 Overview

Allogeneic mesenchymal stem [stromal] cells (MSC) may provide significant clinical advantages over autologous MSCs for the treatment of equine musculoskeletal disease including convenience, timing of administration, and potential selection for desired cellular characteristics. However, safety and efficacy of allogeneic MSCs remains to be proven. Investigation of safety and efficacy includes a multifaceted approach utilizing in vitro assays, experimental in vivo (pre-clinical) trials and clinical trials. Although in vitro assays and some experimental in vivo trials have raised concern over immune recognition and the potential for an immunologic response to allogeneic MSCs, few adverse clinical reactions are reported. Published in vivo preclinical and clinical studies suggest allogeneic MSCs to be safe. Direct comparisons between allogeneic and autologous MSCs are few but are necessary to determine if allogeneic MSCs are a suitable replacement for autologous MSCs in the treatment of musculoskeletal disease of the horse.

1.2 Potential advantages of using allogeneic mesenchymal stem cells

The use of MSCs continues to show promise in the treatment of musculoskeletal disease in the horse. [1-3] In the last 10 years, intense debate has occurred over the safety and efficacy of allogeneic MSCs for equine musculoskeletal disease. Expansion of autologous MSCs is labor intensive and costly. Autologous MSCs expansion may take several weeks and in some cases is not feasible due to an immediate need for treatment. The ability to expand and bank allogeneic

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[1] A version of this manuscript has been submitted to the *Equine Veterinary Journal*: Colbath AC, Dow SW, McIlwraith CW, Goodrich LR. Mesenchymal stem cells for treatment of musculoskeletal disease in horses: relative merits of allogeneic versus autologous stem cells.

MSCs makes them a clinically and commercially attractive alternative to autologous MSCs for treatment of musculoskeletal diseases. Other advantages of allogeneic MSCs include the ability to establish well-characterized cell lines. Thus, a more uniform cellular therapy product may allow for a more predictable response and thus advance research efforts focused on elucidating mechanism of action.

Evidence is accumulating that donor characteristics influence MSCs health. MSCs from young individuals appear to have enhanced healing properties and single-cell transcriptomes reveal differences in gene expression. [4; 5] However, even within donor and gender matched controls, individual variation exists in proliferative ability, differentiation ability and gene expression. [6] Allogeneic MSCs would provide an "off-the-shelf" therapy that could be characterized and potentially selected for desired healing properties.

Although the conceptual advantages of allogeneic MSCs use is obvious, the safety of allogeneic MSCs must be established before clinical use. There have been a number of potential issues raised with respect to the use of allogeneic MSCs, and most revolve around the perceived risk of adverse immunological reactions to foreign MHC antigens expressed by MSCs. However, there are now a number of published studies involving the systemic and local administration of allogeneic MSCs in humans, with little evidence of adverse immunological reactions. [7-9] Similarly, studies in dogs have also failed to reveal evidence of significant adverse immunologic reactions following allogeneic MSCs administration with many studies suggesting clinical efficacy. [10-13] The lack of immune responses against allogenic MSCs likely results from their inherent immune suppressive properties and low levels of MHC expression. [14-16] Immunological reactivity against allogeneic equine MSCs has been demonstrated in vitro, and in subcutaneous injection models. [17; 18] However, the relevance of

these models to the use of allogeneic MSCs for treating musculoskeletal injuries (e.g. cartilage and tendon injuries) could be questioned.

Allogeneic MSCs would be unsafe for intra-articular use if they elicited a significant immune response resulting in morbidity or mortality of the patient, and undesirable if they resulted in a significant decrease in efficacy compared to autologous MSC. Investigating the equine joint reaction to allogeneic MSCs requires a multifaceted approach beginning with in vitro assays and progressing through experimental in vivo trials into clinical trials. The authors will not discuss the sources of allogeneic MSCs separately but will rather group the literature surrounding allogeneic MSCs regardless of cell source. The objective of this literature review is to present the current literature on allogeneic MSC use in the horse with special emphasis on the progression from in vitro assays to experimental models to clinical trials.

1.3 In vitro studies investigating safety of allogeneic mesenchymal stem cells for clinical use

In vitro assays for cellular immune response have been a mechanism by which to measure immunomodulation of MSCs. The most commonly performed assay is the modified mixed leukocyte reaction (MLR) where peripheral blood leukocytes of one horse are mixed with stem cells of another horse. [19] Immunomodulatory ability of MSCs is elucidated by measuring lymphocyte proliferation. If allogeneic MSCs result in an increase in lymphocyte proliferation, the cells are believed to be causing an immune response. If allogeneic MSCs result in a decrease in lymphocyte proliferation following exogenous lymphocyte stimulation, then allogeneic MSCs are considered to have an anti-inflammatory or immune suppressive effect. In addition to examining lymphocyte proliferation, assays are used to measure inflammatory cytokines secreted by lymphocytes such as interferon gamma (IFNγ), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα).

Several studies using MLRs have shown a significant decrease in lymphocyte proliferation as a result of co-culture with allogeneic MSCs. [16; 20-22] Early studies by Paterson et al. (2014) found MSCs caused a decrease in production of IL-6 and IFNγ by peripheral blood mononuclear cells (PBMCs). [21] Although these results are promising, neither study reported the haplotype of the allogeneic horses or the expression of major histocompatibility complex I and II by the MSCs.

Major histocompatibility complex are genes which encode cell surface proteins used to recognize and present foreign material. The MHC complex on a cell interacts with T cells. MHC I molecules are recognized by CD8+ T cells which cause destruction of cells through cellular immunity. [22] The MHC II molecules are recognized by CD4+ T cells and therefore result in acquired immunity. [22] The MHC region in horses is designated as equine leucocyte antigen (ELA). The ELA system is complex with greater than 300 blood types and approximately 15 distinct MHC I haplotypes established through serology.[23] With this in mind, a study by Ranera et al. (2016) selected horses with mismatched MHC I and MHC II haplotypes for performing MLRs. This important study showed that BMDMSCs of a mismatched haplotype were able to significantly reduce lymphocyte proliferation in vitro. [24] However, this study did not assess expression of MHC II by BMDMSCs. MHC II may be conditionally expressed on MSCs, and there are conflicting reports on the level of MHC II expression by MSCs. In some studies, MSCs have a low level of MHC II or lack MHC II expression. [14; 15] Other researchers have found a large variation in the expression of MHC II by MSCs. [17; 25] In vitro studies have shown if allogeneic haplotype-mismatched MSCs which express MHC II are mixed with lymphocytes they result in lymphocyte stimulation. [17] Further, these haplotype-mismatched MSCs may undergo cytotoxic cell death. [26] The seemingly simple solution would

be to screen MSCs for MHC II expression prior to use. However, MSCs may alter their MHC II expression depending on environmental exposure. The presence of IFNγ appears to increase the expression of MHC II by MSCs. [17; 25] However, the presence of interleukin-1β does not. [25] However, MHC I and MHC II expression may not be the only factor in immune recognition or immune evasion. A study by El Haddad et al. (2011) found mouse MSCs produce serine protease inhibitor which allows allogeneic MSCs to escape host surveillance. [27] Therefore, one could argue that in vitro assays may fall short in appropriately modeling these multiple, complex mechanisms of immune evasion; further, even if MHC II is expressed, MSCs may have other mechanisms for immune suppression.

Although in vitro results of exogenous cytokine stimulation of MSCs suggest the use of allogeneic MSCs could result in immune recognition and stimulation, it is important to recognize that in vitro conditions are contrived and may not mimic biological processes. Further, the complexity of the immune system and cell-cell interaction cannot be replicated in vitro. For example, when inflammatory synovial fluid was added to MSCs in culture there was no significant changes in MHC I or MHC II expression although exogenous cytokine administration (in the same study) increased MHC I and MHC II expression. [28] Regulation is highly complex and increases in MHC I or MHC II may also be compensated by an increase in gene expression of other immunoregulatory molecules. [29] Further, inflammation causes down-regulation of stem cell migration related genes and increases gene expression of cellular adhesion molecules in vitro. [28] This indicates the propensity of MSCs to localize to sites of inflammation. The connection between in vitro MSCs characteristics and in vivo activity has not been well established. Only a single study has correlated improved stemness as defined by trilineage differentiation and MHC I expression with a decrease in immunogenicity. [30] Those that argue

against the use of allogeneic MSCs due to immune recognition indicate that allogeneic MSCs may be removed from the body potentially resulting in decreased efficacy. [26] However, there is currently no data to suggest that MSCs persistence correlates with efficacy. In vivo studies investigating the use of allogeneic MSCs are imperative for understanding the complex interaction between allogeneic MSCs and their environment.

1.4 In vivo, experimental (pre-clinical), studies to investigate safety of allogeneic mesenchymal stem cells for clinical use

Initial in vivo studies used intravenous and subcutaneous administration to assess the equine immune system's reaction to allogeneic MSCs. Multiple intravenous injections resulted in no adverse clinical effects. [31] In the same study, bone marrow-derived allogeneic MSCs resulted in a peripheral increase in CD8+ T cells but there was no increase in splenic CD8+ T cells. The authors concluded that allogeneic MSCs were well tolerated and should be further explored. [31] Likewise, intravenous administration of equine cord blood-derived allogeneic MSCs resulted in no adverse clinical effects, no changes in serum cytokines, and no absolute changes in number of blood leucocytes or lymphocytes, but caused an increase in CD4+ and CD8+ T cells at 1 week post-administration. [32] Interestingly, however, when the same group examined intra-articular administration of equine cord blood-derived allogeneic MSCs, the cells resulted in a decrease in synovitis. [33] In conclusion, initial studies found allogeneic MSCs to be well tolerated with no adverse clinical effects but resulted in changes in lymphocyte subsets.

Other studies focusing on subcutaneous administration of MSCs have led to varying results. A study by Pezzanite et al. (2015) identified the formation of a "wheal" after allogeneic stem cell injection and the development of allo-antibodies; however, no comparison to autologous MSCs was made. [18] In another study by Carrade et al. (2011), no immediate or

delayed sensitivity reaction was seen after 2 intradermal injections, and there was no difference in the degree of wheal formation caused by autologous or allogeneic MSCs within the same individual. [34] Although subcutaneous administration allowed for assessment of allo-antibody production, subcutaneous injection of MSCs has little clinical relevance. Both studies described little to no adverse reactions to administration of allogeneic MSCs and, therefore, more clinically relevant studies were warranted.

Allo-antibody formation as reported by Pezzanite et al. (2015) was also reported in 37% horses after intravenous, intra-tendinous, intra-arterial and intra-ocular administration. [35] However, no adverse clinical responses could be attributed to the development of allo-antibodies. In addition, anti-bovine antibodies were identified in 89% of horses but, again, were not associated with adverse clinical events. [35] The formation of allo-antibodies indicates MSCs may be recognized by the equine host's immune system. However, without evidence of clinical effects, the clinical relevance of host recognition is to be questioned.

1.5 Intra-tendinous administration of allogeneic MSCs

There are a limited number of experimental trials that have been conducted using allogeneic MSCs in models of tendonitis, but the results have been promising (Table 1.1). An in vivo trial in normal superficial digital flexor tendon revealed no adverse or inflammatory reaction as a result of allogeneic MSC administration as measured on physical, morphological, thermography or ultrasonographic assessment. [36] Although in vitro studies have raised concern over allogeneic stem cell destruction due to immune response, allogenic stem cells were found in experimentally induced tendon lesions for 34 days post-implantation with no difference in the number or distribution of allogeneic MSCs when compared to autologous MSCs and there was no evidence of a cell mediated immune response to allogeneic MSCs. [37] These experimental

studies do not indicate efficacy but seem to suggest safety and persistence of cells. MHC II expression was not reported. However, allogeneic MSC implantation into normal tendons or induced tendon lesions did not result in a measurable inflammatory or immune response and allogeneic MSCs appeared to survive for over 30 days. Appropriately, these encouraging experimental studies were followed by clinical trials.

No clinical trials using allogeneic MSCs have reported adverse effects following treatment (Table 1.2). Studies using umbilical cord and adipose derived MSCs for tendonitis report a success rate of 77% and 89.5% respectively as defined by return to previous level of work. [38; 39] In addition, a study utilizing tenogenically-induced allogeneic blood MSCs found that 85.7% of horses had returned to their previous level of competition at 24 months post-injury; a meta-analysis showed that the prescribed cell based therapy had a lower re-injury rate than conventional therapies. [40] With no adverse events reported in studies of allogeneic MSCs use for the treatment of tendon and ligament disease and studies suggesting efficacy, the treatment appears to warrant further investigation and direct comparison to autologous MSCs use.

1.6 Intra-articular administration of allogeneic MSCs

A larger number of experimental trials have been reported using allogeneic stem cells in joints versus tendons and ligaments (Table 1.1). Intra-articular administration has multiple experimental advantages including the ability to non-invasively and serially collect joint fluid for examination, ability to record response to flexion, and to measure joint distention as well as lameness. For these reasons, intra-articular administration studies routinely use a combination of clinical signs (lameness, effusion, response to flexion) and synovial cytologic data (nucleated cell count, differential, total protein, synovial cytokines) to measure immunologic response.

A few experimental trials have evaluated the response of the normal joint to allogeneic stem cells with slightly differing results. An initial study by Carrade et al. (2011) found no difference in the clinicopathologic findings following intra-articular administration of allogeneic versus autologous MSCs. [41] In contrast, Pigott et al. (2013) found an increase in joint inflammation reflected by an increase in nucleated cell count and total protein at 24-48 hours post-injection with allogeneic MSCs compared to autologous MSCs. [42] However, the arthroscopic examination and synovial biopsy of these joints at 60 days found no evidence of articular pathology and the remaining synovial monocytic inflammation was no different between the autologous versus allogeneic MSCs treatment groups. [43] These initial studies differed regarding an increased nucleated cell count at 24-48 hours, but neither identified a marked nor sustained inflammatory response to administration of autologous versus allogeneic BMDMSCs. As none of these studies assessed efficacy, it is unclear whether a transient increase in inflammation is detrimental to the efficacy of allogeneic MSCs. However, no marked clinical adverse events were recorded. Therefore, additional studies in allogeneic MSCs safety together with efficacy is warranted.

Some researchers and clinicians have raised concerns over an induced immune response to allogeneic MSCs following repeated MSCs injections. However, studies that have evaluated the immunological consequences of multiple doses of allogeneic MSCs have not identified adverse clinical events. Analysis of synovial fluid cellular responses to allogeneic MSCs injection have yielded conflicting results. For example, in one study, lymphocytes isolated from synovial fluid from allogeneic MSCs treated animals was re-exposed to allogeneic MSCs and there was no immune response to re-exposure. [43] A study comparing the equine joint response to allogeneic and autologous MSCs found the second injection of allogeneic MSCs resulted in

less clinical synovitis, compared to that of a second injection of autologous MSCs. [44] In contrast, another study found a significant but transient increase in nucleated cell count following the second injection of allogeneic stem cells when compared to a second injection of autologous MSCs. [45]

Differences in study design make these results difficult to directly compare and interpret. Different doses of MSCs were administered, and different joints were used. For example, Ardanaz et al. (2016) administered 25 million pooled-allogeneic MSCs into the antebrachiocarpal joint, and the comparison was made to autologous MSCs administered into the tibiotarsal joint. [44] Although using the same horses removed inter-horse variability in the response to intra-articular administration of MSCs therapies, different joints are known to react with variability to the same inflammatory stimuli. [46] Joswig et al. (2017) administered 10 million MSCs (allogeneic versus autologous) into metacarpophalangeal joints. [45] In this study, the treatments were administered into the fetlock joint of different horses. By using the same joint for administration of MSCs, the authors controlled for potential differences in an individual joint's response to intra-articular administration but inter-horse variability to intra-articular administration was not controlled. An additional concern in comparing studies is treatment preparation and culturing or administration of MSCs with xenogenic proteins. MSCs are routinely culture expanded with the use of fetal bovine serum. Administration of cells grown in FBS resulted in the development of anti-bovine antibodies in 89% of horses, [35] as well as increased pain and elevated nucleated cell counts when administered intra-articularly. [45] A 24-hour culture period without xenogenic protein (FBS) is necessary for removal of xenogeneic proteins prior to treatment administration. [45] However, this is rarely performed and varies between studies which adds an additional variable. Despite difficulty in directly comparing these

various intra-articular, pre-clinical studies, the only reported potentially adverse reaction was a transient increase in nucleated cell count in one study following the second administration of allogeneic MSCs. Further, despite this transient increase in nucleated cell count, no adverse clinical reaction was recorded. These results are compelling for further examination of allogeneic MSCs for intra-articular use. Future studies should evaluate allogeneic and autologous MSCs while controlling for inter-horse variation in response to intra-articular treatments, variations in individual joint responses to treatments, and the presence or absence of xenogeneic proteins within the MSCs treatment.

1.7 Effect of allogeneic MSCs in experimentally induced inflammatory joints

There is in vitro evidence that MSCs may act differently in or be primed by inflammatory environments. [17; 25] Therefore, when assessing the safety and potential efficacy of allogeneic MSCs, experimental models of inflammation may be particularly informative. Models examined include the use of Lipopolysaccharide (LPS), recombinant interleukin-1β or amphotericin B to induce severe inflammation and model osteoarthritic processes. Only three studies have examined the effect of MSCs in an in vivo inflammatory joint environment; each used a different method to induce inflammation. When umbilical cord blood derived MSCs were administered into joints subjected to LPS, allogeneic MSCs resulted in a significant reduction in inflammation. [33] Likewise, when allogeneic MSCs were used in an amphotericin-B model of joint inflammation, clinical and synovial inflammatory signs were significantly reduced, and a second injection of allogeneic cells yielded no adverse reactions. [47] Neither study compared the effect to autologous MSCs; therefore, a difference in efficacy between autologous and allogeneic MSCs could not be elucidated. Therefore, both studies reported anti-inflammatory effects of allogeneic MSCs and there were no adverse effects with allogeneic MSC use. These studies

support the safety of allogeneic MSCs use in inflammatory joints and urge further clinical investigation.

1.8 Allogeneic MSCs in clinical trials

Several clinical trials using allogeneic MSCs have already been reported (Table 1.2). No clinical trials report any significant adverse effects, and many indicate efficacy. The authors are aware of 4 studies using allogeneic MSCs for tendon and ligament disease. Each study utilized a different cell source. Umbilical cord derived allogeneic MSCs administered as 2-10 million per lesion resulted in a 77% return to work. [38] A study utilizing adipose-derived MSCs resulted in an 89.5% return to previous level of competition. [39] Lange-Consiglio et al. (2013) compared the use of allogeneic amnionic derived MSCs versus BMDMSCs for tendon injury; amnion-derived MSCs resulted in a better clinical outcome then BMDMSCs, but neither allogeneic cell source led to significant adverse effects. [48] Further, despite concern over the possible increased immunogenicity of pre-conditioned allogeneic MSCs, tenogenically-induced allogeneic blood derived MSCs in PRP were shown to reduce the injury rate 2 years post-treatment. [40] Although all results are positive and no adverse reactions were recorded, none of the mentioned studies compared the efficacy of allogeneic MSCs to autologous MSCs.

Clinical trials using intra-articular allogeneic MSCs studies are less common. The authors are aware of two clinical studies reporting intra-articular allogeneic MSCs. Both studies were designed to gain approval for an allogeneic cellular product for commercialization. In the first study, 39 horses were administered allogeneic adipose-derived MSCs for the treatment of osteoarthritis; this was a carefully constructed parallel-group, blinded, randomized and controlled clinical trial which revealed a significant reduction in lameness even 90 days following treatment. Importantly, no adverse effects were reported. [49] The second study utilizing intra-

articular allogeneic MSCs evaluated chondrogenically-induced blood-derived MSCs. This study was a randomized, multi-center, double-blinded and placebo-controlled study in which 50 horses received allogeneic MSCs in equine allogeneic plasma and resulted in extremely promising results; in weeks 3 to 18, treated animals had decreased lameness scores, decreased flexion test responses and decreased effusion scores compared to the placebo control. In addition, more treated horses had returned to their previous level of work at 1 year post-treatment. [50] However, again, neither study compared the treatment effect between allogeneic MSCs and autologous MSCs.

1.9 Conclusions

Despite early controversy over whether allogeneic MSCs induce harmful inflammatory responses, the preponderance of evidence suggests that in fact allogeneic MSCs are no more inflammatory than autologous MSCs in the context of treating musculoskeletal injuries. In support of this conclusion, both experimental and clinical trials utilizing allogeneic MSCs have yielded largely, positive results. Even with the promising clinical results, the question of whether allogeneic MSCs are more or less safe and effective then autologous MSCs has been left unanswered due to a lack of direct comparative studies of autologous to allogeneic MSC use. Therefore, carefully designed in vitro studies and in vivo comparisons of allogeneic and autologous MSCs are warranted.

