THE MACROSCOPIC, HISTOLOGIC, AND IMMUNOMODULATORY EFFECTS OF INTRAVENOUS ALLOGENEIC EQUINE UMBILICAL CORD BLOOD-DERIVED MULTIPOTENT MESENCHYMAL STROMAL CELL THERAPY ON EXPERIMENTALLY CREATED LIMB WOUNDS IN THE HORSE

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ABSTRACT

BACKGROUND: Limb wound on horses are often slow to heal and are prone to developing exuberant granulation tissue (EGT) and close primarily through epithelialization, which results in a cosmetically inferior and less durable repair. In contrast, wounds on the body heal rapidly and primarily through contraction and rarely develop EGT. Intravenous (IV) multipotent mesenchymal stromal cells (MSCs) are promising. They home and engraft to cutaneous wounds and promote healing in laboratory animals, but this had not been demonstrated in the horse. Furthermore, the clinical safety of administering >1.00 x 10^8 allogeneic (allo) MSCs IV to a horse has not been determined.

PILOT PROJECT: A proof-of-principle pilot project was performed with two horses that were administered 1.02 x 10^8 fluorescently labelled allo-cord blood-derived MSCs (CB-MSCs) following surgical wound creation on the forelimb and thorax. Results confirmed preferential homing and engraftment to wounds with persistence of CB-MSCs at 33 days following wound creation, without clinically adverse reactions to the infusion.

SECONDARY PROJECT: A minor secondary project was developed from the pilot project, where the mRNA of the inflammation-associated proteins β-arrestin-2 (βarr2), CXC ligand (CXCL) 8, CXC receptor (CXCR) 2, CXCL10, and CXCR3 was compared in limb and thoracic wounds. Results suggest that there are differences in expression between βarr2 and CXCL8/CXCR2 in limb and thoracic wounds.

MAJOR PROJECT:

Materials and Methods: Wound were surgically created on the forelimbs of treatment and control horses. 1.51 – 2.46 x 10^8 allo-CB-MSCs were administered to treatment horses 12 hours after wound creation and were monitored for clinically adverse reactions during infusion. Control horses were administered diluted cellular suspension medium (HTS-FRS) only. Biopsies of the wounds were collected on days 0, 1, 2, 7, 14, and 28 from treatment and control horses. The biopsies tissue was dived – a portion was snap frozen and analyzed using multiplex mRNA assays for proinflammatory (TNFα, CXCL8), anti-inflammatory (IL-4, IL-8), inflammation resolving INFγ, CXCL10), profibrotic (TGFβ1, TGFβ2), and anti-fibrotic (TGFβ3) cytokines, and the other portion was evaluated histologically and given a

combined repair and inflammation score. The wounds were photographed on days 7, 14, and 28 and evaluated for wound size and total percentage of wound closure by contraction and epithelialization using planimetric analysis. Day of wound closure was recorded when the granulation tissue was covered with epithelium. Rate of wound closure, percentage of contraction, percentage of epithelialization, and mean fold change of cytokines was evaluated using generalized estimating equations for an overall treatment effect over all days ($P \leq 0.2$) and pairwise comparison for treatment effect at individual days ($P \leq 0.05$).

Results: 3/6 (50%) treatment horses and 1/6 (17%) control horses experiences clinically adverse responses during injection. Day to wound closure was not significantly improved (treatment 26+/- 4 days, control 27 +/- 3 days; two sample T-test, P=0.702), although overall wound size (GEE; P=0.145) was decreased, characterized by overall increased contraction (GEE; P=0.145) and decreased epithelization (GEE: P=0.015) with significantly less epithelization on day 14 (GEE, PWC; P=0.0173). Histologic repair score was not improved and virtually identical between groups, thus no static analysis was performed. There was overall decrease in proinflammatory (GEE; P=0.032), anti-inflammatory (GEE; P=0.022), inflammation resolving (GEE; P=0.033) and profibrotic (GEE; P=0.191) cytokines with significantly less proinflammatory cytokines on day 2 (GEE; P=0.0003). There was no difference in antifibrotic cytokines.

CONCLUSIONS: Administered $>1.51 \times 10^8$ IV allo-CB-MSCs suspended in HTS-FRS can induce infusion reactions in recipients which may be caused by antigenic stimulation or the suspension medium. IV allo-CB-MSC therapy did not improve time to wound closure or histologic repair scores, although overall wound size was smaller due to increased contraction and decreased epithelization. IV allo-CB-MSCs decreased expression of all cytokines except for antifibrotic cytokines. Although minor improvements in wound healing were measured, the advantages of IV allo-CB-MSC therapy likely do not justify the risks of infusion reactions. Investigation into other methods of MSC delivery and therapies are warranted.

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DEDICATION

This thesis is dedicated to my family.

To my husband, Floyd, for his never ending love and support. To my son, Ira, for always cheering me up with his goofy antics and being understanding when mommy was again home late or gone early. To my parents, sisters, Grandma Kitz, in-laws, aunties, uncles, cousins, nieces, and nephews, for their immense pride, celebration in my small successes, and continual support in my crazy career-woman goals. To my late Grandma Kurtenbach, for igniting a love of all things horsey and from whom all we cousins inherited her passion and hard-working spirit.

And finally, to the many ponies of my childhood, who taught me more than any person could the art of persistence, patience, perseverance, tenacity, and compromise. Sometimes the best life lessons are taught by those who can't speak.

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LIST OF ABBREVIATIONS

Allo	Allogeneic
Allo-MSCs	Allogeneic MSCs
Auto-MSCs	Autologous MSCs
βarr2	Beta-arrestin-2
CB-MSCs	Umbilical cord blood-derived MSCs
CD	Cluster of differentiation
CMV	Cytomegalovirus
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
DMEM	Dulbecco modified Eagle medium
EDTA	disodium ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
EGT	Exuberant granulation tissue
ELR	Glutamate-leucine-arginine motif
GEE	Generalized estimating equations
GPCR	G-protein coupled receptor
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HTS-FRS	Hypothermasol-FRS
IFN	Interferon
IFNγ	Interferon-gamma
IL	Interleukin
ISCT	International Society for Cellular Therapy
IV	Intravenous
MC-III	Third metacarpus
MFC	Mean fold change
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MOI	Multiplicity of infection

mRNA	Messenger ribonucleic acid
MSCs	Multipotent mesenchymal stromal cells
PBS	Phosphate buffered saline
PMN	Polymorphonuclear cell
RLP	Regional limb perfusion
TGFβ	Transforming growth factor-beta
TIMP	Tissue inhibitor of metalloproteinase
TNFα	Tumour necrosis factor-alpha

1. INTRODUCTION

Horses and humans have been intimately connected for many thousands of years, with the first evidence of domestication having occurred over five thousand years ago[1,2]. At first, the horse was domesticated for a source of food, but eventually its usefulness for transportation and labour was realized and, with the introduction of the harness and collar, revolutionized agriculture and advanced society[1].

Domesticating horses necessitates restraining them in enclosures to keep them in close proximity, but their innate