Table 1.1. Experimental (preclinical) in vivo trials utilizing allogeneic MSCs.

Study reference	**Cell type**	**Study type**	**Sample size (per group)**	**Experimental or Clinical Study?**	**Outcome measures**	**Were adverse effects reported?**	**Were favorable effects reported?**
TENDON/ LIGAMENT							
Brandão et al. (2018)	Allogeneic adipose- derived MSCs	Randomized, controlled, double-blinded	n=6	Experimental	Clinical, morphology, thermography, ultrasound	No	Not assessed

Guest et al. (2008)	Allogeneic bone marrow-derived MSCs	Controlled	n=1 limb/treatment	Experimental	Post-mortem, histopathology	No	Not assessed
INTRA-ARTICULAR							
Broeckx et al. (2019)	Allogeneic chondrogenic-induced MSC (from peripheral blood)	Randomized, double-blinded, placebo-controlled	n=6	Experimental (osteochondral fragment-groove model)	Clinical, synovial fluid cytology/ biomarkers	No	Yes
Barrachina et al. (2018)	Allogeneic bone marrow-derived MSC	Randomized, controlled, double blinded	Treated, n=7 Control, n=4	Experimental (Amphotericin-B)	Clinical, synovial, imaging	No	Yes
Mariñas-Pardo et al. (2018)	Allogeneic adipose derived MSC	Randomized, multicenter, placebo-controlled, double-blinded	Safety study, n=8	Safety: Experimental	Safety: Clinical, synovial, hematology Efficacy: Clinical	No	Yes
Broeckx et al. (2018)	Allogeneic chondrogenically induced MSCs (from peripheral blood)	Randomized, double blinded, placebo controlled	Treated, n=8 Control, n=8	Experimental	Clinical, hematology, biochemical analysis, post-mortem histology, immunochemistry	No (same effect at saline)	Not assessed
Joswig et al. (2017)	Allogeneic bone marrow-derived MSC	Randomized, controlled study	Treated, autologous, n=6 Treated, allogeneic, n= 6	Experimental	Clinical, synovial	Yes, increasing NCC after second injection	Not examined
Ardanaz et al. (2016)	Allogeneic bone marrow-derived MSCs	Randomized, controlled, double-blinded	n= 6 limbs/treatment	Experimental	Clinical, synovial, ultrasound	No	Yes
Williams et al. (2016)	Allogeneic umbilical cord blood derived MSC	Randomized, blinded	n=6 limbs/treatment	Experimental	Clinical, synovial	No	Yes
Pigott et al. (2013)	Allogeneic bone marrow-derived MSC + platelet rich plasma	Randomized, controlled, blinded	n= 6 limbs/treatment	Experimental	Clinical, synovial fluid	Yes, greater inflammatory response for allogeneic MSC	Not examined
Carrade et al. (2011)	Autologous and allogeneic placentally-derived MSC	Randomized, controlled, blinded	Treated, allogeneic, n=16 limbs Treated, autologous, n=11 limbs	Experimental	Clinical, synovial fluid	No	Not examined

Table 1.2. Clinical trials using allogeneic MSCs.

Study reference	**Cell type**	**Study type**	**Sample size (per group)**	**Experimental or Clinical Study?**	**Outcome measures**	**Were adverse effects reported?**	**Were favorable effects reported?**
TENDON/ LIGAMENT							
Beerts et al. (2017)	Allogeneic tenogenically induced MSCs (from peripheral blood) + platelet rich plasma	Uncontrolled study	Treated, SL, n=68; treated, SDFT, n=36	Clinical	Clinical	No	Yes
Vandenberghe et al. (2015)	Allogeneic tenogenically induced MSC	Single case report	n=1 horse	Clinical	Clinical, ultrasound	No	Yes

	(from peripheral blood)						
Lange-Consiglio et al. (2013)	Autologous bone marrow-derived MSCs and allogeneic amnion derived MSCs	Randomized	Amnion, n=51, bone marrow, n=44	Clinical	Clinical, ultrasound	No	Yes
INTRA-ARTICULAR							
Broeckx et al. (2019)	Allogeneic chondrogenic-induced MSC (from peripheral blood)	Randomized, multicenter, double-blinded, placebo-controlled	Treated, n=75, Control, n=25	Clinical	Clinical	No	Yes
Mariñas-Pardo et al. (2018)	Allogeneic adipose derived MSC	Randomized, multicenter, placebo-controlled, double-blinded	Efficacy trial, treated, n= 39, Control, n=33	Clinical	Safety: Clinical, synovial, hematology Efficacy: Clinical	No	Yes
Van Loon et al. (2014)	Allogeneic umbilical cord blood derived MSC	Retrospective	n=52	Clinical	Clinical	No	Yes

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Chapter 2: Research Overview and Specific Aims

2.1 Research Overview

Mesenchymal stem cells (MSCs) hold much promise in equine orthopedics. Evidence is mounting regarding the benefits this population of cells provides in treatment of tendonopathy, desmitis, and cartilage repair. [1-3] Although there are many sources of MSCs including adipose, umbilical cord blood or tissue, placental, muscle and tendon, the most intensively studied and clinically utilized in the horse currently are bone marrow-derived MSCs (BMDMSCs). BMDMSCs are easily collected from either the ilium or the sternum and subsequently expanded over a period of 2-4 weeks, at which time an average of 10 to 20 million BMDMSCs are administered to the diseased tissue. While culture expansion techniques have improved in efficiency over the last several years and reports of ideal small volumes of aspirates have somewhat reduced expansion times, the delay from aspiration to administration of treatment remains a short coming of autologous, culture expanded BMDMSCs. [4-7] Furthermore, the expense of individual culture expansion due to materials and labor result in costs that, for some, render this treatment cost prohibitive.

Although studies have been conducted using autogenous and allogeneic cells, limited studies have examined immune properties of allogeneic BMDMSCs, and no published studies have closely compared the immune properties of allogeneic and autogenous BMDMSCs in vitro. Further, few studies have directly compared the response of the normal joint to autologous and allogeneic BMDMSCs while controlling for inter-horse variability, and no studies have compared the inflamed joint response to autologous and allogeneic BMDMSCs in a recombinant interleukin-1β (rIL-1β) model of synovitis.

This information would be valuable to clinicians in that an "off the shelf" therapy of BMDMSCs may offer a point of care treatment without the delay associated with culture expansion as well as a potentially more affordable therapy. Therefore the goals of the research described in this dissertation include to: 1) determine whether there are differences in immunogenicity between autologous and allogeneic BMDMSCs using both in vitro and in vivo assays; 2) compare the immunosuppressive properties of autologous and allogeneic BMDMSCs and elucidate underlying mechanism(s) by which BMDMSCs generate immunosuppressive properties in vitro; 3) determine whether autologous and allogeneic BMDMSCs elicit different inflammatory responses following intra-articular (IA) injection in horses; and 4) determine whether autologous and allogeneic BMDMSCs elicit different responses or have a difference in suppressing inflammation following intra-articular administration in a rIL-1β model of synovitis. Chapter 3 investigates the immunosuppressive ability of autologous and allogeneic BMDMSCs and the mechanism by which immunosuppression is achieved in vitro. Chapter 4 examines the normal equine joint response to allogeneic and autologous BMDMSCs. Chapter 5 describes a preliminary study to properly develop the in vivo rIL-1β model for comparing autologous and allogeneic BMDMSCs. Specifically, the study compares the middle carpal joint and tibiotarsal joint response to the same dose of rIL-1β. Results of this investigation influenced the study design described in Chapter 6. Finally, Chapter 6 explores the inflamed joint response to allogeneic and autologous BMDMSCs.

2.2 Specific Aim 1 (Chapter 3: Autologous and Allogeneic Equine Mesenchymal Stem Cells Exhibit Equivalent Immunomodulatory Properties In Vitro)

BMDMSCs are known to have immune suppressive effects. These immune suppressive or anti-inflammatory effects are likely to add to the clinical value and efficacy of these cells. Although equine allogeneic BMDMSCs have been evaluated in multiple studies using modified mixed leucocyte reactions, no study has directly compared the immunosuppressive ability of allogeneic and autologous BMDMSCs. [8; 9] Therefore, the specific aim of this chapter was to compare the immunogenic and the immunosuppressive properties of autologous BMDMSCs to allogeneic BMDMSCs in vitro and identify a mechanistic explanation for their immunosuppressive properties.

2.3 Specific Aim 2 (Chapter 4: Allogeneic versus autologous intra-articular mesenchymal stem cell injection within normal horses: clinical and cytological comparisons suggest safety)

The potential clinical advantages of using allogeneic BMDMSCs are many including timing of administration and selection of cells with desired characteristics. However, allogeneic BMDMSCs must be proven to be safe for intra-articular administration prior to clinic use. The few studies that have examined the effect of the intra-articular administration of allogeneic stem cells have administered allogeneic stem cells alone or have evaluated allogeneic versus autologous cells in different cohorts of animals. [10-12] This can be difficult to interpret, as individual reactions to intra-articular treatments can vary significantly horse to horse. In addition, individual joints may react differently to intra-articular medications. [13] Therefore, studies which compare allogeneic and autologous treatments administered within the same individual but into different joints can also be difficult to interpret. [14] The specific aim of this chapter was to compare the clinical and synovial fluid response of the normal equine joint to autologous and

pooled-allogeneic BMDMSCs while controlling for individual variation and joint variations in response to intra-articular injections.

2.4 Specific Aim 3 (Chapter 5: Induction of synovitis using interleukin-1 beta: are there differences in the response of middle carpal joint compared to the tibiotarsal joint?)

Interleukin-1β (IL-1β) is an inflammatory cytokine present in naturally-occurring osteoarthritis. [15; 16] Equine recombinant interleukin-1β (rIL-1β) is readily available from a commercial vendor and results in a potent but short-term synovitis when administered intra-articularly. [17-20] However, when comparing the literature, it appears that administration into different joints may lead to diverse degrees of response. [17; 18; 20] Further, no previous studies have described the cytological response to rIL-1β in the tibiotarsal joint. Therefore, the specific aim of this chapter was to describe cytological and clinical responses of the tibiotarsal joint to the administration of 75ng of rIL-1β and to compare the clinical and cytological responses of the tibiotarsal joint versus middle carpal joint when administered the same amount of rIL-1β.

2.5 Specific Aim 4 (Chapter 6: Allogeneic and autologous equine bone marrow-derived mesenchymal stem cells are safe but ineffective following a single and repeated intra-articular injections for reducing acute inflammation in an experimental interleukin-1β model of synovitis)

MSCs are influenced by their local tissue environment. In vitro studies suggest that inflammatory proteins such as interferon gamma may cause increased expression of MHCII and potentially result in immune recognition of allogeneic BMDMSCs. [21; 22] In contrast, other studies have suggested that priming BMDMSCs with exposure to inflammatory cytokines may cause BMDMSCs to have a more anti-inflammatory phenotype. [23; 24] Therefore, it is imperative that the safety of allogeneic BMDMSCs be evaluated in both the normal joint and the

inflamed joint environment. In addition, pre-clinical studies modeling joint inflammation may help determine the efficacy of allogeneic and autologous BMDMSCs for joint disease in the horse. Therefore, the specific aim of this chapter was to compare the inflamed joint response to autologous versus allogeneic BMDMSCs injections, and to determine if either treatment generated an anti-inflammatory effect.

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Chapter 3: Autologous and Allogeneic Equine Mesenchymal Stem Cells Exhibit Equivalent Immunomodulatory Properties In Vitro[2]

3.1 Overview

The use of allogeneic bone marrow-derived mesenchymal stem cells (BMDMSCs) may provide an effective alternative to autologous BMDMSCs for treatment of equine musculoskeletal injuries. However, concerns have been raised regarding the potential safety and effectiveness of allogeneic BMDMSCs. We conducted studies to assess the immunological properties of equine allogeneic BMDMSCs compared to those of autologous BMDMSCs. For assessment of inherent immunogenicity, the relative ability of allogeneic and autologous BMDMSCs to stimulate spontaneous proliferation of equine lymphocytes was compared. The immune suppressive activity of the two cell types was evaluated by adding autologous or allogeneic BMDMSCs to activated lymphocytes and assessing suppression of lymphocyte proliferation and IFNɣ production. Fifty-six allogeneic and 12 autologous combinations were evaluated. Studies were also done to elucidate mechanisms by which equine MSCs suppress lymphocyte function. Potential mechanisms evaluated included production of prostaglandin E_2 (PGE_2), nitric oxide, transforming growth factor-beta (TGFß), and Indoleamine 2,3-dioxygenase. We found that autologous and allogeneic BMDMSCs both induced mild but equivalent levels of

[2] The first aim of this work was to compare the immunogenic and the immunosuppressive properties of autologous BMDMSCs to allogeneic BMDMSCs in vitro and identify a mechanistic explanation for their immunosuppressive properties. This chapter includes the complete published manuscript for this aim, Autologous and allogeneic equine mesenchymal stem cells exhibit equivalent immunomodulatory properties in vitro (Aimee C. Colbath, Steve W. Dow, Jennifer N. Phillips, C. Wayne McIlwraith, Laurie R. Goodrich, *Stem Cells and Development*, Vol. 26, Issue 7, 2017). My contributions to this publication included performing laboratory assays, statistical analysis and the writing the majority of the manuscript. Minimal modifications were made to meet formatting requirements and table and figure numbers have been modified. This article is reproduced with permission from Mary Ann Liebert, Inc., New Rochelle, NY.

spontaneous lymphocyte activation *in vitro*. *In vitro* assays assessing the ability of BMDMSCs to suppress activated lymphocytes, both allogeneic and autologous BMDMSCs suppressed T cell proliferation and IFNɣ production to an equal degree. The primary mechanism of equine BMDMSCs suppression of T cells was mediated by prostaglandin E_2. We concluded that allogeneic and autologous BMDMSCs are equivalent in terms of their immune modulatory properties, and stimulated peripheral blood mononuclear cells (PBMCs) appear to trigger the immune suppressive properties of MSCs. Therefore, both cell types appear to have equal potency in modulating inflammatory processes related to acute or chronic musculoskeletal injuries in the horse.

3.2 Introduction

Musculoskeletal injuries cause extensive morbidity and mortality in the equine industry with 23-36% of racehorses sustaining a musculoskeletal injury each year. [1] These tissues are slow to heal and bone marrow-derived mesenchymal stem cells (BMDMSCs) have shown promise as a treatment for tendonopathy, desmopathy and joint injury in racehorses, sport horses and western performance horses. [2-4] An *in vivo* study suggested these cells may travel to the site of damaged tissue and demonstrated their ability to localize to joint structures including cruciate ligaments, menisci and cartilage. [5]

There are many sources of equine mesenchymal stem cells (MSCs), but BMDMSCs are by far the most commonly used as a clinical treatment in the horse. [6; 7] BMDMSCs can be easily collected from either the ilium or the sternum of horses, using minimal restraint or sedation. A recent publication by Lombana *et al.* (2015) was unable to identify a difference in cell characteristics between BMDMSCs from iliac and sternal origin. [8] BMDMSCs for clinical use are typically culture expanded over a period of 2-4 weeks, at which time 1 to 2 X 10^6 cells

BMDMSCs are typically administered to diseased tissues (eg, joints or injured tendons or ligaments). [6]

When autologous BMDMSCs are administered intra-articularly, the risk of immune reaction is minimal but not negligible (~9% rate of reactions). [7] Likewise, a study utilizing intra-articular allogeneic MSCs reported a low occurrence of joint inflammation subsequent to injection (1.8%). [9] Culture expansion of autologous BMDMSCs for clinical use has multiple clinical disadvantages including the delay in obtaining cells, the expense of the procedure, and the effects of donor age on MSC functionality. [10; 11] Expansion techniques have improved in efficacy over the last several years, and studies have suggested that only small volumes of bone marrow aspirate are necessary. [12] However, the delay from aspiration to administration of the treatment remains a short-coming of autologous, culture expanded BMDMSCs. [11-13] In addition, autologous bone marrow expansion is costly due to high labor and material costs.

A readily available source of allogeneic BMDMSCs would allow clinicians to choose the optimal time of injection following injury without the restriction of individual culture and expansion. In addition, a cryopreserved supply of BMDMSCs could reduce material costs and labor associated with cell expansion. Finally, there is speculation in the regenerative medicine field that MSCs from aged or injured patients may have reduced regenerative abilities. [10; 11] Therefore, allogeneic BMDMSCs obtained from young, healthy animals may provide a more optimal cell source for routine clinical usage. Moreover, thorough cell characterization and quality controls are possible with allogeneic MSCs.

Allogeneic BMDMSCs may have several potential advantages but further research is necessary to ensure that allogeneic BMDMSCs are safe and effective for clinical use. Evidence is mounting that BMDMSCs have potent anti-inflammatory and chondroprotective effects. [14;

15] Experimental osteoarthritis studies have demonstrated an increase in comfort level after administration of BMDMSCs. [14] Likewise, a rabbit model of osteoarthritis using anterior cruciate ligament transection (ACLT) revealed less cartilage loss, decreased surface abrasion, increased cartilage content and improved histologic scores when rabbits were administered intra-articular allogeneic BMDMSCs. [15] In terms of their anti-inflammatory properties, it is important to determine if allogeneic BMDMSCs produce the same degree of immune modulation as that of autologous, as this could be an important characteristic for clinical use. [16] Guest et al. (2008) assessed the safety and survival of allogeneic MSCs in surgically induced lesions of the equine superficial digital flexor tendon, and reported no detectable difference in the apparent immunogenicity of autologous versus allogeneic BMDMSCs. [17] Intra-articularly administered allogeneic umbilical cord blood - derived MSCs also appear to reduce inflammatory infiltrate in LPS stimulated joints, [18] though there was no comparison with autologous MSCs in this study.

Clinically, the use of allogeneic MSCs has been described in studies by Broeckx et al. (2014) and Van Loon et al. (2014) where clinical cases of equine degenerative joint disease and equine tendon and ligament injury were treated with allogeneic MSCs and minimal adverse effects were reported. [9; 19-21] Likewise, a phase II clinical trial using allogeneic BMDMSCs in human patients with knee osteoarthritis has reported promising results of increased comfort and cartilage health. [22] Furthermore, fifty-five people received allogeneic MSCs following partial medial meniscectomy resulting in significantly increased meniscal volume and increased comfort compared to a hyaluronate control. [23] A number of *in vitro* studies in other species indicate clearly that allogeneic BMDMSCs are immune modulatory. [24-35] However, studies to directly compare the immune modulatory properties of autologous and allogeneic equine BMDMSCs have not, to our knowledge, been reported previously.

Therefore, the aim of the current study was to compare the immunomodulatory properties of autologous and allogeneic equine BMDMSCs, and to identify mechanism(s) for the immune suppressive properties of allogeneic BMDMSCs.

3.3 Materials and Methods

The experimental protocol described complied with the policies of the Institutional Animal Care and Use Committee at Colorado State University (Protocol 12-3483).

<u>Bone marrow collection and stem cell expansion.</u> Sternal bone marrow aspirates were collected from adult horses using 1000U/ml of heparin as previously described. [13] Nucleated cells were then maintained on polystyrene culture flasks in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1M HEPES buffer in 5% CO_2 at 37°C.

Once stem cell colonies (MSCs) were established, cells were harvested using Accumax™ cell dissociation solution (Sigma-Aldrich®, St. Louis, MO) as per the manufacturer's instructions. Cells were then maintained in culture in Minimum Essential Medium (MEM) Alpha Medium (αMEM) with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1M HEPES buffer in 5% CO_2 at 37°C through a second passage. BMDMSCs were then cryopreserved in freeze media consisting of 95% fetal bovine serum and 5% dimethyl sulfoxide at a concentration of $10x10^6$cells/ml.

<u>Blood collection and preparation of PBMC.</u> Equine peripheral blood mononuclear cells (PBMCs) were prepared from whole, anticoagulated blood collected by jugular venipuncture and density gradient centrifugation as previously described. [25] PBMCs were cryopreserved in 95% fetal bovine serum and 5% dimethyl sulfoxide at a concentration of $10x10^6$ cells/ml and stored in

liquid nitrogen prior to use. For analysis, PBMCs were labeled with mouse anti-horse CD5 (Clone CVS5; Abd Serotec®, Raleigh, NC) and mouse anti-human CD3 (Clone CD3-12; Abd Serotec®, Raleigh, NC), and secondarily labeled with anti-mouse FITC (Abd Serotech®, Raleigh, NC). CD3 and CD5 analysis was used to create a gate for lymphocytes. This gate was then used to select lymphocytes in future assays.

Lymphocyte proliferation assay. MSCs were recovered from cryopreservation and cultured in DMEM with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1M HEPES buffer in 5% CO_2 at 37°C. MSCs were then harvested and placed in a 48 well plate at a concentration of 50,000 cells per well and cultured overnight. At least 12 hours following plating of the BMDMSCs, PBMCs were removed from liquid nitrogen, thawed in a 37°C hot water bath, and washed with phosphate buffered saline. PBMCs used as negative controls were treated with1µM hydroxyurea for 1 hour prior to labeling with carboxyfluorescein succinimidyl ester (Cell Trace™; Thermo Fisher Scientific, Waltham, MA). All other PBMCs were immediately labeled with 5µM CFSE and added to MSCs at ratios of 1 MSC per 10 PBMCs, 1 MSC per 50 PBMCs, 1 MSC per 100 PBMCs, 1 MSC per 500 PBMCs, 1 MSC per 1000 PBMCs, and 1 MSC per 2000 PBMCs. 4 allogeneic and 4 autologous combinations were analyzed for each ratio of MSCs to PBMCs. All cells were incubated in DMEM with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1M HEPES buffer for 4 days in 5% CO_2 at 37°C. Cell co-cultures were maintained for 4 days. Flow cytometry was performed using the CyAN™ ADP flow cytometer (Beckman Coulter®, Brea, CA) to measure lymphocyte cell proliferation using CFSE staining by FITC fluorescence. Fluorescence data was analyzed using Summit Software (Beckman Coulter®, Brea, CA).

MSC titration for suppression of lymphocyte proliferation. MSCs were recovered from cryopreservation and cultured in DMEM with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1M HEPES buffer in 5% CO_2 at 37°C. MSCs were then harvested and placed in a 48 well plate at a concentration of 50,000 cells per well and cultured overnight. At least 12 hours following plating of the BMDMSCs, PBMCs were removed from liquid nitrogen, thawed in a 37°C hot water bath, and washed with phosphate buffered saline. PBMCs used as negative controls were treated with 1µM hydroxyurea for 1 hour prior to labelling with CFSE. All other PBMCs were immediately labeled with 5µM CFSE and stimulated with 2.5µg/ml concanavalin A. MSCs were added to PBMCs at a ratio of 1 MSC per 10 PBMCs, 1 MSC per 100 PBMCs, and 1 MSC per 1000 PBMCs. 56 allogeneic and 12 autologous combinations were evaluated. During the last 4 hours of incubation, the co-cultures were treated with 25 ng/ml Phorbol 12-myristate 13-acetate, 1uM Ionomycin (Sigma-Aldrich, St. Louis, MO) and 10ng/ml Brefeldin A (BioLegend®, San Diego, CA). After which the cells were fixed in 2% paraformaldehyde. Cells were then permeabilized with 1% saponin buffer prior to staining using an intracellular mouse anti-bovine IFNɣ:Alexa Fluor®647 (Clone CC302; AbD Serotech®, Raleigh, NC). Flow cytometry was performed using the CyAN™ ADP flow cytometer (Beckman Coulter®, Brea, CA) to measure lymphocyte cell proliferation using CFSE staining by FITC fluorescence and intracellular IFNɣ production by APC647 fluorescence. Fluorescence data was analyzed using Summit Software (Beckman Coulter®, Brea, CA).

Assessment of mechanism of lymphoycte suppression by MSC. MSCs were recovered from cryopreservation and cultured in DMEM with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1M HEPES buffer in 5% CO_2 at 37°C. MSCs were plated at a concentration of 50,000 cells per well in a 48 well plate overnight. Allogeneic PBMCs were collected the

following day by jugular venipuncture and processed using density gradient centrifugation. [25] Cells were used immediately without cryopreservation. PBMCs used as negative controls were treated with 1µM hydroxyurea for 1 hour prior to labeling with CFSE. All other PBMCs were immediately labeled with 5µM CFSE and stimulated with concanavalin A (2.5µg/ml). MSCs were tested at various ratios of MSC:PBMCs (1:5, 1:10, 1:100, 1:1000) to identify an optimal ratio, which was determined by be 1:10. To assess mechanisms of MSC inhibition of T cells, known biochemical inhibitors of immune suppressive pathways in other species were evaluated. [16; 34-36] The inhibitors were used at previously published concentrations for indomethacin, [16] L-NMMA, [32] and 1-MT [33] and at two times the IC50 for SD208. [36]

Inhibitor concentrations included 10µM indomethacin (PGE_2 inhibitor, Sigma-Aldrich®, St. Louis, MO), 1mM 1-Methyl-D-tryptophan (1-MT, indoleamine inhibitor, Sigma-Aldrich®, St. Louis, MO), 300µM N^G-Monomethyl-L-arginine (L-NMMA,nitric oxide inhibitor, Abcam, Cambridge, MA) and 0.1µM SD208 (TGFβ inhibitor, Sigma-Aldrich®, St. Louis, MO).

After 96 hours in culture the supernatants were removed from each of the wells and stored at -80°C for analysis. The cells were then fixed with 2% paraformaldehyde. Flow cytometry was performed using the Gallios™ flow cytometer (Beckman Coulter®, Brea, CA) to measure lymphocyte cell proliferation using CFSE staining by FITC fluorescence. Fluorescence data was analyzed using FlowJo (Version 10.0.8, Ashland, OR).

ELISA assay for PGE_2. PGE_2 levels in the supernatants of the untreated MSCs and PBMC co-culture as well as the wells treated with indomethacin were evaluated using the Prostaglandin E_2 Parameter Assay Kit (R&D Systems® Minneapolis, MN) as per the

manufacturer's recommendations. Samples were read using a microplate reader (Molecular Devices®, SpectraMax M3, San Jose, CA) at 540 nm and quantified with a standard curve.

MHCII expression of MSCs exposed to lymphocyte conditioned media. This assay was performed to analyze the effects of MSCs exposure to PBMC conditioned media from stimulated or unstimulated PBMCs, on expression of MHCII by MSCs. Allogeneic PBMCs were collected by jugular venipuncture and processed using density gradient centrifugation. [25] 500,000 PBMCs were added to each well of a 48 well plate. PBMCs were cultured in DMEM with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1M HEPES buffer in 5% CO_2 at 37°C. Stimulated PBMCs were exposed to10µg/ml of concanavalin A for 96 hours. After 96 hours, supernatants were collected from both stimulated and unstimulated PBMC wells.

Four BMDMSC cell lines, at passage 3, were recovered from cryopreservation and cultured in DMEM with 10% fetal bovine serum (Hyclone, Sigma-Aldrich®), 1% penicillin-streptomycin, and 1M HEPES buffer in 5% CO_2 at 37°C. When MSCs were 70-80% confluent they were dissociated from culture using Accumax™ and resuspended in a single cell solution at 1x10^6 cells per ml of media (DMEM with 1% HEPES buffer). Cells were then added to a 96 well plate at a seeding density of 25,000 cells per well. Cells were allowed to attach overnight. The following day, cells were exposed to conditioned media from previously unstimulated or stimulated PBMC cultures. Conditioned media was diluted with control media to concentrations of 0% (control), 10%, 50%, and 100% conditioned media. After 96 hours, supernatants were removed and all wells were washed with phosphate buffered solution prior to dissociation using Accumax™. Cells were resuspended in complete media prior to two concurrent washes and staining with a monomorphic mouse anti-horse MHCII:RPE antibody (Clone CVS20; BioRad®, Hercules, CA). All wells were blocked with 5% equine serum for 10 minutes prior to antibody

exposure. Mouse IgG1: PE (Clone IS5-21F5; Miltenyl Biotech, Cambridge, MA) was utilized as an isotype control. Cells were analyzed for MHCII expression using the FL2 channel. Results were reported as the % of cells expressing MHCII.

Statistical Analysis. The continuous data was evaluated for normality. Immunogenicity data was evaluated using a non-parametric test due to low sample size. A Mann-Whitney test was used to compare immunogenicity outcomes to the PBMC positive and negative controls. Likewise, immune suppression proliferation and interferon gamma data were found to be non-normal and were evaluated using a Mann-Whitney test in order to compare proliferation of PBMCs co-cultured with MSCs and the positive control (stimulated lymphocytes). For evaluating the cell mechanism data, a Kruskal-Wallis test was used with a Dunn's Multiple Comparisons Test. Finally, MHCII expression was evaluated using a two-way ANOVA with Tukey's multiple comparison test. gMFI data was found to be non-normal and was compared using a Mann Whitney test. A P-value of 0.05 was used to determine statistical significance. GraphPad Prism v 6.0 (La Jolla, CA) was used to perform the statistical analysis.

3.4 Results

Relative immunogenicity of allogeneic and autologous MSC. To assess the ability of MSCs to stimulate spontaneous proliferation of resting T cells, both allogeneic and autologous MSCs were co-cultured with PBMCs at ratios of 1 MSC to 10 PBMC, 1 MSC to 50 PBMC, 1 MSC to 100 PBMC, and 1 MSC to 1000 PBMC for 96 hours, and T cell proliferation assessed by flow cytometry. We observed a small but statistically significant increase in lymphocyte proliferation when both autologous and allogeneic MSCs were cultured with T cells at a ratio of 1MSC per 10 PBMC ($P<0.05$) (Figure 3.1). Importantly, however, the degree of T cell proliferation induced by autologous MSCs was not statistically different from that of allogeneic

MSCs. Similar results were observed at other MSC to T cell ratios (data not shown). Thus, we concluded that equine allogeneic MSCs were not inherently more immunogenic than autologous MSCs in terms of T cell activation.

Figure 3.1: Lymphocyte proliferation assay: Relative immunogenicity of allogeneic and autologous MSC. Allogeneic and autologous MSCs were non-immunogenic at low ratios of MSC:PBMC. No difference was noted between the immunogenicity of allogeneic and autologous MSCs regardless of dilution. A mild increase in lymphocyte proliferation was noted when autologous BMDMSCs were co-cultured with unstimulated lymphocytes at a ratio of 1 MSC per 10 PBMCs, and when allogeneic BMDMSCs were co-cultured with unstimulated lymphocytes at a ratio of 1 MSC per 10 PBMC and 1 MSC per 50 PBMC. Boxes indicate the average value +/- SEM. P values < 0.05 are marked by *.

Suppression of T cell proliferation by autologous and allogeneic MSC. Next, studies were conducted to determine whether allogeneic MSCs suppressed activated T cell responses to the same degree as autologous MSCs. We found that there was a significant and statistically equivalent degree of suppression of T cell proliferation by both allogeneic and autologous BMDMSCs (Figure 3.2). For example, at an MSC to T cell ratio of 1:10, there was significant decrease in lymphocyte proliferation induced by both autologous and allogeneic MSCs.

However, there was no statistically significant difference in the degree of lymphocyte proliferation suppression induced by the two different MSC populations (autologous versus allogeneic).

Figure 3.2: **Immune suppression assay (lymphocyte proliferation).** Allogeneic and autologous BMDMSCs were immune suppressive, demonstrated by a decrease in lymphocyte proliferation compared to the positive control. No difference was noted between allogeneic and autologous MSCs regardless of concentration. The greatest immune suppression was noted at 1 MSC per 10 PBMCs. Boxes indicate the average value +/- SEM. P values <0.01 are marked by **.

In addition, co-culture with autologous and allogeneic BMDMSCs both resulted in significantly decreased IFNɣ production by T cells (<0.05) (Figure 3.3). However, there was no statistically significant difference in the degree of suppression of IFNɣ production by autologous BMDMSCs when compared to suppression induced by allogeneic BMDMSCs regardless of dilution.

Figure 3.3: Immune suppression assay (IFNɣ expression). IFNɣ expression decreased with increasing ratios of BMDMSCs to PBMCs. The greatest decrease in IFNɣ expression was observed at a ratio of 1 MSC per 10 PBMCs. P values < 0.05 are marked by *. Boxes indicate the average value +/- SEM. P values <0.001 are marked by ***, and P values < 0.0001 marked by ****.

<u>Mechanisms of T cell immune suppression by equine MSC.</u> T cell proliferation and INFɣ production assays, along with specific inhibitors of known pathways of MSC suppression in other species, were used to interrogate possible pathways of immune suppression in the equine system. We found that incubation of MSCs with activated T cells in the presence of indomethacin (an inhibitor of the cyclooxygenase pathway) resulted in significant (P<0.01) reversal of MSC-induced T cell proliferation suppression (Figure 3.4). Moreover, we also found that incubation of MSCs with activated equine T cells resulted in production of significant amounts of PGE_2 *in vitro* (Figure 3.5). The amount of PGE_2 in the supernatant of the MSC-T cell co-cultures was significantly reduced by the addition of indomethacin (P<0.05) (Figure 3.5). In contrast there was no effect of addition of inhibitors of the NO pathway (L-NMMA), the TGFβ pathway (SB208) or the IDO pathways (1-MT), when added at concentrations known to

reverse MSC suppressive effects in other species [16; 32; 33] or at two times the IC50 for SD208. [36]

Figure 3.4: Immune mechanism assay. Inhibition of potential mediators of immune suppression by 1MT (indoleamine pathway), SD208 (TGFβ pathway), LNMMA (nitric oxide pathway), and indomethacin (PGE_2 pathway) revealed a reversal of immune suppression only when co-cultures were treated with indomethacin. The lines indicate the mean +/- SEM. P values <0.01 are marked by **.

Figure 3.5: PGE_2 levels in supernatants from BMDMSC/PBMC co-cultures and indomethacin treated co-cultures. PGE_2 levels were significantly increased in BMDMSC/PBMC co-cultures compared to co-cultures treated with indomethacin. The lines indicate the mean +/- SEM. P values <0.05 is marked by *.

<u>MHCII expression of MSCs after co-culture with stimulated PBMCs.</u> Flow cytometry was used to evaluate the effects of cytokines released from activated T cell cultures on MHCII expression by MSCs. MHCII expression by MSCs exposed to conditioned media from unstimulated PBMCs or MSCs exposed to conditioned media from stimulated PBMCs was determined using MSCs from 4 different horses. It should also be noted that the PBMCs were collected from horses unrelated to the MSC donors. The level of MHCII expression was found to be significantly increased in MSCs exposed to 50% and 100% conditioned media from stimulated PBMCs (Figure 3.6a). Exposure of MSCs to conditioned media from unstimulated PBMCs (100%) also caused an increase in MHCII expression compared to MSCs grown in control media (Figure 3.6b). Less than 2% of MSCs expressed MHCII when incubated in control media, and expression of MHCII remained under 10% for all conditions (Figure 3.6a,b).

Figure 3.6a: MHCII expression of MSCs following exposure to conditioned media from stimulated PBMCs. MHCII expression was found to increase in MSCs exposed to conditioned media from stimulated PBMCs when 50% and 100% conditioned media was used. P value <0.01 is marked by **, and P value <0.001 is marked by ***.

Figure 3.6b: **MHCII expression of MSCs following exposure to conditioned media from unstimulated PBMCs.** MHCII expression was found to increase in MSCs exposed to

conditioned media from unstimulated PBMCs when 100% conditioned media was used. P value <0.01 is marked by **.

3.5 Discussion

Autologous BMDMSCs are extensively used for the treatment of osteoarthritis, tendonitis, and desmitis in the horse. However, little is known about the immune modulatory properties of allogeneic equine BMDMSCs. Previous studies have shown that equine allogeneic MSCs are immune modulatory *in vitro* [16; 37; 38] but these studies did not directly compare their immune suppressive properties to those of autologous MSCs in side-by-side assays. Without such a direct comparison, the relative immune modulatory effectiveness of the two cell sources cannot be determined with certainty.

To determine whether equine allogeneic MSCs can activate resting T cells, allogeneic BMDMSCs were mixed with resting lymphocytes from unrelated donor animals (allogeneic reaction), and with lymphocytes from the same animal (autologous reaction). A small degree of lymphocyte activation was observed with both MSC types, but the degree of activation was similar with both. Thus, allogeneic MSCs do not appear to be capable of generating strong allo-reactive T cell responses in previously untreated horses and the small degree of activation with allogeneic MSCs is not more than autologous MSCs.

Previous *in vitro* studies using human and equine BMDMSCs support this finding; Mancheño-Corvo *et al*. (2015) demonstrated an increase in the immune suppressive properties of MSCs pre-activated with IFNɣ, suggesting local inflammation is an important trigger for MSC immune suppression. [28] Likewise, Paterson *et al.* (2014) revealed a decrease in the production of inflammatory cytokines (IL-6, INF, TNF) by antigen stimulated PBMCs when they were co-cultured with MSC supernatants. [39] In agreement with *in vitro* studies, [28] a recent *in vivo*

equine study, demonstrated that allogeneic umbilical MSCs cause a decrease in inflammation on joints treated with LPS. [18] A local inflammatory environment may be important for the activation of MSC ant-inflammatory mechanisms and should be further investigated.

In our study, co-culture of allogeneic and autologous BMDMSCs with stimulated lymphocytes resulted in equivalent immune suppression as defined by a decrease in lymphocyte proliferation. Our study indicates that the source of BMDMSCs (allogeneic vs autologous) is not an important variable in determining the degree of immune suppression elicited *in vitro*. This finding could have significant clinical implications, as allogeneic BMDMSCs may provide a more convenient and less expensive product for the treatment of musculoskeletal disease in the horse. These findings suggest that further *in vivo* studies are warranted to compare the behavior of allogeneic and autologous cells within the inflamed joint environment.

The pathways of the immune suppressive properties of human, murine, and canine BMDMSCs have been previously investigated. [29; 40; 41] Our study examined the role of TGFß, PGE_2, indoleamine and reactive nitrogen species as mediators of immune suppression and found that only PGE_2 was an important mediator of immune suppression by allogeneic equine BMDMSCs. This finding is in agreement with a previous study that investigated interleukin-6 (IL-6), nitric oxide (NO), and prostaglandin E_2 (PGE_2) as mediators of immune suppression by allogeneic BMDMSCs [16] and found only PGE_2 to be important.

We also observed that the concentration of PGE_2 was significantly increased in co-cultures of allogeneic BMDMSCs and PBMCs as a result of MSC cytokine production, confirming its upregulation during immune suppression. PGE_2 has been extensively studied in conditions of acute and chronic inflammation. PGE_2, a naturally occurring prostaglandin, suppresses acute inflammation by influencing neutrophils, macrophages, and T cells. [42]

Understanding how allogeneic BMDMSCs create an anti-inflammatory environment is important to harnessing their full potential as a therapeutic modality and directing their clinical use. This study was able to identify a single paracrine factor, PGE_2, utilized by allogeneic BMDMSCs.

We found no significant effect of inhibitors of the nitric oxide pathway (L-NMMA), the TGFβ pathway (SD-208) or the IDO pathways (1-MT) when added to the MSCs and T cell co-cultures. Previous equine studies have demonstrated L-NMMA and indomethacin to be active against the nitric oxide and indomethacin pathways, respectively. [16] However, other equine studies have been unable to detect a difference in the level of TGFβ in co-cultures of MSCs and lymphocytes when compared to lymphocytes alone, suggesting TGFβ is unlikely to be involved as with immune modulation. [37] We performed a TGFβ-1 ELISA (R&D Systems, Human TGF-β1 Immunoassay) which revealed no difference in the levels of TGFβ in the co-culture system with and without SD-208 (data not shown). In addition, 1-MT has not been previously used as a modulator of indoleamine secretion in the horse. However, in agreement with our findings, previous studies have not detected indoleamine in equine co-cultures of MSCs and PBMCs, suggesting this pathway may not be functionally active. [37]

Our study focused on detecting soluble, paracrine factors associated with immune modulation by BMDMSCs. Recent literature suggests that BMDMSCs may utilize multiple pathways to achieve immune suppression including a combination of direct cell-to-cell contact and paracrine stimulation. [43] Although multiple mechanisms are likely involved, a recent *in vitro* study using human MSCs suggests that the majority of immune modulation occurs through paracrine secretion, as opposed to the effects of direct cell-to-cell contact between lymphocytes and MSCs. [44]

Previous studies have used intra-articular administration of allogeneic MSCs to treat joint disease in horses. [9] Broeckx *et al*. (2014) used immature and chondrogenically induced allogeneic MSCs in 165 horses with a complication rate of only 1.8% consisting entirely of joint flares, [9] a rate similar to that published for intra-articular administration of autologous BMDMSCs. [7] In humans, a multicenter phase II clinical trial using allogeneic MSCs for knee osteoarthritis, has reported improvement in algofunctional indices and cartilage quality compared to a hyaluronic acid control. [22] Likewise, a rabbit model of osteoarthritis has reported an increase in cartilage health with intra-articular BMDMSC administration, demonstrating allogeneic stem cell survival and engraftment. [15] Evidence is mounting that allogeneic MSCs may be safe and effective *in vivo*.

Recent publications have examined the expression of major histocompatibility complex II (MHCII) by equine BMDMSCs. [45-47] Expression of MHCII may lead to immune recognition and, therefore, is a potential concern for allogeneic use. Direct contradiction exists in the literature with the majority of publications demonstrating a lack of MHCII expression by BMDMSCs, [17; 45; 47] while an alternate publication reports MHCII expression in 11 of 13 lines of BMDMSCs. [46] In our laboratory, MSCs are routinely MHCII negative (data not shown). However, a prior study by Schnabel *et al.* (2014), found that exposure of MSCs to 100ng/ml of recombinant IFNɣ would cause MSCs that lacked MHCII expression to markedly increase expression of MHCII. [46] Lymphocytes in culture produce IFNɣ, and stimulation with concanavalin A causes an increase in IFNɣ production. Therefore, it was our expectation that MSCs would increase their expression of MHCII when exposed to conditioned media from concanavalin A stimulated PBMCs and unstimulated PBMCs. As expected, we found an increase in MHCII expression after MSCs were exposed to conditioned media from PBMCs. However,

despite evidence of an increase in MHCII expression after MSCs are exposed to conditioned media PBMCs, 56 allogeneic co-culture combinations were found to demonstrate immune suppression *in vitro*. This indicates that either the utilized allogeneic BMDMSCs had low MHCII expression despite co-culture with stimulated lymphocytes or that MHCII status cannot be directly linked with the ability to cause immune modulation in mixed lymphocyte reactions.

Based on the findings from our study, we suggest that further research should be conducted *in vivo* to compare the relative clinical benefits of the anti-inflammatory and immune modulating properties of allogeneic BMDMSCs compared to autologous BMDMSCs. If allogeneic BMDMSCs are found to have comparable immune-modulating properties to autologous BMDMSCs *in vivo*, they may provide a beneficial off-the-shelf therapy for musculoskeletal disease in horses, eliminating the need for time-consuming, individual culture techniques and the added expense of individual culture.

In conclusion, allogeneic BMDMSCs and autologous BMDMSCs appear to be equally immune suppressive *in vitro.* It also appears that equine MSCs principally use the cyclo-oxygenase pathway for suppression of T cell function.

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Chapter 4 - Allogeneic versus autologous intra-articular mesenchymal stem cell injection within normal horses: clinical and cytological comparisons suggest safety[3]

4.1 Overview

Allogeneic bone marrow-derived mesenchymal stem cells (BMDMSCs) could provide multiple advantages over autologous BMDMSCs, including creating an "off-the-shelf" treatment together with the ability to control for donor variation. The objective of this randomized-controlled study was to compare the clinical and synovial fluid response of the normal equine joint to autologous and pooled-allogeneic BMDMSCs while controlling for individual variation and joint variations in response to intra-articular injections. We hypothesized that, by controlling for individual animal and joint variation, we could identify differences between allogeneic versus autologous BMDMSCs in non-inflamed joints. Bone marrow was harvested from eight horses. Autologous BMDMSCs were culture expanded, cryopreserved and thawed immediately prior to administration. For allogeneic BMDMSC treatments, four horses' BMDMSCs were culture expanded, pooled, cryopreserved and thawed immediately prior to use. Ten million (autologous or pooled-allogeneic) BMDMSCs were administered into contralateral forelimb metacarpophalangeal joints so that every autologous and allogeneic injection could be compared within the same animal. Clinical parameters included subjective lameness, objective lameness (Lameness Locator™), response to flexion, joint circumference and joint effusion.

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[3] The second aim of this work was to compare the normal joint reaction to autologous BMDMSCs and allogeneic BMDMSCs in vivo. This chapter includes the complete published manuscript for this aim, Allogeneic versus autologous intra-articular mesenchymal stem cell injection within normal horses: clinical and cytological comparisons suggest safety (Aimee C. Colbath, Steve W. Dow, Leone S. Hopkins, Jennifer N. Phillips, C. Wayne McIlwraith, Laurie R. Goodrich, *Equine Veterinary Journal*, 2019 May 23). My contributions to this publication included performing laboratory assays, statistical analysis and the writing the majority of the manuscript. Minimal modifications were made to meet formatting requirements and table and figure numbers have been modified. This article is reproduced with permission from John Wiley and Sons, Inc., Hoboken, NJ.

Arthrocentesis was performed for assessment of the nucleated cell count, differential cell count, total protein, and synovial concentrations of prostaglandin E2 (PGE_2) and c-reactive protein (CRP). All parameters were measured at baseline, 6, 12, 24, 72, 168, and 336 hours post-injection. No difference was detected in any parameters between forelimb metacarpophalangeal joints administered autologous or pooled-allogeneic BMDMSCs. This study did not attempt to measure efficacy of BMDMSCs for musculoskeletal disease and should be followed by properly controlled, efficacy trials. The study did not identify any clinical or cytological differences in the normal joint response to allogeneic or autologous BMDMSCs. A larger study to prove equivalence is warranted as allogeneic BMDMSCs may be a feasible alternative to autologous BMDMSCs.

4.2 Introduction

Evidence is mounting that mesenchymal stem cells may be an effective treatment for equine osteoarthritis, tendonitis, desmitis, and wound healing. [1-4] Equine mesenchymal stem cells (MSCs) may be obtained from multiple tissues including bone marrow, adipose tissue, and synovium [5; 6]; adipose-derived and bone marrow-derived mesenchymal stem cells are commonly used in musculoskeletal disease treatment. These cells have anti-inflammatory properties and produce cytokines and growth factors which may influence endogenous tissue healing. [7-11]

Culture expansion of autologous bone marrow derived MSCs (BMDMSCs) requires a minimum of a 2-3 week period causing a delay in treatment, and adds to the expense and risk of individual bone marrow harvest. [12-14] Allogeneic stem cells have the distinct advantage of being an "off-the-shelf treatment" allowing the potential for immediate therapy at the time of diagnosis. In addition, there is evidence that age and disease may affect the healing properties of

MSCs. [15-17] Thus, allogeneic BMDMSCs could be harvested from young, healthy donors and be preemptively characterized for cytokine production, chondrogenic ability, or other desired properties.

Despite the potential advantages of allogeneic BMDMSCs, theoretical concerns regarding the potential immune-mediated inflammatory effects of these cells have been raised. [18] At present, the relative immunogenicity of autologous versus allogeneic BMDMSCs in horses is incompletely understood, especially with regards to the ultimate target organs (i.e., joints, tendons, ligaments). Recently, it was reported that allogeneic umbilical-derived mesenchymal stem cells injected into the joints of horses with LPS-induced synovitis were no less effective than autologous MSCs. [8] In addition, intra-articular allogeneic MSCs have been used in multiple clinical studies in humans and horses with little to no significant side effects. [19-21] Nonetheless, it has also been reported that allogeneic MSCs are recognized immunologically in horses. [13; 18; 22] However, immune recognition does not necessarily equate to increased inflammation in the joint. Moreover, activated MSCs have evolved multiple different mechanisms of immune suppression and modulation. [9; 23; 24] Therefore, the significance of allo-antibodies following MSC injection remains unknown. For example, there appears to be little correlation between the presence of allo-antibodies and adverse reactions to MSC injection. [13; 18; 22]

A few equine studies have sought to determine the intra-articular response to allogeneic stem cells, but these studies have administered allogeneic stem cells alone or have evaluated allogeneic versus autologous cells in different cohorts of animals. [8; 18; 25] Individual reactions to intra-articular treatments can vary significantly horse to horse. Joint flare can be experienced with any intra-articular medication and individual predisposition to joint flare is still poorly

understood. [26] With such individual variation, it is difficult to compare the effect of allogeneic and autologous BMDMSCs on the joint when each treatment is administered into different animals and in small groups. A recent study suggests that xenogenic factors (eg, fetal bovine serum) incorporated during the MSC cell culture process may be responsible for some joint inflammation, [18] but even individual reactions to xenogenic factors remain difficult to predict. Likewise, individual joints may react differently to intra-articular medications. [27] Further, Colbath et al. (2018) found differences in total nucleated cell counts, neutrophil infiltration, and total protein between tarsocrural and middle carpal joints administered rIL-1β. Therefore, studies which compare allogeneic and autologous treatments administered into different joints must be evaluated with caution. [28]

To control for individual variability and joint-related variability, this study used the same horses for each treatment and administered allogeneic and autologous cells at the same timepoint into contralateral metacarpophalangeal joints. The objective was to determine the intra-articular response to the administration of autologous and allogeneic BMDMSCs while controlling for individual animal and joint variation in reaction to intra-articular treatment administration. We hypothesized that, by controlling for individual animal and joint variation, we could identify differences between allogeneic versus autologous BMDMSCs in non-inflamed joints.

4.3 Materials and Methods

Eight horses (sixteen joints) were used in a randomized-controlled study. An a priori power analysis was based on prior joint studies with described differences in clinical parameters as well as cytokine levels, total protein and nucleated cell counts in the joint fluid and a pilot study which evaluated the variability in response to the intra-articular administration of PBS (data not shown). [5; 29; 30] With a power of 0.8 and an alpha error rate of 0.05, the power

calculation suggested that 8 horses would be sufficient for identification of a difference in NCC of 15 x 10^3 cells/ul, a 1-grade change in lameness, a 1.5 grade change in effusion, and a 2.5 gm/dL difference in total protein. All horses had an absence of joint effusion in the metacarpophalangeal joint (MCPJ) or metatarsophalangeal joint (MTPJ) and were determined to be sound prior to entering the study with no response to joint flexions. Horses were of mixed breed and ranged in age from 2-5 years old. Treatment limbs were randomized, and all investigators and staff were unaware of treatment assignment with the exception of the first author. This work was conducted under the approval of the Institutional Animal Care and Use Committee of Colorado State University (15-5810A).

Each of eight horses received one treatment (allogeneic vs. autologous) into a forelimb metacarpophalangeal joint and the opposite treatment (allogeneic vs. autologous) into the contralateral forelimb at the same time point. In addition, a hindlimb metatarsophalangeal joint was used to assess the effect of multiple arthrocentesis with no treatment. All treatments were administered as a total of 10 million cells in 1ml of equine serum. All joints were clipped and aseptically prepared before administration and treatments were administered using aseptic technique.

MSC isolation, culture, expansion, cryopreservation

All horses were sedated with detomidine (0.01 mg/kg IV) and butorphanol (0.01 mg/kg). The sternum was clipped and aseptically prepared. Bone marrow was aspirated from each horse and aseptically collected into heparinized syringes as previously described. [12] Five milliliters of bone marrow aspirate were obtained in each of three syringes. Red blood cells were removed from bone marrow aspirates using centrifugation. Bone marrow was then cultured overnight in low-glucose DMEM, 10% fetal bovine serum (FBS), 10,000 U/ml of penicillin-streptomyocin-

amphotericin B (PSA) and 1N HEPES. After 24 hours, the media was changed and colonies were allowed to form over the next 7-10 days. Once colonies were formed, cells were dissociated with Accumax™ and cells were cultured in αMEM supplemented with 10% FBS, 10,000 U/ml PSA, 1N HEPES, and 2 ng/ml of fibroblast growth factor (FGF). The cells were passaged three times in monolayer prior to being cryogenically preserved. Autologous cells were cryopreserved in 95% autologous serum and 5% DMSO. For allogeneic BMDMSC treatments, horses were split into two groups of 4 horses. BMDMSCs were combined from each of the four horses and cryopreserved in doses of 10x10^6 BMDMSCs/ml. The serum used for cryopreservation was also pooled from each of the four horses.

Evaluation of clinical response to treatment

A physical examination and lameness evaluation was performed, and joints were evaluated for joint circumference, joint effusion, and heat at 0, 6, 12, 24, 72, 168 (1 week), and 336 (2 weeks) post-injection hours (PIH). Subjective lameness evaluation was performed by a board-certified equine surgeon (AC) and objective lameness evaluation was conducted using a wireless motion analysis system (Lameness Locator™).

Joint circumference (cm) was measured three times, consecutively, at the same location at each time point; the three values were averaged for each time point. The location of joint circumference measurement was chosen by palpation (as the middle of the joint pouch) prior to beginning the study and marked by clipping the hair. Joint effusion was given a subjective clinical grade with grade 0 indicating no effusion, grade 1 indicating slight effusion, grade 2 indicating mild effusion, grade 3 indicating moderate effusion, and grade 4 indicating severe effusion. All measurements were conducted by a single observer.

Synovial fluid analysis

Synovial fluid was harvested prior to treatment (0 PIH) and 6, 12, 24, 72, 168, and 336 PIH. Arthrocentesis was performed aseptically following clinical assessment. Horses were sedated using detomidine hydrochloride and butorphanol tartrate (0.01 mg/kg IV). Synovial fluid was immediately placed in plain glass tubes and processed within 1 hour of collection. A portion of the aspirate was used for direct smear and cytospin analysis prior to hyaluronidase digestion and analysis for total nucleated cell count using an automated cell counter. Total protein content was determined using a refractometer. Differential neutrophil, monocyte, lymphocyte and eosinophil counts were evaluated using direct smear and cytospin analysis. The remainder of the synovial fluid was centrifuged for 10min at 1000xg and the supernatants were stored at -80°C in Eppendorf tubes until ELISA analysis could be performed. Multiple aliquots were frozen to prevent freeze-thaw cycles.

Enzyme-linked immunosorbent assays

Synovial Prostaglandin E2 (PGE_2) was evaluated as previously described. [31] Briefly, a solid-state extraction was performed using C2 ethyl mini-columns prior to quantification using a commercially available equine specific PGE_2 Enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, Farmingdale, NY). Synovial C-reactive protein (CRP) was evaluated using a commercially available ELISA kit (ICL Laboratories, Portland, OR).

Statistical analysis

Clinical data (excluding objective lameness data) and synovial fluid data were compared using a two-way ANOVA for repeated measures with time defined as the within subjects factor, and the joint (TTJ versus MCJ) defined as a between-subjects effect. For objective lameness

data, a one-way ANOVA was used to evaluate a significant difference from 0mm at any time point. Significance was set at $P<0.05$. Simple effects between rows were analyzed using a Tukey's multiple comparisons test. Normality was assessed by evaluating diagnostic plots of the residuals for each variable. Log transformation was performed for nucleated cell count data. Statistical analysis was conducted using GraphPad Prism (version 7.03, Portland, OR).

4.4 Results

Clinical responses to BMDMSC injection

Heart rate, respiratory rate and temperature were measured at each time point in each horse prior to lameness examination. No change was detected in temperature, respiration or heart rate after injection of the forelimbs in any animal (Figure 4.1).

Figure 4.1. Physical examination following intra-articular injection of autologous and allogeneic BMDMSCs into contralateral joints. There was no significant difference between baseline (0hr) and any time point following intra-articular injection of autologous and allogeneic BMDMSCs into contralateral joints. Bars indicate the mean for each time point and the standard deviation of the mean.

Lameness was assessed subjectively using the AAEP grading scale and objectively using a wireless motion analysis system (Lameness Locator™) at each time point prior to sedation and arthrocentesis (Figure 4.2). Despite being screened for lameness prior to the start of the study, a single horse was determined to have a grade 1/5 forelimb lameness at the onset of the study.

Therefore, results are reported as a change in lameness from baseline. No difference was detected in subjective lameness score between limbs injected with autologous versus allogeneic BMDMSCs. A single horse that received autologous BMDMSCs had a lameness that increased by 2 AAEP lameness grades during the study period. Likewise, a single horse that received allogeneic BMDMSCs had a lameness that increased by 3 AAEP lameness grades during the study period. Otherwise, all lameness increases were a single grade for both allogeneic and autologous treatments. The mean subjective lameness grade pre-treatment and 2 weeks following treatment was 0/5 for both autologous and allogeneic BMDMSCs. The median subjective lameness grade was 0 for both treatment groups at all time points.

Figure 4.2. Change in AAEP grading scale (subjective lameness) and vector sum (mm) (objective lameness) following intra-articular injection of allogeneic and autologous BMDMSCs into contralateral joints. Vector sum (mm) was assigned a negative sign for the allogeneic limb and a positive sign for the autologous limb. No significant difference was found in change in subjective (AAEP grading scale) or objective (vector sum) lameness at any time point.

The Lameness Locator™, wireless motion analysis system, was used to complement subjective lameness assessment. A vector sum (mm) was recorded at each time point. In short, the vector sum is a measurement (mm) which corresponds to the displacement of the horse's head and is used to determine forelimb lameness. In the unmanipulated data, a negative vector

sum corresponded to a left forelimb lameness and a positive vector sum corresponded to a right forelimb lameness. However, the data was then processed so that all autologous limbs were labeled as a positive vector sum and all allogeneic limbs were labeled as a negative vector sum. The vector sum for each time point was then compared to baseline and reported as the change in vector sum (mm). There was no significant difference in the change in vector sum (mm) when compared to 0 mm at any time point, indicating no difference in lameness between treatment groups. The number of limbs treated with autologous or allogeneic BMDMSCs which were reported lame is described in Table 4.1 for both subjective lameness assessment (AAEP scale) and objective lameness assessment (Lameness Locator®).

Table 4.1. A comparison of the number of horses which were found to be lame by subjective or objective lameness assessment at each time point.

HOUR	Lame Allogeneic Limbs by Objective Assessment	Lame Allogeneic Limbs by Subjective Assessment	Lame Autologous Limbs by Objective Assessment	Lame Autologous Limbs by Subjective Assessment
0	2	0	3	2
6	5	3	3	2
12	4	3	3	3
24	3	2	4	2
72	3	3	5	3
168	4	0	2	2
336	4	0	2	1

Flexions were performed at each time point with no difference detected between the response to flexion in limbs injected with allogeneic versus autologous BMDMSCs. Joint size was assessed by both joint circumference and by a subjective joint effusion score (Figure 4.3). The metacarpophalangeal joints injected with autologous and allogeneic BMDMSCs did not show any significant increase in joint circumference. The largest mean change in joint

circumference was measured at 72 PIH for both groups (mean, autologous: 0.53cm; allogeneic: 0.63cm). The untreated metatarsophalangeal joint also had its greatest increase in joint circumference when joint aspiration was performed at 72 hours (mean: 0.48 cm) (Figure 4.6). There was a significant increase in subjective joint effusions scores for limbs injected with autologous BMDMSCs up to 72 PIH with the peak increase in effusion score at 24 PIH (maximum change in effusion score: 1.625). Limbs injected with allogeneic BMDMSCs showed a significant increase in subjective joint effusion over baseline until 24 PIH (maximum change in effusion score: 1.8). The untreated metatarsophalangeal joint experienced a maximum mean increase in joint effusion score at 24 PIH (mean: 0.94 cm). Despite a subjective increase in joint effusion over baseline in both groups, no difference was detected between groups at any timepoint.

Figure 4.3. Change in joint circumference and joint effusion score. There was no significant difference between the change in circumference (cm) or joint effusion score in limbs treated with allogeneic versus autologous BMDMSCs. Bars indicate the standard deviation of the mean.

Synovial fluid analysis

There was no difference in the total nucleated cell count in joints injected with allogeneic versus autologous BMDMSCs at any time point (Figure 4.4). The nucleated cell count was

significantly increased over baseline for autologous and allogeneic BMDMSCs by 6 hours post-injection [mean, autologous: 4.08 x 10^6/uL (P=0.04); allogeneic: 3.38 x 10^6/uL (P=0.03)]. The highest NCC occurred at 24 PIH for both treatment groups (mean, autologous: 15.74 x 10^6/uL; allogeneic: 12.26 x 10^6/uL). Metacarpophalangeal joints injected with autologous BMDMSCs continued to have a significantly elevated NCC (mean: 4.0 x 10^6/uL) through 72 hours post-injection. Metatarsophalangeal joints that received no treatment but underwent repeated arthrocentesis never exceeded a NCC of 4.9 x 10^6/ul (Figure 4.5).

Figure 4.4. Nucleated cell count and total protein following intra-articular allogeneic and autologous BMDMSCs. No significant differences were found between the nucleated cell count and total protein in joints administered allogeneic and autologous BMDMSCs. Bars indicate the mean for each time point and the standard deviation of the mean.

No difference was detected in the total protein of limbs injected with allogeneic versus autologous treatments at any time point (Figure 4.4). The total protein in the synovial fluid was increased from 6 hours to 24 hours post-injection for both limbs injected with allogeneic and autologous BMDMSCs (P<0.0001). The maximum mean synovial total protein in the autologous BMDMSC treated limbs occurred at 24 PIH (3.9 gm/dL). While, maximum mean synovial total protein in the allogeneic limbs occurred at 12 PIH (3.8 gm/dL). By 72 hours, the total protein was not significantly increased over baseline for either limb. Interestingly, metatarsophalangeal

joints that received no treatment but underwent repeated arthrocentesis had a maximum mean total protein concentration of 4 gm/dL at 24 PIH (Figure 4.5).

Figure 4.5. Nucleated cell count, total protein and joint circumference following repeated arthrocentesis of the metatarsophalangeal joint. Bars indicate the mean for each time point and the standard deviation of the mean.

Differential cell counts revealed a significant increase over baseline of monocytes in joints treated with allogeneic and autologous BMDMSCs at 12, 24, and 72 hours post-injection and neutrophils at 6, 12, 24 and 72 hours post-injection. The maximum mean neutrophil count was seen at 24 PIH for both autologous (mean: 9.1 x10^6/uL; P<0.0001) and allogeneic (mean: 7.3 x10^6; P<0.0001) BMDMSC treated limbs. Likewise, both autologous and allogeneic BMDMSCs treated limbs experienced the maximum mean monocyte count in the synovial fluid at 24 PIH (mean, autologous: 5.5 x10^6/uL, (P=0.0003); allogeneic: 8.5 x10^6/uL, (P<0.0001)). The untreated limbs which underwent serial arthrocentesis showed a maximal mean neutrophil count of 2.4 x 10^6 at 24 PIH, and a maximal mean monocyte count of 2.8 x10^6 at 72 PIH. Lymphocytes were significantly increased over baseline at 24 hours post-injection in limbs treated with allogeneic BMDMSCs only and never exceeded 1 x10^6/uL (P=0.04). Eosinophil counts were significantly increased over baseline for both metacarpophalangeal joints injected with allogeneic (P= 0.04) and autologous (P=0.04) BMDMSCs at 12 hours post-injection but

remained at low levels (mean, autologous: 0.05 x10^6/uL; allogeneic: 0.09 x10^6/uL). No difference in differential cell counts was detected between allogeneic and autologous treatments at any time point (Figure 4.6). In the untreated metatarsophalangeal joints, lymphocyte and eosinophil counts remained low (maximal mean lymphocyte count: 0.11 x10^6; maximal mean eosinophil count: 0.03 x 10^6/uL) (data not shown).

Figure 4.6. Differential cell counts following intra-articular allogeneic and autologous BMDMSCs. No significant differences were found between the monocytes, neutrophils, lymphocytes, or eosinophils in joints administered allogeneic and autologous BMDMSCs. Bars indicate the mean for each time point and the standard deviation of the mean.

Synovial fluid biomarkers

No differences were detected in the change in synovial fluid levels of PGE_2 and CRP (Figure 4.7) between treatment groups. Neither the limbs injected with autologous BMDMSCs, those injected with allogeneic BMDMSCs, nor the untreated limbs showed a significant increase in CRP or PGE_2 from baseline.

Figure 4.7. Change in synovial PGE2 and CRP. No significant differences were found in the change in synovial PGE2 or CRP for joints administered allogeneic and autologous BMDMSCs. Bars indicate the mean for each time point and the standard deviation of the mean.

4.5 Discussion

This study examined the clinical and cytological effects of allogeneic and autologous bone marrow-derived mesenchymal stem cells administered into normal equine metacarpophalangeal joints. Importantly, this study sought to control for individual variation in response to intra-articular treatments, as well as differing joint responses to intra-articular treatments, by administering treatments into contralateral joints at the same time point.

Our study did not identify any significant differences in the clinical, cytological or biomarker response of the metacarpophalangeal joint to intra-articular injection of allogeneic versus autologous BMDMSCs. Previous studies which administered treatments (allogeneic versus autologous MSCs) into different joints or different cohorts of horses are in agreement with these results. [18; 25; 28] In addition to cell counts and differential cytology results which have been previously reported, our study investigates a synovial inflammatory mediator (PGE_2) and an acute phase protein (CRP). These results provide additional evidence that pooled-allogeneic BMDMSCs produce no greater joint inflammation over autologous BMDMSCs.

A single horse in each treatment group had a clinically significant increase in lameness. When subjective lameness was assessed, the horse that was lame on the autologous MSC-treated limb had a 2-grade increase in lameness while the horse that was lame in the allogeneic MSC-treated limb had a 3-grade increase in lameness. All other changes in lameness were a maximum of 1-grade on the AAEP lameness scale and the median lameness grade was 0 for both groups at all time points. A corresponding increase in vector sum (mm) is seen for both the horse that was found lame in the autologous MSC-treated limb and allogeneic MSC-treated limb. Overall, subjective and objective lameness data revealed no significant differences between groups at any time point. In addition, no differences were found in the response to flexion or the character or quantity of cellular infiltration or total protein. Subjective lameness and subjective joint effusion scores were reported as ordinal variables but analyzed using a repeated measures 2-way ANOVA to take into consideration related, non-independent groups, sampled over multiple time points. Although this analysis is consistent with multiple previous publications, [32-36] a non-parametric analysis could have been performed on this data. Due to the potential controversial nature of this analysis, both subjective lameness and subjective effusion were also assessed by continuous variables (vector sum and joint circumference), and the authors confirmed that analysis of the groups at 6 hours, 12 hours, and 24 hours using a non-parametric statistical test (Wilcoxon matched pairs signed rank test) was also unable to identify a difference between groups (data not shown).

There was no difference in the synovial fluid NCC, total protein or differentials of metacarpophalangeal joints administered autologous versus allogeneic BMDMCs at any time point. Untreated limbs, autologous BMDMSC treated limbs, and limbs treated with allogeneic BMDMSCs, had a similar increase in total protein. However, both autologous and allogeneic

BMDMSC treatments resulted in an increase in NCC for at least 24 hours post-injection (characterized by an increase in neutrophils and monocytes) while the untreated metatarsophalangeal joints which underwent multiple arthrocentesis had only a marginal increase in NCC. Both autologous and allogeneic BMDMSCs resulted in a transient synovial inflammation. These results indicate that a single injection of autologous and allogeneic BMDMSCs may be well tolerated clinically despite an equivalent, initial, mild inflammation.

Lameness was measured both subjectively by a board-certified large animal surgeon as well as objectively using the Lameness Locator® motion analysis system. Contralateral forelimbs were chosen (instead of administration into one forelimb and one hindlimb) for multiple reasons. Firstly, the Lameness Locator® system produces different measurements for the forelimb versus hindlimb and forelimb and hindlimb lameness is determined by different thresholds, making direct comparisons difficult. Secondly, for subjective assessments, hindlimb lameness may be more difficult to assess with agreement in inter-evaluator, subjective evaluation varying greater in hindlimbs versus forelimbs. [37] Thirdly, hindlimb lameness can also influence forelimb lameness but in a less predictable fashion then contralateral forelimb lameness. By administering both treatments at the same time point in contralateral limbs we eliminated inter-horse variability in the response to intra-articular injection. This, however, did create a limitation for lameness analysis. Namely, if both treatments produced lameness simultaneously in contralateral limbs the lameness could have been masked. However, our goal was to compare the treatments to each other. We were looking for a *difference* between the effects of the treatments. Therefore, if both treatments had caused a lameness the result would have still been the same, a net of no difference between the treatments.

Unfortunately, stem cell tracking studies in the horse have primarily concentrated on injection of MSCs into tendon lesions. These studies have shown a large movement of stem cells into the blood 24 hours following administration, [38] although some cells are found within the lesions even 9 weeks post-injection. [39] The joint environment is unique with a barrier of synovial lining and small capillaries present within the synovium. Therefore, it is difficult to translate tracking results from tendon injections to intra-articular administration. In other species, studies that have focused on tracking MSC following intra-articular administration indicate retention within the joint. [40-42] Most recently, a study in rats which received intra-articular administration of MSCs showed a significant retention of stem cells in non-inflammatory joints for 21 days post-injection. [43] Likewise, a study in sheep found MSCs detectable 12 weeks following intra-articular injection. [42] Stem cell tracking remains difficult, but it is likely that the majority of stem cell are retained in the joint. That said, the potential for stem cell movement remains a limitation of the study. However, the authors of this study felt that the ability to control for individual variation with intra-articular administration outweighed the potential for potential stem cell migration to the contralateral joint.

Pooled allogeneic BMDMSCs were used to expose each individual horse to multiple other horses' stem cells increasing the likelihood of eliciting a potential immune reaction. Still, pooled-allogeneic of BMDMSCs elicited no greater inflammation then autologous BMDMSCs. This study demonstrates minimal reaction of the non-inflammatory joint to a single administration BMDMSCs with no greater reaction whether injection is allogeneic or autologous MSCs. This cannot be directly extrapolated to the inflammatory joint which retains stem cells longer and may activate an anti-inflammatory phenotype. [43; 44] Likewise, multiple injections

of allogeneic or autologous mesenchymal stem cells may result in a different cytological or clinical response as found by Joswig et al. (2017). [18]

The objective of the current study was to compare the non-inflamed joint's response to autologous versus allogeneic BMDMSCs. Therefore, a clinically relevant dose was selected. Clinically, we commonly use 10 x 10^6 BMDMSCs in the metacarpophalangeal joint, middle carpal joint and tarsocrural joint. In a previous study, treatment of the middle carpal joint with 10x10^6 BMDMSCs resulted in an improvement in PGE_2 levels indicating a potential decrease in inflammation. [5] Larger doses (20 x 10^6) have been used in stifle joints with a clinical effect. [26] However, there remains little agreement on the appropriate dose for intra-articular BMDMSC administration in the horse. Higher doses may have further increased inflammation. An additional study would be needed to assess the effect of dose.

Culture expansion methods and cryopreservation may have a significant effect on the ultrastructure, immunophenotype, and transcription factor expression of mesenchymal stem cells. [45; 46] All cells in this study were culture expanded simultaneously under the same initial conditions, and allogeneic cells were pooled immediately before cryopreservation. Fetal bovine serum was used during the culture period prior to cryopreservation. At the time of cryopreservation, the cells were stored in the horses' own serum (autologous) or an equal mixture of allogeneic serum. Cryopreservation was performed in 5% DMSO and 95% serum to minimize the effects of cryopreservation on cellular health and function. [47] The culture expansion methods and cryopreservation were chosen to mimic our clinical process. Fetal bovine serum has been implicated in individual inflammatory reactions [18]. Although treatments were cryopreserved in equine serum to minimize xenogenic contamination, no wash-out period was performed. Therefore, small amounts of fetal bovine serum likely remained within the cells and

could have contributed to joint inflammation. [18] However, because all treatments were given in the same animal, individual inflammatory reactions to fetal bovine serum would have been equal between treatments.

Recent publications have raised concern over equine BMDMSCs expressing major histocompatibility complex II (MHCII). [14; 48] Authors indicate that expression of MHCII may lead to immune recognition and, therefore, result in an immune reaction to allogeneic cells. [14] However, the majority of studies of equine BMDMSCs have reported a lack of MHCII expression by BMDMSCs. [14; 49; 50] Pooled-allogeneic BMDMSCs in this study were not tested for MHCII expression. However, BMDMSCs in our laboratory have a routinely low to negative level of expression of MHCII. [9]

In summary, although theoretical concerns have been raised over immune recognition of allogeneic BMDMSCs, this study demonstrates no significant difference in the response of normal equine joints to pooled-allogeneic versus autologous BMDMSCs. These results provide further information compared to previous studies by controlling for individual and joint variation to intra-articular injection as well as measuring additional synovial fluid inflammatory markers. This information further supports the use of allogeneic cells for musculoskeletal disease in the horse and should be coupled with controlled, clinical, efficacy trials.

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Chapter 5: Induction of synovitis using interleukin-1 beta: are there differences in the response of middle carpal joint compared to the tibiotarsal joint?[4]

5.1 Overview

The effects of recombinant IL-1β (rIL-1β) have been described for the middle carpal joint (MCJ). However, we are unaware of any studies that have described the cytological response of the tibiotarsal joint (TTJ) to rIL-1β or compared the clinical and cytological responses of the MCJ to the TTJ following the administration of intra-articular rIL-1β. Such information is critical for researchers planning to use rIL-1β to create acute synovitis models in horses. The objective of the study was to compare the clinical and cytological responses of the MCJ to the TTJ following administration of rIL-1β. Twelve horses were used for the study. Eight horses received 75ng of rIL-1β into the MCJ and four horses received 75ng of rIL-1β into the TTJ. Clinical and cytological outcome parameters including lameness, joint circumferences, joint effusion score, total nucleated cell count, cellular differentials, C-reactive protein, and prostaglandin-E2 concentrations which were determined at baseline and multiple post-treatment time points over a 336h period (2 weeks). rIL-1β administered into the TTJ resulted in a significantly greater respiratory rate at 24 hours and heart rate at 12 hours when compared to rIL-1β administered into the MCJ. In addition, the TTJ had a significantly greater increase in joint circumference at 24 post-injection hour (PIH) and subjective effusion grade at 24 PIH and 336

[4] The third aim of this work was to report the tibiotarsal joint response to rIL-1β and compare the tibiotarsal joint response to rIL-1β with the middle carpal joint response to the same of dose of rIL-1β. This chapter includes the complete published manuscript for this aim, Induction of synovitis using interleukin-1 beta: are there differences in the response of middle carpal joint compared to the tibiotarsal joint? (Aimee C. Colbath, Steve W. Dow, Jennifer N. Phillips, C. Wayne McIlwraith, Laurie R. Goodrich, *Frontiers in Veterinary Science*, Aug 31;5:208, 2018). My contributions to this publication included performing laboratory assays, statistical analysis and the writing the majority of the manuscript. Minimal modifications were made to meet formatting requirements and table and figure numbers have been modified. This article is reproduced with permission from Frontiers Media, Seattle, WA.

PIH. The MCJ had significantly higher total protein concentration at 6 PIH, and a significantly higher neutrophilic infiltration than the MCJ at 6 PIH and 168 PIH. This study establishes that the same intra-articular dose of rIL-1β elicits significantly different clinical and cytological responses in the MCJ compared to the TTJ in the equine model of intra-articular synovitis. In addition, clinical and cytological evidence of synovitis may persist up to or greater than 1 week following intra-articular administration of rIL-1β.

5.2 Introduction

Interleukin-1β (IL-1β), an inflammatory cytokine, has been used in multiple in vivo and in vitro inflammatory models of equine synovitis. [1-7] IL-1β has been detected in both human and equine naturally-occurring osteoarthritis (OA), and causes the production of other destructive mediators of OA including matrix metalloproteases (MMPs) and prostaglandin-E_2.[2; 5; 8; 9] Further, treatments directed at reducing IL-1β, such as interleukin-1 receptor antagonist protein, have resulted in improved clinical outcomes and reduced joint destruction. [10-12]

Recombinant interleukin-1β (rIL-1β) produces a reliable, reproducible, short-term synovitis in the equine middle carpal joint (MCJ). [2] The recombinant, equine-specific, cytokine is readily available from a commercial vendor and easily reconstituted for intra-articular administration. A study by Ross et al. (2012) comparing the inflammatory response elicited by rIL-1β to that of lipopolysaccharide describes the clinical and cytologic effects of 100ng of rIL-1β administered into the MCJ. However, an additional study by Toth et al. (2014) describing the use of rIL-1β in the stifle reports more severe lameness than described for the MCJ. Further, a study conducted by Carmalt et al. (2011) revealed that various joints may respond differently to inflammation.

A recent study [7] and the experiences of the authors of this current study with rIL-1β in the tibiotarsal joint (TTJ) led to the question whether the TTJ may have a different clinical and cytological response to the administration of rIL-1β than described for the MCJ. We felt this was an important question because previous studies have assumed the response to a treatment agent is equivalent between MCJ and TTJ and have drawn conclusions regarding the immunomodulatory ability of treatments such as mesenchymal stem cells using TTJ and MCJ as equivalent joints to investigate treatments. [13; 14] Further, variability in the TTJ and MCJ joint is important when determining the dose of rIL-1β appropriate for research studies, while comparing treatment responses and evaluating treatment strategies and clinical responses. Therefore, the first objective of the current study was to determine the clinical and cytological response of the TTJ to the administration of 75ng of rIL-1β. We hypothesized that there would be a cytological response that was reflective of the lameness parameters and that the response would be acute (less than 3 days). The second objective was to compare the cytological and clinical responses of rIL-1β administered into the TTJ versus the MCJ. We hypothesized that administration of rIL-1β in the TTJ would result in a greater inflammatory response when compared to the MCJ.

5.3 Materials and Methods

Experimental Design

Twelve horses were utilized for the study. Initial lameness examinations were conducted two weeks prior to the start of the study. Eight horses were administered 75ng of commercially available rIL-1β (R&D systems, Minneapolis, MN) into the MCJ with no other treatment. After a 4 week wash out period these same 8 horses entered a subsequent study with administration of rIL-1β and a treatment into the TTJ (data not shown). The investigators became aware that the two joints being investigated, the MCJ and TTJ, may respond differently to the same dose of rIL-

1β. Therefore, the investigators designed and executed the current study, comparing the response of MCJ and TTJ to the same dose of rIL-1β alone with no concurrent treatment (Figure 5.1); four additional horses were administered 75ng of rIL-1β into a single tibiotarsal joint.

N = 12 horses

Middle carpal joint:
75ng rIL-1β

8 horses

Tibiotarsal joint:
75ng rIL-1β

4 horses

Figure 5.1. Experimental design. Twelve horses were enrolled in the study. Eight horses received 75ng rIL-1β into the middle carpal joint. Four horses received 75ng rIL-1β into the tibiotarsal joint. No other treatments were administered prior to or within 4 weeks following rIL-1β administration.

An *a priori* power calculation was performed using Lenthe's power calculator (https://homepage.divms.uiowa.edu/~rlenth/Power/index.html) based on the means and standard deviations for the nucleated cell counts (NCC) and total proteins obtained from the first 8 horses enrolled in the study. The *a priori* power calculation found that four additional horses would produce a power of 0.8, accounting for an alpha error rate of 0.5, if the difference in total protein was 1 gm/dL and the difference in NCC was 34 x 10^3 cells/uL. When comparing the MCJ and TTJ response to rIL-1β, the initial data suggested a difference in mean NCC of approximately 40

x 10^3 and a difference in total protein of 1.2 gm/dL. Therefore, 4 additional horses were used to investigate the same dose of rIL-1β administered into the TTJ (without a concurrent treatment) (Figure 5.1).

All horses were determined to be sound by two ACVS board-certified large animal surgeons on a straight line at the trot prior to enrollment in the study. Horses had no joint effusion present in the MCJ or TTJ and no response to flexion. Horses ranged in age from 2-5 years old (mean age: 3.625 years) and were mixed breed. Treatment limbs were randomized using a random number generator (www.random.org), and all investigators and staff were unaware of treatment assignment with the exception of the first author. This work was conducted under the approval of the Institutional Animal Care and Use Committee of Colorado State University (15-5810A). The treatment (75ng of rIL-1β) were diluted in phosphate buffered saline and administered as 1ml. All joints were clipped and aseptically prepared before administration of rIL-1β, and treatments were administered using aseptic technique.

Evaluation of clinical response to treatment

A physical examination including heart rate, respiratory rate and temperature and lameness evaluation was performed, and joints were evaluated for joint circumference, and joint effusion at 0, 6, 12, 24, 72, 168 (1 week), and 336 (2 weeks) post-injection hours (PIH). Subjective lameness examination was conducted by trotting animals, in-hand, and graded using the AAEP lameness scale (https://aaep.org/horsehealth/lameness-exams-evaluating-lame-horse). Subjective lameness was reported as the mean change in lameness for each time point. The change in lameness was calculated for each horse at each time point by subtracting any baseline lameness observed at 0 PIH.

At each time point joint circumference (cm) was measured three times, consecutively, at the same location (at the point of greatest circumference). This location was determined in the normal joint prior to the initiation of the study and marked by clipping hair at the location of measurement. The three values were averaged for each time point. Joint effusion was given a subjective clinical grade with grade 0 indicating no effusion, grade 1 indicating slight effusion, grade 2 indicating mild effusion, grade 3 indicating moderate effusion, and grade 4 indicating severe effusion.

Synovial fluid analysis

Synovial fluid was harvested prior to treatment (0 PIH) and 6, 12, 24, 72, 168, and 336 PIH. Arthrocentesis was performed aseptically following clinical assessment. Horses were sedated using detomidine hydrochloride (0.01 mg/kg IV) and butorphanol tartrate (0.01 mg/kg IV). Synovial fluid was immediately placed in plain glass tubes and processed within 1 hour of collection. A portion of the aspirate was used for direct smear and cytospin analysis prior to hyaluronidase digestion and analysis for total nucleated cell count using an automated cell counter. Total protein content was determined using a refractometer. Differential neutrophil, monocyte, lymphocyte and eosinophil counts were evaluated using direct smear and cytospin analysis. The remainder of the synovial fluid was centrifuged for 10min at 1000xg and the supernatants were stored at -80°C in Eppendorf tubes until ELISA analysis could be performed. Multiple aliquots were frozen to prevent freeze-thaw cycles.

Enzyme-linked immunosorbent assays

Synovial Prostaglandin E2 (PGE_2) was evaluated as previously described.[2] Briefly, a solid-state extraction was performed using C2 ethyl mini-columns prior to quantification using a

commercially available equine specific PGE_2 Enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, Farmingdale, NY). Synovial C-reactive protein was evaluated using a commercially available ELISA kit (ICL Laboratories, Portland, OR).

Statistical analysis

Clinical (subjective and objective lameness, joint circumference, joint effusion) and synovial fluid data (nucleated cell count, total protein, differential cell counts) were compared using a two-way mixed ANOVA for repeated measures with time defined as the within subjects factor, and the joint (TTJ versus MCJ) defined as a between-subjects effect. Significance was set at $P<0.05$. Simple effects between treatments were analyzed using a Tukey's multiple comparisons test. Normality was assessed by evaluating diagnostic plots of the residuals for each variable. Log transformation was performed for nucleated cell count data. Statistical analysis was conducted using the R "lsmeans" statistical package (version 3.3.3).

5.4 Results

Clinical responses

Physical examination parameters (heart rate, respiratory rate and temperature) were measured at each time point. Although temperature was not different between groups, rIL-1β administered into the TTJ resulted in a greater respiratory rate at 24 hours ($P=0.0013$) (mean, MCJ: 17 bpm vs. TTJ: 26 bpm) and a greater heart rate at 12 hours ($P=0.0018$) (mean, MCJ: 38 bpm vs. TTJ: 56 bpm) when compared to horses receiving rIL-1β in the MCJ (Figure 5.2).

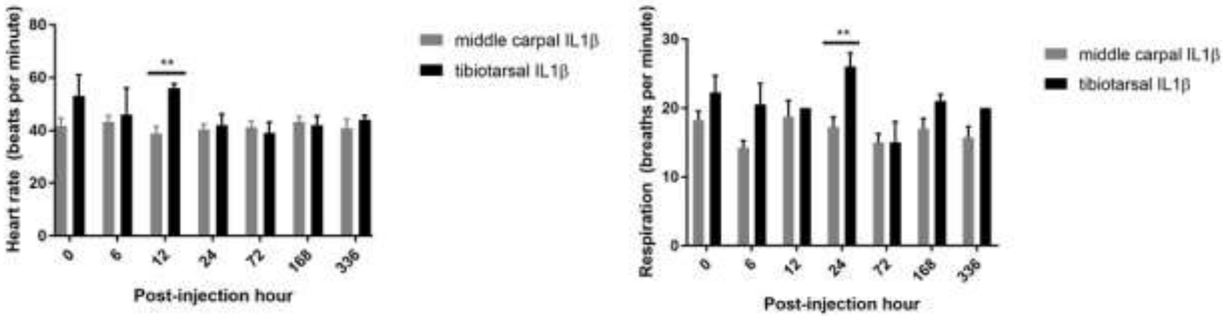

Figure 5.2. Heart rate and respiratory rate of horses prior to and following rIL-1β administration into the middle carpal and tibiotarsal joint. An increase in mean heart rate and respiratory rate is seen in horses receiving rIL-1β into the TTJ at 12 and 24 PIH, respectfully, when compared to the MCJ. Error bars represent the standard error of the mean (SEM) and significance is indicated by ** (P<0.001).

Although horses were evaluated for lameness (in-hand at the trot) two weeks prior to starting the study and determined to be sound by two ACVS board certified large animal surgeons using the AAEP grading scale, one horse in each group was found to have a grade 1 lameness at baseline. Therefore, each horse's lameness was calculated at each timepoint as a change in AAEP lameness grade from baseline. Interestingly, both horses with a grade 1/5 lameness at baseline were found to have no lameness two weeks following rIL-1β administration. Therefore, change in lameness for these horses was reported as a value of "-1" at 168 PIH. In all horses administered rIL-1β into the MCJ, subjective lameness scores increased by 6 PIH (P=0.0013) (mean change, MCJ: 3). In contrast, horses administered rIL-1β into the TTJ showed a significant increase in subjective lameness by 12 PIH compared to baseline measurements (P<0.0001) (mean change, TTJ: 3.12). Lameness continued above baseline, for both groups, until 72 hours post-injection. There was no difference between the change in lameness when rIL-1β was administered in the MCJ versus the TTJ at any time point (Figure 5.3).

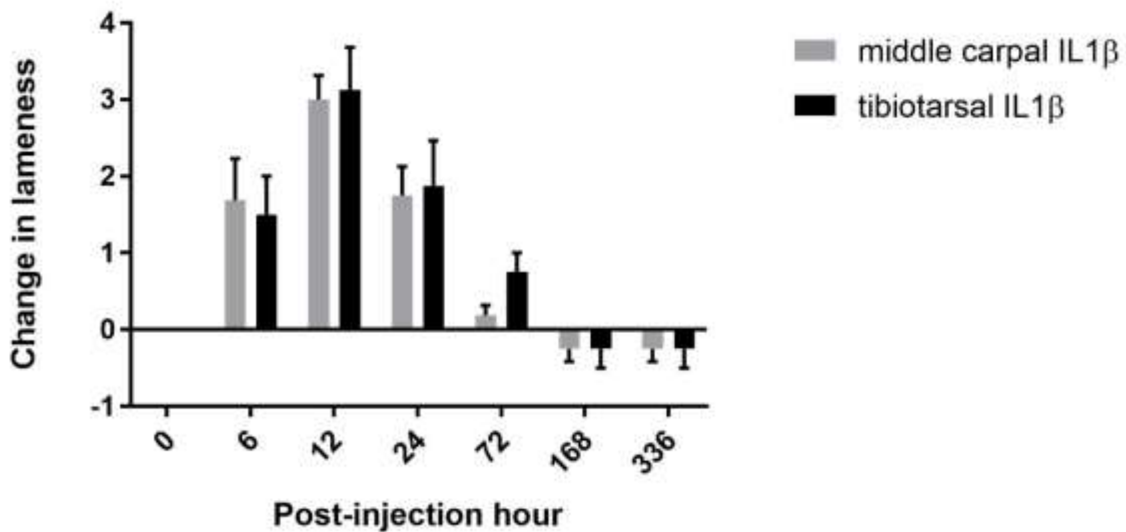

Figure 5.3. Change in subjective lameness score. There was no difference detected in the mean change of AAEP lameness scores when horses were administered rIL-1β in the MCJ or TTJ. Error bars represent the SEM.

The mean joint circumference at baseline for the MCJ and TTJ were 26.81 +/- 0.912 cm and 31.12 +/- 1.46 cm, respectively. The change in joint circumference was measured over time for both groups. Both treatment groups demonstrated an increase in joint circumference by 72 PIH (mean change, MCJ: 2.07 cm (P=0.0233) vs. TTJ: 2.96 cm, (P=0.0036)) with the TTJ showing increased joint circumference at 24 PIH (P=0.002) (mean change, TTJ: 3.75 cm). Change in joint circumference was greater for the horses administered rIL-1β into the TTJ at 24 hours when compared to horses administered rIL-1β into the MCJ (Table 5.1) (mean change, MCJ: 1.21cm vs. TTJ: 3.75 cm) (P=0.0015). For both treatment groups, an increase in subjective effusion grade was noted at 6 PIH (P<0.05) (mean change, MCJ: 1.75 (P<0.0001) vs. TTJ: 1.50 (P=0.0017)). Horses receiving rIL-1β into the TTJ had a greater change in subjective effusion grade versus the MCJ at 24 PIH (Table 5.1) (mean change, MCJ: 2.07 vs. TTJ: 3.25) (P=0.0096) and 336 PIH (mean change, MCJ: 2.50 vs. TTJ: 1.25) (P=0.0274).

Table 5.1. Joint circumference and effusion scores following IL1β administration. Significant differences between the MCJ and TTJ are noted by * (P<0.05) and ** (P<0.01).

	Middle carpal joint mean (+/- SD)	**Tibiotarsal joint mean (+/- SD)**	**P-value**
Change in effusion score			
0 PIH	0 (+/- 0)	0 (0 +/- 0)	1.0
6 PIH	1.75 (+/- 0.46)	1.5 (+/- 0.58)	0.58
12 PIH	2.38 (+/- 0.52)	3.0 (+/- 0.82)	0.16
24 PIH	2.06 (+/- 1.08)	3.25 (+/- 0.50)	**0.01***
72 PIH	1.25 (+/- 0.89)	2.0 (+/- 0)	0.09
168 PIH	1.0 (+/- 1.07)	1.25 (+/- 0.50)	0.57
336 PIH	0.25 (+/- 0.89)	1.25 (+/- 0.50)	**0.03***
Change in circumference (cm)			
0 PIH	0 (0 – 0)	0 (0 – 0)	1.0
6 PIH	0.65 (+/- 0.54)	0.41 (+/- 1.7)	0.74
12 PIH	0.94 (+/- 0.57)	1.52 (+/- 1.93)	0.44
24 PIH	1.21 (+/- 1.17)	3.75 (+/- 2.03)	**0.002****
72 PIH	2.07 (+/- 2.53)	2.96 (+/- 0.98)	0.24
168 PIH	0.91 (+/- 0.68)	1.97 (+/- 0.78)	0.16
336 PIH	0.45 (+/- 0.56)	1.55 (+/- 0.95)	0.15

Synovial fluid analysis

Synovial fluid was analyzed for total nucleated cell count (NCC) and total protein, and percent neutrophils, monocytes, lymphocytes, and eosinophils were calculated using a

differential cytology determined by cytospin or direct smear. Six of eight horses' receiving rIL-1β in the MCJ had a NCC peak at 6 hours and the remaining two horses peaked at 12 hours. All horses that received rIL-1β into the TTJ had a NCC peak at 12 PIH. The NCC was higher in the MCJ at 24 PIH (P=0.0005) (mean NCC, MCJ: 56.25 $x10^3/\mu l$ vs. TTJ: 5.96 $x10^3/\mu l$) and 72 PIH (P=0.04) (mean NCC, MCJ: 5.03 $x10^3/\mu l$ vs. TTJ: 0.98 $x10^3/\mu l$) when compared to the TTJ joint (Figure 5.4). Despite a higher NCC in the MCJ, neutrophilic infiltration occurred faster in the TTJ resulting in a significantly larger percentage of neutrophils in the TTJ versus MCJ at 6 PIH (P=0.007) (% neutrophils, MCJ: 64.13% vs. TTJ: 93.50%). Likewise, the monocytic population remained higher in the MCJ synovial fluid versus the TTJ synovial fluid at 6 PIH (P=0.0264) (% monocytes, MCJ: 27.37% vs. TTJ: 6.50%) (Figure 5.4). In addition, the TTJ experiences a longer duration of neutrophilic inflammation resulting in a significantly greater percentage of neutrophils at 168 PIH (1 week) versus the MCJ (P=0.0061) (% neutrophils, MCJ: 8.88% vs. TTJ: 38.75%). The total protein increased faster in the MCJ, resulting in a significant increase from baseline at 6 PIH (P<0.0001). Conversely, a significant increase in total protein was not detected in the TTJ until 12 PIH (P<0.0001). The total protein in the MCJ was significantly greater than that of the TTJ at 6 PIH (P=0.0228) (mean total protein, MCJ: 4.33 g/dL vs. TTJ: 3.20 g/dL) (Figure 5.4).

Figure 5.4. Cytologic analysis following rIL-1β administration. Total nucleated cell count (NCC) was higher at 24 PIH and 72 PIH when rIL-1β was administered into the MCJ. In contrast, the percent of neutrophils was increased in the TTJ when compared to the MCJ at 6 PIH and 168 PIH. Error bars represent SEMs and significance is indicated by * (P<0.05), **(P<0.01) and ***(P<0.0001).

<u>Synovial fluid biomarkers</u>

No significant differences were detected in synovial fluid levels of PGE_2 and C-reactive protein between treatment groups (Figure 5.5).

Figure 5.5. Synovial fluid biomarkers. There was no significant difference in synovial PGE_2 or synovial CRP levels between MCJ and TTJ at any time point.

5.5 Discussion

This study was performed to clarify differences between injecting equivalent doses of rIL-1β in the TTJ compared to the MCJ because subjectively, a previous report as well as clinical observations by the authors of the current study, suggested that these joints may respond differently to the same dose of rIL1-β. [7] Further, no other reports reveal the longitudinal, clinical and cytological changes that occur without intervention (such as joint lavage, or biopsies) when rIL-1β is administered into the MCJ or TTJ. The results of this work highlight the differences between the response to rIL-1β in commonly studied joints used for modeling synovitis and provide a reference of respective joint and systemic reactions to rIL-1β. Although synovial biopsies and arthroscopic examination would have provided additional information [2; 7], they also require invasion of the joint capsule and/or joint lavage which could significantly change the cytological parameters measured. Therefore, we excluded these procedures to obtain a two-week assessment of clinical and cytological findings without confounding results with biopsy or surgical lavage which would be used to assess gross and histological changes in response to rIL-1β.

The present study revealed the greatest increase in both TTJ and MCJ circumference (synovial effusion) was at 24 PIH. In contrast, a recent study that utilized standing arthroscopy to perform biopsy samples 10 hours following administration of rIL-1β into the tibiotarsal joint reported a decrease in synovial effusion at 24 PIH (following arthroscopic biopsy) when compared to 4 PIH. Without arthroscopic lavage, the current study demonstrated the maximum increase in effusion score for both TTJ and MCJ was at 24 hours. Additionally, significant ($P<0.05$) effusion was detected as late as 336 PIH in the tibiotarsal joint. This finding was unexpected as other studies report a shorter period of post injection effusion. [2; 7] Likewise,

neutrophilic inflammation was still present in the tibiotarsal joint at 168 PIH. Therefore, previous studies [2; 7] may have underestimated the effect of rIL-1β on the duration of neutrophilic inflammation due to surgical lavage that was performed to assess the joint.

Interleukin-1 beta promotes multiple inflammatory mediators including nitric oxide, PGE_2, chemokines, adhesion molecules, matrix metalloproteinases, and multiple cytokines leading to synovitis, cartilage destruction and ultimately osteoarthritis. [2; 5; 8; 9] There is significant precedent for the use of rIL-1β to induce inflammation *in vitro* assays [5; 15-17]; however, only a few studies have reported the utility of rIL-1β for *in vivo* studies. [1; 2; 4; 7; 18] Ross et al. (2012) provided the first description of rIL-1β to induce acute synovitis in the MCJ of the horse. Four additional horse studies have followed; one that also utilized the carpus, two which sought to induce acute synovitis in the equine stifle, and a recent study using rIL-1β in the tibiotarsal joint. [1; 4; 7; 18] The first study used 100ng of rIL-1β in the joint in the carpus, [2] while a pilot study used 100ng of rIL-1β in the stifle, [4] and later, the same group conducted a study using 200ng of rIL-1β in the stifle. [1] Most recently, a study initially used 100ng of rIL-1β in the tibiotarsal joint prior to reducing the dose to 50ng. [7] No studies have compared different joint responses within the same individual to equivalent doses of rIL-1β.

The TTJ, like the MCJ, has distinct advantages for joint studies, including its accessibility, and a large volume of synovial fluid for sampling. A previous study compared the TTJ to the MCJ to analyze joint responses to a therapeutic intervention (stem cells). [13] Another study assumed the MCJ and TTJ would react similarly to lipopolysaccharide if treatment dose was adjusted for relative joint volume. [14] However, our study indicates that it would be inappropriate to assume that the MCJ and the TTJ would respond similarly to a treatment agent. This is important when determining both study design and drawing conclusions with regard to

intra-articular treatments based on cytological differences. The study also reinforces the importance in determining individual joint responses to an inflammatory agent such as rIL-1β or lipopolysaccharide.

No differences were measured between the change in subjective lameness when rIL-1β was administered in the TTJ and MCJ. A post-hoc sample size calculation supports an equivalent lameness between treatment groups, as over 1200 horses would be needed to find a one-degree difference in subjective lameness using the observed standard deviation with 80% power. But, horses in which the TTJ was injected with rIL-1β, had a significantly higher heart rate and respiratory rate at 12 and 24 PIH than horses receiving rIL-1β in the MCJ which may indicate an increased pain level. Limitations in the range of values (0-5) within the AAEP scale may have decreased our ability to detect more subtle differences. Despite the limitations of lameness scale, our study supports a similar duration (72 hours) and a degree of lameness between the TTJ and MCJ when the AAEP lameness grading scale is used.

Recombinant IL-1β is known to cause a substantial synovitis characterized by rapid neutrophilic infiltration. [2] The level of neutrophilic inflammation has been described in the MCJ but no other joints.[2] Our study is the first to characterize the cytologic response of the TTJ to rIL-1β for 336 PIH and further, to report responses without interceding with joint lavage and/or cartilage and synovial biopsies. We highlight here how the MCJ and TTJ responded differently to the same dose of rIL-1β and provide researchers data concerning the responses of the MCJ and the TTJ to rIL-1β. Finally, this may also suggest how the TTJ and MCJ may respond differently in the clinical setting to acute, non-septic, inflammation or how joint type may affect the progression of osteoarthritis.

Total cellular infiltration as a result of rIL-1β administration was significantly lower in the TTJ compared to the MCJ at 12 hours PIH. The sampling times of the current study were slightly different than those performed previously by Ross et al. (2012). [2] However, the previous study of the MCJ found a mean NCC at 4 PIH (134.30 x 10^3) and 8 PIH (170 x 10^3), [2] similar to those reported here for 6 PIH (110.60 x 10^3) and 12 PIH (176.15 x 10^3). As expected from previous studies, the increased NCC is a result of neutrophil infiltration into the joint, where neutrophils compose greater than 70% of the MCJ NCC at 12 hours, and greater than 90% of the TTJ NCC at 12 hours. In the results, we reported both total NCC and the percentage of each cell type instead of reporting total differential cell counts. This was done as reporting total cell numbers for differential cell types such as neutrophils or monocytes would have disguised an important difference between groups. Namely, the percent neutrophils were higher in the TTJ despite a lower NCC. By 24 hours, the NCC was statistically and substantially higher in the MCJ versus the TTJ (mean NCC, MCJ: 56.25 x10^3μl vs. TTJ: 5.96 x10^3/μl) and stayed consistently higher through 72 PIH (mean NCC, MCJ: 5.03 x10^3/μl vs. TTJ: 0.98 x10^3/μl). Although the TTJ had a lower total NCC compared to the MCJ, a greater percentage of neutrophils composed the inflammatory infiltrate in the TTJ at 6 PIH and 168 PIH. In summary, there was a higher percentage of neutrophils but lower total NCC in the TTJ compared to the MCJ. This may be attributed to an increased synovial fluid produced in the TTJ. The TTJ had a more rapid increase in joint circumference than the MCJ and a greater increase in joint circumference at 24 PIH. Likewise, the subjective joint effusion scores of the TTJ were significantly higher than the MCJ at 24 PIH. Synovial fluid, an ultrafiltrate, likely caused a "dilutional" effect in the TTJ resulting in a decreased total NCC despite a higher percentage of neutrophils.

The MCJ has a synovial continuation with the carpometacarpal joint and the TTJ has a synovial continuation with the proximal intertarsal joint. The TTJ appears to accommodate a larger volume of fluid then the MCJ (Colbath AC, *unpublished data*). Although both the MCJ and TTJ have dorsal and palmar/plantar extensions, the palmar extension of the MCJ is firmly attached to the third carpal bone. Both the dorsal and palmar/plantar pouches of the MCJ and TTJ are lined by synovium. The volume of the MCJ and TTJ have not been compared in the literature. However, in one study, arthrocentesis of the TTJ resulted in 6.25 – 21 ml of synovial fluid (mean: 10ml +/- 1.2ml). [19] Our clinical and arthroscopic experience indicates that the tibiotarsal joint has a larger joint volume and greater synovial lining pliability when compared to the MCJ. Interleukin-1β results in the production of many cytokines produced by synoviocytes including interleukin-8 which is a chemokine that initiates neutrophilic activation and recruitment. [20; 21] The larger TTJ joint pouch lending to greater synovial surface area, may result in larger amounts of subsequent neutrophilic migration into the joint. In addition to differences in the NCC between the MCJ and TTJ, the MCJ had a faster increase in total protein and a greater total protein at 6 PIH when compared to the TTJ. Again, this could be explained by a greater increase in synovial fluid, an ultrafiltrate, in the TTJ when compared to the MCJ.

The initial volume of the TTJ may be greater than the MCJ for the same dose of rIL-1β; however, the change in lameness is similar. Conversely, physical examination characteristics (heart rate and respiration) suggest potentially greater pain associated with rIL-1β administration in the TTJ. The increase in pain may be explained by increased synovial fluid production, leading to an increase in joint circumference and effusion resulting in stretching of the joint capsule and a greater pain response from joint distention.

Different cohorts of horses were utilized instead of a washout model, as previous equine rIL-1β studies had not established the duration of effect without biopsy or lavage. Synovial biopsies were not taken during the study period. However, two horses that were administered rIL-1β into the TTJ were euthanized for a different study and synovial biopsies were taken at the time of euthanasia, approximately 98 days post-injection. At the time of necropsy, one horse had an increased synovial cellular infiltration, intimal hyperplasia, and subintimal fibrosis compared to the un-injected TTJ. These results would indicate a model employing a "washout period" may be inappropriate unless the washout period is lengthy or joint lavage is performed.

All horses received the same dose of rIL-1β. This was done to provide a comparison between the joint response to the same dose of rIL-1β. Alternatively, the dose could have been titrated to the estimated volume of the joint but this would be difficult and was beyond the scope of this study. All rIL-1β in this study was from the same lot and stored and reconstituted identically. This is important as different lots and methods of storage and reconstitution may lead to varying activity levels. [1; 2; 7] A future study could also compare the response of both TTJ and MCJ to a dose escalation of rIL-1β.

Although an *a priori* power calculation was performed and our sample size was adequate to detect statistical differences in both clinical (other than lameness) and cytological parameters including heart rate, respiratory rate, joint effusion and differential cell counts, the small sample size remains a limitation of the study. However, due to the small standard deviations in the observed cytological and clinical parameters, post-hoc power calculations revealed the statistical power to exceed 80% for all parameters excluding heart rate and total protein concentration. Further, the post-hoc power calculation for total protein exceeded 70%.

In conclusion, we had hypothesized that administration of rIL-1β in the TTJ would result in an acute (< 3 days) cytological and clinical response and that inflammation would be greater in the TTJ when compared to the MCJ. Our hypotheses were partially correct; the inflammation could not be characterized as acute. However, the TTJ does have a longer-lasting inflammatory response characterized by greater neutrophilic inflammation when compared to the MCJ. Although lameness subsided within 3 days, neutrophilic inflammation persisted in the TTJ (and was significantly greater than the MCJ) at 1-week post-injection, and effusion was still detectable in the TTJ at two weeks post-injection. These results indicate that a greater than 2-week washout period is necessary when administering IL-1β into the TTJ. Although the TTJ experienced a longer duration of effusion and neutrophilic inflammation, the total NCC were lower in the TTJ at 24 and 72 PIH when compared to the MCJ. This study provides important clinical and cellular parameters for future investigations in which researchers plan to utilize rIL-1β in an equine model of intra-articular inflammation. Previous studies have used the MCJ as a control for treatments administered into the TTJ. [13] The current study provides evidence of varying cytological responses between the TTJ and MCJ and suggests that these joints should not be considered similar in the clinical and cytological responses. In addition, this is the first study to describe the clinical effects, cytology, total protein, and inflammatory mediators resulting from the administration of rIL1β into the equine TTJ or MCJ for 336 PIH.

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Chapter 6: Allogeneic and autologous equine bone marrow-derived mesenchymal stem cells are safe but ineffective following a single and repeated intra-articular injections for reducing acute inflammation in an experimental interleukin-1β model of synovitis[5]

6.1 Overview

Allogeneic and autologous bone marrow-derived mesenchymal stem cells (BMDMSCs) have been administered in equine joints for their anti-inflammatory effects. However, allogeneic BMDMSC offer multiple clinical and practical advantages. Therefore, it is important to determine the relative effectiveness of allogeneic versus autologous BMDMSCs. The objective of this randomized-controlled study was to compare the inflamed joint response to autologous versus allogeneic BMDMSCs injections, and to determine if either treatment generated an anti-inflammatory effect. Bone marrow was harvested from eight horses. Autologous BMDMSCs and pooled-allogeneic BMDMSCs were culture expanded, cryopreserved and thawed immediately prior to administration. Ten million autologous BMDMSCs were administered with 75ng rIL-1β into one tibiotarsal joint and the contralateral tibiotarsal joint received allogeneic BMDMSC plus 75ng rIL-1β. Repeat injections were performed with the same treatment administered into the same joint. Four additional horses received 75ng rIL-1β alone in a single tibiotarsal joint. Clinical parameters (lameness, joint circumference and joint effusion) and synovial fluid parameters including nucleated cell count (NCC), differential cell count, total protein (TP), prostaglandin E_2 (PGE_2) and C-reactive protein (CRP) were measured at baseline, 6, 12, 24, 72, 168, and 336 hours post-injection. No difference was detected between autologous and

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allogeneic treatment groups with respect to subjective lameness, joint effusion, joint circumference, NCC, TP, differential cell count, CRP or PGE_2. Neither autologous nor allogeneic treatments resulted in an improvement in clinical or cytological parameters over that elicited by rIL-1β alone. This study revealed allogeneic and autologous BMDMSCs resulted in an equivalent clinical and cytological response. Allogeneic and autologous BMDMSCs were equally ineffective in reducing the inflammatory response from rIL-1β-induced joint inflammation in horses.

6.2 Introduction

Bone marrow-derived mesenchymal stem cells (BMDMSCs) have shown promise in the treatment of inflammatory musculoskeletal conditions including osteoarthritis, desmitis and tendonitis. [1-6] In vitro studies have documented the anti-inflammatory effects of both allogeneic and autologous BMDMSCs. [7; 8]

Interleukin-1β (IL-1β) is an important inflammatory mediator in naturally-occurring, equine, osteoarthritis (OA) which within the joint environment results in the production of matrix metalloproteases and prostaglandin-E_2. [9; 10] Because of this, treatments directed against IL-1β such as IL-1β receptor antagonist protein have resulted in improved clinical outcomes and disease modifying effects. [11; 12] Although BMDMSCs have been increasingly used as an anti-inflammatory joint therapy, no studies have investigated the effect of BMDMSCs in an inflammatory model of disease. These controlled, experimental models are important for assessing BMDMSCs as an anti-inflammatory therapy.

Researchers have long argued over the use of allogeneic BMDMSCs for intra-articular injection. Allogeneic BMDMSCs have been used to treat horses with joint disease without inducing obvious negative effects. [1; 13] Experimental studies comparing allogeneic and

autologous BMDMSCs have yielded conflicting results. [14-17] A study by Joswig et al. (2017) suggested that allogeneic BMDMSCs may be more inflammatory. However, the study showed only a single statistically significant difference between allogeneic and autologous BMDMSC, with a higher nucleated cell count (NCC) for a single day following the second injection of BMDMSCs. [15] This study and other studies have compared allogeneic and autologous BMDMSCs by administering the treatment in different cohorts of animals. With variable individual reactions of horses to intra-articular treatments, this can be difficult to interpret. Therefore, the present study sought to compare allogeneic and autologous BMDMSCs within the same cohort of animals.

The objectives of this study were three-fold. First, the authors sought to compare the reaction of the inflammatory joint to allogeneic and autologous BMDMSCs. Second, we sought to determine whether a repeat injection changed the intra-articular response to BMDMSC injection within inflamed joints. Third, we sought to determine whether autologous or allogeneic BMDMSCs elicit an equivalent anti-inflammatory effect when injected into joints with recombinant equine interleukin-1β (rIL-1β) induced inflammation. We hypothesized that there would be no difference in the reaction of the inflamed joint to autologous versus allogeneic BMDMSCs after a single injection or two repeated injections, and that both autologous and allogeneic BMDMSCs would result in an anti-inflammatory effect.

6.3 Materials and Methods

Animals

Eight horses (sixteen joints) were used in the study. Horses were of mixed breed and ranged in age from 2-5 years old. All horses were determined to be sound prior to entering the

study with no response to joint flexions and no effusion present in the tibiotarsal joints. Treatment limbs were randomized, and all investigators and staff were unaware of treatment assignment with the exception of the first author. This work was conducted under the approval of the Institutional Animal Care and Use Committee of Colorado State University (15-5810A).

BMDMSC isolation, culture, expansion, cryopreservation

All horses were sedated with detomidine (0.01 mg/kg IV) and butorphanol (0.01 mg/kg IV),the sternum was clipped and aseptically prepared, and 5ml of bone marrow was aspirated into 3 heparinized syringes for each horse as previously described. [18] Centrifugation was used to remove red blood cells from bone marrow aspirates, and bone marrow was cultured overnight in low-glucose DMEM, 10% fetal bovine serum (FBS), 10,000 U/ml of penicillin-streptomyocin-amphotericin B (PSA) and 1N HEPES. After 24 hours, the media was removed and replaced, and colonies were allowed to form over the next 7-10 days. Established colonies were then dissociated with Accumax™, and cells were transferred to new flasks and cultured in αMEM supplemented with 10% FBS, 10,000 U/ml PSA, 1N HEPES, and 2 ng/ml of fibroblast growth factor (FGF). The cells were passaged three times in monolayer and then cryogenically preserved in 95% serum and 5% DMSO. Autologous cells were cryopreserved in 95% autologous serum. Allogeneic cells were combined into two groups of four horses at the time of cryopreservation and serum was pooled from all four of the horses for cryopreservation.

Treatment groups

Commercially available equine recombinant IL-1β (R&D systems, Minneapolis, MN) was used. The autologous treatment (AUTO) limb was given 75ng of rIL-1β with 10 million autologous BMDMSCs in 1ml of freeze media. The allogeneic treatment (ALLO) limb was

given 75 ng of rIL-1β with 10 million pooled-allogeneic BMDMSCs in 1 ml freeze media. Eight horses received one treatment in one tibiotarsal joint and the other treatment in the contralateral tibiotarsal joint a week later. All treatments were administered into the dorsal pouch of the tibiotarsal joint. Treatments were then repeated at 2 weeks in the same limb they were given previously (again, with a one-week interval between treatments). Whether the horse received allogeneic or autologous BMDMSCs for the first treatment was determined randomly using a random number generator (www.random.org). Four additional horses were treated once with 75 ng of rIL-1β in the dorsal pouch of the tibiotarsal joint with no other treatment (previously published data).[19] These horses underwent the same clinical and cytological analysis as the AUTO and ALLO treatment groups and were used to evaluate the effect of BMDMSCs on the synovitis created by the rIL-1β model. Only the first injection of AUTO and ALLO were compared to rIL-1β alone. The study design is described in Figure 6.1.

Figure 6.1. Experimental design. Eight horses received two injections of AUTO and ALLO BMDMSCs with rIL-1β in contralateral tibiotarsal joints at 1 week intervals. The first treatment (AUTO vs. ALLO) was randomly chosen. In this example, AUTO BMDMSCs were given first.

Four additional horses were administered rIL-1β alone in the tibiotarsal joint. Each animal was assessed at baseline then 6, 12, 24, 72, 168 and 336 hours after injection.

Treatment Administration

All joints were clipped and aseptically prepared before administration and treatments were administered using aseptic technique. Horses were sedated with detomidine hydrochloride (0.01 mg/kg IV) and butorphanol tartrate (0.01 mg/kg IV). Clinical assessment and arthrocentesis were performed prior to treatment administration. Treatments were administered through the same needle without needle manipulation. rIL-1β was always given first, followed by the appropriate BMDMSC treatment (ALLO or AUTO).

Evaluation of clinical response to treatment

Clinical evaluations were performed at 0, 6, 12, 24, 72, 168 (1 week), and 336 (2 weeks) post-injection hours (PIH) including a physical examination and lameness. Clinical assessment and arthrocentesis were performed prior to treatment administration. At each time point, joints were evaluated for joint circumference, joint effusion, and heat. A board-certified equine surgeon (AC) performed all subjective lameness evaluations. Subjective lameness was graded using the AAEP lameness scale; half points were awarded at the discretion of the evaluator. [20] Wireless motion analysis system (Lameness Locator®) was used for objective lameness evaluation.

The location of joint circumference measurement was chosen by palpation (as the middle of the joint pouch) prior to beginning the study and marked by clipping the hair. Joint circumference (cm) was measured three times, consecutively, at the same location on the limb at each time point. For each time point, the three values were averaged. Joint effusion was given a subjective clinical grade with grade 0 indicating no effusion, grade 1 indicating slight effusion,

grade 2 indicating mild effusion, grade 3 indicating moderate effusion, and grade 4 indicating severe effusion. All measurements were conducted by a single observer.

Synovial fluid analysis

At each time point (0, 6, 12, 24, 72, 168, 336 PIH), arthrocentesis was performed aseptically following clinical assessment. Horses were sedated using detomidine hydrochloride (0.01 mg/kg IV) and butorphanol tartrate (0.01 mg/kg IV) and synovial fluid was harvested from the dorsal pouch of the tibiotarsal joint prior to treatment. Synovial fluid was immediately placed in plain glass tubes and processed within 1 hour of collection. A portion of the aspirate was used for direct smear and cytospin analysis for determination of differential neutrophil, monocyte, lymphocyte and eosinophil counts. Hyaluronidase digestion was performed prior to using an automated cell counter to determine total nucleated cell count (NCC). Total protein (TP) content was determined using a refractometer. The remainder of the synovial fluid was centrifuged for 10min at 1000xg and the supernatants were stored at -80°C in Eppendorf tubes. Multiple aliquots were frozen to prevent freeze-thaw cycles until c-reactive protein (CRP) and Prostaglandin-E2 (PGE_2) analysis could be performed.

Enzyme-linked immunosorbent assays

Synovial Prostaglandin E2 (PGE_2) was evaluated as previously described, [21] using a commercially available equine specific PGE_2 Enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, Farmingdale, NY). Synovial C-reactive protein (CRP) was evaluated using a commercially available ELISA kit (ICL Laboratories, Portland, OR). Synovial PGE_2 and CRP were evaluated at baseline, 24 hours, 72hours and 168 hours following injection of AUTO, ALLO, and rIL-1β alone.

Statistical analysis

An a priori power analysis was performed. The power calculation was based on prior joint studies with described differences in clinical parameters, as well as synovial cytokine levels, total protein and nucleated cell counts. [6; 19; 21-23] The power calculation suggested that 8 horses would achieve a power of 0.8 and an alpha error rate of 0.05. Clinical and synovial fluid data were compared using a two-way ANOVA for repeated measures with time defined as the within subjects factor, and the treatment (AUTO vs. ALLO) defined as a between-subjects effect. In order to compare the eight horses given BMDMSCs versus the 4 horses only administered rIL-1β, a two-way ANOVA (without repeated measures) was performed. Means were compared between treatments at each time point using Sidak's multiple comparison test. Significance was set at $P<0.05$. Normality was assessed by evaluating diagnostic plots of the residuals for each variable. Log transformation was performed for nucleated and differential cell count data. Statistical analysis was conducted using GraphPad Prism (version 7.03).

6.4 Results

Physical examination

No difference was detected in the temperature, respiratory rate or heart rate between AUTO and ALLO treatment groups at any time point following the first or second injection (Figure 6.2). In addition, there was no difference in the temperature, respiratory rate or heart rate between the horses which received a single dose of rIL-1β alone compared to the AUTO and ALLO treatment groups (Figure 6.2).

Figure 6.2. Physical examination parameters. There was no difference in heart rate, respiratory rate, or temperature between AUTO and ALLO and those receiving BMDMSCs with rIL-1β at any time point after the first or second injection. Significance was set at P<0.05. Individual data points are plotted. Bar graphs indicate the mean and standard deviation.

Lameness

After the first injection, no difference was found in change in subjective lameness between AUTO and ALLO treatments at any time point (Table 6.1). Consistent with the rIL-1β model of synovitis, both treatment groups showed an increase in subjective lameness score by 6 hours post-injection (P<0.0001) and continued 24 hours post-injection for the AUTO treatment group (P<0.0001) and 72hours post-injection for the ALLO treatment group (P=0.02). When the subjective lameness scores of the AUTO and ALLO group were compared to horses receiving rIL-1β alone, the AUTO (P=0.008) and ALLO (P=0.04) treatment groups had a greater increase in subjective lameness score at 6 hours post-injection (Table 6.1).

Table 6.1. Change in subjective lameness score. Increase in subjective lameness score did not differ between AUTO and ALLO at any time point for the first or second injection. When the first injection of AUTO and ALLO were compared to a single injection of rIL-1β alone, there was a significant increase in lameness at 6 hours for both the AUTO (P=0.0082) and ALLO group (P=0.0387). The second injection of AUTO and ALLO was not compared to rIL-1β alone as only a single injection of rIL-1β was performed. Significance was set at P<0.05.

Time	*1st injection:* **Autologous+75ng rIL-1β** mean (sd)	*1st injection:* **Allogeneic+75ng rIL-1β** mean (sd)	*2nd injection:* **Autologous+75ng rIL-1β** mean (sd)	*2nd injection:* **Allogeneic+75ng rIL-1β** mean (sd)	*Single injection:* **75ng rIL-1β** mean (sd)	**P-value**
6	2.6[c] (0.7)	2.0[b] (1.9)	3.7 (1.1)	4.3 (0.3)	1.5[a] (1.0)	P(ab)=0.04 P(ac)=0.008
12	3.8 (0.4)	3.6 (0.7)	3.9 (0.9)	4.1 (0.2)	3.1 (1.1)	
24	3.6 (0.5)	3.3 (0.7)	2.7 (1.1)	3.2 (0.5)	1.9 (1.2)	
72	0.8 (1.0)	1.0 (1.1)	0 (0.9)	0.4 (0.5)	0.8 (0.5)	
168	0.4 (1.1)	0.5 (1.1)	0 (0.8)	0.6 (1.0)	0 (0.5)	

Following the second injection, no difference was found in change in subjective lameness between AUTO and ALLO treatments at any time point (Table 6.1). Both treatment groups showed an increase in subjective lameness score for 24 hours post-injection ($P<0.0001$).

For Lameness Locator® data, the DiffMax and DiffMin for each time point were compared to baseline and reported as the change in DiffMax and DiffMin (mm). For the first injection, the change in DiffMax and DiffMin were greater in the AUTO group versus the ALLO group at 6 hours post-injection (DiffMax: $P=0.0147$, DiffMin: $P=0.04$) (Figure 6.3). After the second injection, the change in DiffMax and DiffMin were greater for the AUTO group versus the ALLO group at 12 hours (DiffMax: $P=0.0001$, DiffMin: $P=0.002$) (Figure 6.3). There was no significant difference in the lameness locator measurements (DiffMin, DiffMax) between the animals treated with only rIL-1β and those treated with rIL-1β and BMDMSCs (Figure 6.3).

Figure 6.3. Lameness Locator®. Six hours following the first injection, the change in DiffMax (P=0.0147) and DiffMin (P=0.0428) was greater in the autologous group versus the allogeneic treatment group. Twelve hours following the second injection, the change in DiffMax (P=0.0001) and DiffMin (P=0.002) was greater for the autologous treatment group versus the allogeneic treatment. P<0.05, P<0.01, and P<0.001 are signified by *,**, and *** respectively. No significant difference was found between the horses receiving rIL-1β alone and those receiving AUTO BMDMSCs with rIL-1β at 6 hours following the first injection (DiffMin, P= 0.0622; DiffMax, P=0.0694). Individual data points are plotted. Bar graphs indicate the mean and standard deviation.

<u>Joint circumference and effusion score</u>

Joint circumference and a subjective joint effusion score were used to evaluate joint distention post-injection. Following the first injection, no difference was detected in the change in joint circumference between the AUTO and ALLO treatment groups at any time point (Figure 6.4). Consistent with the rIL-1β model of synovitis, joint circumq1erence was significantly increased 6 hours post-injection for both treatment groups (AUTO P<0.0001, ALLO: P=0.0007). Change in joint circumference continued to be increased from baseline for both groups through

336 hours following the first injection (P<0.0001). Joint circumferences were not improved in the AUTO or ALLO groups compared to control joints administered rIL-1β alone (Figure 6.4).

Figure 6.4. Change in joint circumference. There was no significant difference in the change in joint circumference (cm) between AUTO and ALLO at any time point following the first or second injection. There was no significant difference in change in joint circumference between horses which received rIL-1β alone and those which received BMDMSCs and rIL-1β. Significance was set at P<0.05. Individual data points are plotted. Bar graphs indicate the mean and standard deviation.

Following the second injection, there was no significant difference in the change in joint circumference between AUTO and ALLO treatment groups at any time point (Figure 6.4). Joint circumference was significantly increased for animals in the AUTO treatment group by 12 hours (P<0.0001) and the ALLO treatment group by 6 hours (P=0.0001).

Subjective joint effusion score was assessed at every time point by the same observer. After the first injection and second injection, no difference was detected between AUTO and ALLO treatment groups at any time point (Table 6.2). However, both groups showed a significant change in subjective joint effusion score from baseline by 6 hours following the first injection (P<0.0001) and second injection (P<0.0001). There was no improvement in joint effusion score in treatment groups administered BMDMSCs versus rIL-1β alone (Table 6.2).

Table 6.2. Change in subjective effusion score. There was no significant difference in the change in subjective joint effusion score between AUTO and ALLO at any time point following the first or second injection. No difference was found in the change in subjective effusion score between horses receiving rIL-1β alone and those receiving BMDMSCs with rIL-1β. Significance was set at P<0.05.

Time	***1st injection:*** **Autologous + 75ng rIL-1β mean (cm)**	***1st injection:*** **Allogeneic + 75ng rIL-1β mean (cm)**	**2nd injection: Autologous + 75ng rIL-1β mean (cm)**	**2nd injection: Allogeneic + 75ng rIL-1β mean (cm)**	***Single injection:*** **75ng rIL-1β** mean (sd)
6	2.1 (0.6)	1.8 (0.9)	1.9 (0.5)	2.1 (0.6)	1.5 (0.6)
12	3.4 (0.4)	2.7 (0.5)	3.0 (0)	2.6 (0.5)	3.0 (0.8)
24	3.1 (0.9)	2.6 (0.5)	2.1 (0.4)	1.9 (0.4)	3.3 (0.5)
72	2.0 (0.5)	1.4 (0.9)	1.0 (0)	1.1 (0.4)	2.0 (0.0)
168	1.6 (0.5)	1.1 (0.4)	0.4 (0.5)	0.7 (0.8)	1.3 (0.5)
336	1.1 (0.4)	0.6 (0.5)	0.1 (0.4)	0.4 (0.5)	1.3 (0.5)

<u>Synovial fluid analysis</u>

Arthrocentesis was performed at each time point and synovial fluid was assessed for NCC, TP and differential cell counts. There was no significant difference in the NCC between AUTO and ALLO treatment groups at any time point after the first or second injection (Figure 6.5). Following the first injection, both AUTO and ALLO treatment groups had an increase in NCC compared to baseline 6 hours post-injection (P<0.0001) which persisted for 72 hours for both treatment groups (P<0.0001). Administration of BMDMSCs (AUTO or ALLO) did not result in a significant decrease in inflammation as determined by the NCC. In fact, the NCC was significantly higher at 24 hours (P=0.009) and 72 hours (P=0.02) in the AUTO treatment group compared to rIL-1β alone. Further, the NCC was significantly higher at 24 hours (P=0.03) in the ALLO group compared to rIL-1β alone (Figure 6.5). Following the second injection, there was a

significant increase in NCC for both groups by 6 hours (AUTO, ALLO: P<0.0001) compared to baseline and persisting for 72 hours post-injection (AUTO, ALLO: P<0.0001).

Figure 6.5. Synovial nucleated cell count and total protein. There was no significant difference in the nucleated cell count or total protein between the AUTO and ALLO treatment groups at any time point following the first injection or second injection. The NCC was significantly higher at 24 hours (P=0.0087) and 72 hours (P=0.0198) in the AUTO treatment group versus rIL-1β alone. The ALLO group had a significantly higher NCC at 24 hours (P=0.0272) compared to rIL-1β alone. There was no significant difference in the TP at any time point between AUTO, ALLO, and rIL-1β alone. Significance was set at P<0.05. Individual data points are plotted. Bar graphs indicate the mean and standard deviation.

Differential cell counts were determined for each time point post-injection. There was no difference in monocyte count or neutrophil count between AUTO and ALLO treatment groups at any timepoint following the first or second injection (Figure 6.6). Monocytes decreased significantly for both AUTO and ALLO treatment groups by 6 hours post-injection (AUTO, ALLO: P<0.0001) and continued to be decreased for 72 hours post-injection (AUTO: P<0.0001,

ALLO: P=0.0101). The AUTO (P=0.0498) and ALLO (P=0.04) treatment groups had a significantly higher monocyte count at 24 hours compared to horses which received rIL-1β alone (Figure 6.6). No other significant differences were found in the differential counts between horses receiving BDMSCs and rIL-1β versus rIL-1β alone. Following the second injection, there was a significant decrease in monocytes for 24 hours following injection (P=0.001) in the ALLO group, and 72 hours (P=0.0008) post-injection in the AUTO group. The neutrophil count was significantly increased for both treatment groups by 6 hours (P<0.0001) and continuing through 72 hours post-injection (P<0.0001) following the first injection. After the second injection, the neutrophils significantly increased by 6 hours (P<0.0001) for both groups and continued to be increased until 72 hours in both groups (P<0.0001) and through 1 week in the ALLO group (P=0.02). There was no difference in lymphocyte or eosinophil counts between AUTO and ALLO at any time point after the first or second injection, and neither lymphocytes nor eosinophils increased significantly from baseline (data not shown).

Figure 6.6. Synovial differential cell count. There was no significant difference in the differential cell counts between AUTO and ALLO treatment groups at any time point following the first or second injection. The monocyte count was significantly higher in the AUTO and ALLO group versus horses receiving rIL-1β alone at 24 hours following the first injection. Significance was set at $P<0.05$. Individual data points are plotted. Bar graphs indicate the mean and standard deviation.

There was no difference in the synovial fluid TP between AUTO and ALLO groups for any time point following the first or second injection (Figure 6.5). Following the first injection, both treatment groups had a significant increase in synovial fluid TP at 6 hours post-injection (AUTO, ALLO: $P<0.0001$) which persisted for 2 weeks following injection (AUTO: $P=0.0002$, ALLO: $P<0.0001$). There was no significant difference in the synovial fluid TP in horses receiving BMDMSCs versus r-IL1β alone (Figure 6.5). As the synovial fluid TP had not returned to baseline by 2 weeks following the first injection, a significant change from baseline was noted in the AUTO group only at 6 hours ($P=0.04$), 12 hours ($P<0.0001$) and 24 hours ($P=0.0002$)

post-injection and in the ALLO group only at 12 hours (P=0.0001) and 24 hours post-injection (P=0.003).

No significant difference was identified in the change in synovial CRP and PGE_2 between AUTO and ALLO treatment groups at any time point following the first or second injection. In addition, there was no significant difference in the change in synovial CRP or PGE_2 when horses received rIL-1β alone versus those which received BMDMSCs and rIL-1β. As expected with intra-articular administration of rIL-1β, synovial PGE_2 levels were increased by 24 hours following the first injection (AUTO: P=0.01, ALLO: P=0.03) and second injection (AUTO: P=0.001, ALLO: P=0.0004) (Figure 6.7). Following the first injection, synovial CRP increased mildly but significantly at 24 hours (compared to baseline) in the AUTO group only (P=0.02). Following the second injection, synovial CRP increased mildly but significantly (compared to baseline) in the ALLO group only (P=0.02) (Figure 6.7).

Figure 6.7. Change in synovial PGE_2 and synovial CRP. There was no significant difference in the change in synovial PGE_2 (pg/ml) or synovial CRP (ng/ml) at any time point following the first or second injection. There was no difference in the change in synovial PGE_2 or CRP between horses receiving rIL-1β alone and those receiving BMDMSCs following the first injection. Significance was set at $P<0.05$. Individual data points are plotted. Bar graphs indicate the mean and standard deviation.

6.5 Discussion

The use of allogeneic BMDMSCs has multiple practical clinical advantages for the treatment of joint injuries and inflammation in horses, including being an off-the-shelf therapy and potentially less expensive, while also increasing the overall availability of stem cell therapy to veterinarians. They also have many potential medical advantages; allogeneic cells may be screened and characterized for their healing abilities prior to administration. Age and disease state negatively affect stem cell health and efficacy; [24; 25] allogeneic cells would surpass these barriers and provide potentially better cells for healing.

Allogeneic stem cells have been evaluated alone and compared to autologous stem cells in vitro and in vivo in horses. [7; 15; 26-28] These studies have yielded conflicting results. A single in vivo study comparing intra-articular injection of allogeneic and autologous BMDMSCs identified an increase in the synovial total nucleated cell count when allogeneic cells were repeatedly administered; however, this was a transient response for a single day and there were no differences in clinical parameters. [15] Likewise, a single study of intravenous administration revealed an increase in CD8+ T cells following injection of allogeneic BMDMSCs but no clinical effects. [26] In fact, large clinical trials of intravenous and intra-articular administration of allogeneic cells have demonstrated no adverse effects. [1; 2; 27; 28] One potential reason for conflicting results in small experimental studies may be the variability in individual horses' responses to any intra-articular medication including the administration of mesenchymal stem

cells (MSCs). [4; 15; 29] Therefore, the authors of the current study sought to compare the intra-articular response after intra-articular injection of autologous and allogeneic BMDMSCs within the same cohort of animals.

Unlike most pharmaceutical drugs, in the case of cellular therapies, living cells can respond and react to their environment. In vitro studies have shown BMDMSCs appear to be primed by inflammation creating a more anti-inflammatory phenotype. [30; 31] However, this may be stimuli dependent as a different in vitro study found BMDMSCs increase expression of inflammatory mediators when exposed to inflammatory stimuli. [32] Therefore, it is imperative that stem cells be evaluated experimentally in both the normal and inflammatory joint environment. Studies in normal joints cannot be effectively extrapolated to predict the results of BMDMSC treatment in the clinical, inflamed joint. In the present study these cells were evaluated in a well-established model of synovitis. [19; 21; 33]

No significant differences between the two treatment groups (allogeneic versus autologous BMDMSC) were identified in clinical parameters including subjective lameness, joint effusion or joint circumference at any time point following the first or second injection. The only clinical difference identified between groups was an increase in objective lameness parameters in the AUTO group compared to the ALLO group at one time point (6 hours) following the first and one timepoint (12 hours) following the second injection. This difference should be interpreted with caution, as the change in subjective lameness grades was not different and no other significant clinical or cytological differences were identified. No differences were found in the synovial fluid NCC, TP, differential cell counts, synovial PGE_2 or synovial C-reactive protein between AUTO and ALLO treatment groups at any time point following the first or second injection. The study found no appreciable difference in the inflammatory joint clinical

or cytological reaction to AUTO versus ALLO stem cells at any time point following the first or second injection.

In our study, AUTO and ALLO BMDMSCs resulted in no clinical or cytological improvement in comparison to joints treated with rIL-1β alone. A single previous study in horses evaluated the use of umbilical derived mesenchymal stem cells in a lipopolysaccharide (LPS) induced inflammatory joint model. This study showed a decrease in nucleated cell counts when MSCs were injected together with LPS in the tibiotarsal joint.[14] Although this study revealed promising data which supported allogeneic stem cell use, an in vitro study with equine BMDMSCs revealed that MSCs may respond differently to different inflammatory stimuli. [32] IL-1β is a cytokine produced by joint tissues (unlike LPS), therefore, the rIL-1β model may be more clinically relevant. [21; 34] In addition, MSCs derived from various tissues may respond differently to inflammation.[35] BMDMSCs are commonly used in equine practice. Therefore, understanding the response of BMDMSCs to joint inflammation is particularly important. The authors are unaware of an additional experimental study comparing autologous and allogeneic MSC in an inflamed joint model.

In the present study, allogeneic and autologous BMDMSCs appeared to transiently increase inflammation when administered concurrently with rIL-1β. This was evident as a transient increase in NCC, monocyte count, and subjective lameness in horses treated with BMDMSCs versus those administered rIL-1β alone. Traditionally, rIL-1β causes a neutrophilic inflammation. [19; 21] Therefore, it is particularly interesting that BMDMSCs have resulted in an increase in monocytes over rIL-1β alone. A sustained monocytic inflammation has been found in previous studies of intra-articular administration of BMDMSCs.[36] This finding does not discount the significant clinical success that has been reported by others, [1; 28] as BMDMSCs

mechanism of action is still largely unknown; anti-inflammatory properties may not be what is responsible for clinical healing. In addition, the clinical effect of a transient monocytic inflammation is unknown. The study does indicate that AUTO and ALLO BMDMSCs are not effective in reducing the severe inflammation induced by rIL-1β at the dose (75ng) administered in this study.

The potential reasons for the lack of response to BMDMSC treatment in this model are several. For example, it is possible the inflammation induced was so severe as to override the milder anti-inflammatory effects of BMDMSC. In our model, a single dose (75 ng) of rIL-1β was utilized. The dose administered was lower than previous doses described in the literature. [21; 34] The lower dose was chosen as previous researchers found the tibiotarsal joint to be more sensitive to rIL-1β administration. [33] In addition, it is difficult to compare doses of rIL-1β between studies. Potency of rIL-1β depends on dilution and storage, and potency is variable between lots due to manufacturing processes and testing. This dose resulted in an acute and severe synovitis. It is possible the inflammation was too great for the anti-inflammatory properties of BMDMSCs and did not effectively mimic the level of inflammation present in a typical osteoarthritic joint. In addition, the acute synovitis caused by rIL-1β may not be the most appropriate for determining long-term efficacy of BMDMSCs. Clinically and experimentally, BMDMSCs appear to have an anti-inflammatory effect, [1; 7] therefore, a lesser degree of synovitis may have resulted in evidence of this anti-inflammatory effect. In addition to controversy regarding dose of rIL-1β, the optimal time for mesenchymal stem cell administration remains unclear. Therefore, BMDMSCs may have been ineffective in reducing inflammation because of inappropriate timing of administration. BMDMSCs may have been more effective if administered prior to the onset of inflammation or following the initial inflammatory phase.

Unfortunately, rIL-1β induced inflammation has a rapid onset and short-term of action. Because of this, the authors did not delay administration of BMDMSCs and administered BMDMSCs concurrently with rIL-1β. Administration prior to onset of inflammation could have been performed but is not clinically applicable.

Opposite treatments (ALLO vs. AUTO) were administered into contralateral tibiotarsal joints at 1-week intervals; repeat treatments (into the same tibiotarsal joint) were administered at 2 weeks (Supplemental Information 1). Baseline lameness returned to a mean of less than 1 out of 5 (AAEP lameness scale) prior to treatment of the contralateral limb. However, we recognize that residual lameness in the contralateral limb may complicate interpretation of the lameness data. Due to this concern, all lameness is reported as a change from baseline lameness (with baseline lameness as the lameness at the time of treatment administration). However, a small degree of residual lameness at the time of contralateral limb treatment is recognized as a limitation of study. The two-week interval for repeat treatment administration was chosen to mimic a clinically relevant inflammation with an initial inflammatory response and gradual resolution followed by a second acute inflammation and gradual resolution. A complete resolution of inflammation was not achieved nor expected prior to the second treatment administration.

The optimal dose of BMDMSCs for inflammation has not been determined. Clinically, the authors routinely administer 10 – 20 x 10^6 cells per joint. [4; 6] The ability of the BMDMSCs to decrease inflammation may also be related to dose. Studies investigating the use of BMDMSCs for human knee osteoarthritis have used much larger doses of BMDMSCs. [37] It is difficult to speculate whether a larger dose of BMDMSCs would result in an anti-inflammatory effect or promote a greater inflammation. A dose escalation and titration of

BMDMSCs may be warranted to further determine the effect of BMDMSCs on rIL-1β induced synovitis.

Culture conditions must be discussed when BMDMSCs are used in any study; both culture expansion methods and cryopreservation can have a significant effect on immunophenotype of mesenchymal stem cells. [38; 39] Although our stem cells were cryopreserved in equine serum, fetal bovine serum was used in our culture period. Recent studies have reported inflammatory reactions because of intra-articular administration following culture expansion in fetal bovine serum. [15] However, because all cells were treated the same and AUTO and ALLO were given in the same animal, individual inflammatory reaction to fetal bovine serum would have been equal between treatments.

Some recent in vitro studies have identified allogeneic MHCII positive cells as immunogenic. [17; 40] However, no in vivo studies have been able to correlate MHCII expression with a negative outcome or intra-articular inflammatory response. In addition, the majority of studies report a lack of MHCII expression by equine BMDMSCs [40-42], and BMDMSCs in our laboratory have a routinely low to absent level of expression of MHCII. [7] Our study did not evaluate the expression of MHCII by pooled allogeneic MSCs. Although the importance of MHCII expression by BMDMSCs administered in vivo is still unknown, not determining MHCII expression by the BMDMSCs is a limitation of our study.

This study used the same cohort of animals to evaluate the response to autologous and allogeneic BMDMSCs. Stem cell tracking studies in equine tendonitis models have identified stem cell migration, [43] and a murine study identified migration of intravenously administered MSCs to inflamed joints. [44] However, recent studies following intra-articular administration of stem cells have shown prolonged retention in the joint. [45-47] In fact, a recent study in rats was

able to identify retention of stem cells in inflammatory (surgical) joints for 10 weeks following intra-articular administration. [48] Likewise, a study in sheep identified MSCs 12 weeks after intra-articular administration. [47] Although a limitation of the present study, the authors felt the possibility of stem cell migration was offset by the importance of controlling for individual variation in response to BMDMSC administration.

In conclusion, the current study did not identify significant clinical or cytological differences between horses treated with AUTO or ALLO BMDMSCs in an rIL-1β model of synovitis. Neither autologous nor allogeneic BMDMSCs reduced inflammation induced by 75 ng of rIL-1β. In fact, a transient increase in NCC, monocyte count, and subjective lameness resulted from BMDMSC treatment. The current study would suggest that BMDMSCs are unsuccessful at mitigating acute, severe, synovitis. However, the inflamed joint response to intra-articular autologous and allogeneic BMDMSCs is equivalent as is the response following repeat injection of either autologous or allogeneic cells. Although the rIL-1β model of synovitis is valuable, joint pathology is multifaceted and future in vivo studies modeling natural joint pathology are necessary to determine the clinical utility of BMDMSCs.

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Chapter 7: Concluding remarks and future directions

7.1 Significance of work

The goal of the research presented in this dissertation was to explore the use of allogeneic BMDMSCs for the treatment of joint disease in the horse. Mesenchymal stem cells are a promising therapy for musculoskeletal injuries in the horse. [1-3] The expansion of autologous MSCs is time consuming, costly and restricts how quickly a patient may be treated. Allogeneic MSCs have many potential advantages including being an "off-the-shelf" treatment that may be carefully selected and characterized prior to use. Before allogeneic MSCs maybe used, it is imperative to determine the safety of these cells. In vitro studies are paramount for understanding immunological processes, but they are rarely able to effectively model the complex biological processes that influence the bodies reaction to cellular therapies. Therefore, the body of work included in this dissertation begins with an in vitro investigation and progresses to pre-clinical models.

Chapter 3 describes a direct in vitro comparison of allogeneic versus autologous BMDMSCs. This chapter reveals no difference in the ability of allogeneic versus autologous BMDMSCs to suppress lymphocyte proliferation. This large in vitro study of fifty-six combinations of allogeneic BMDMSCs complements previous in vitro work, [4-6] but is the first to compare allogeneic and autologous BMDMSCs directly.

With this in vitro work supporting the further investigation of allogeneic versus autologous BMDMSCs, Chapter 4 focuses on the clinical and cytological effects of allogeneic versus autologous BMDMSCs in the normal equine joint. In this pre-clinical model using the normal equine joint, no difference was found in the clinical or cytological responses of the

normal joint to allogeneic versus autologous BMDMSCs. A few equine studies have investigated the intra-articular response to allogeneic stem cells, but these studies have administered allogeneic stem cells alone or have evaluated allogeneic versus autologous cells in different cohorts of animals. [7; 8] By administering allogeneic and autologous BMDMSCs into contralateral forelimb metacarpophalangeal joints, the study was able to control for inter-joint variation as well as inter-horse variation in the response to BMDMSCs administration. This work supported an additional experimental study in a pre-clinical model of joint inflammation.

Chapter 5 describes a preliminary study looking at the tibiotarsal and middle carpal joint response to rIL-1β. Although multiple previous studies have described equine joint responses to rIL-1β, [9-11] no previous study had described the cytological response of the tibiotarsal joint to rIL-1β nor has any study compared different joint responses to the same dose of rIL-1β. This study was necessary to determine the appropriate study design for the study described in Chapter 6. The results indicate the middle carpal joint and tibiotarsal joint respond differently to the same dose of rIL-1β. Therefore, if treatments are administered into the tibiotarsal joint, an appropriate placebo control would also be administered into a tibiotarsal joint. Because of these findings, Chapter 6 describes the administration of allogeneic and autologous BMDMSCs in contralateral, rIL-1β-treated, tibiotarsal joints compared to a separate group of horses administered rIL-1β alone in the tibiotarsal joint. This study, in agreement with the normal joint study described in Chapter 4, finds no significant clinical or cytological difference between the inflamed joint reaction to allogeneic versus autologous BMDMSCs. The study also found no decrease in inflammation as a result of allogeneic or autologous BMDMSCs administration.

The results of this work have potential application to a large audience. The use of MSCs for musculoskeletal disease has increased substantially and is available to ambulatory

practitioners and referral centers alike. The results of this dissertation suggest that there is no difference in the equine joint response to autologous or allogeneic MSCs. Moreover, careful in vivo comparisons of allogeneic and autologous MSC administration have not observed excessive adverse reactions or negative clinical results following allogeneic MSC administration strongly suggesting allogeneic MSCs are safe. However, efficacy of either autologous or allogeneic MSCs remains unproven by this body of work.

7.2 Future directions

What is lacking to help fully resolve the issue of allogeneic versus autologous MSCs are properly designed, randomized, clinical trials comparing treatment outcomes and adverse events in animals treated with allogeneic or autologous MSCs. In the design of such future trials, sufficient attention should be directed to criteria including the number of cells administered, the origin of cells, culture processes, whether the cells have been cryopreserved, and the injection medium. The results of this dissertation in conjunction with future efficacy trials would go a long way towards finally resolving the issue of the use of allogeneic MSCs for treatment of equine orthopedic injuries.

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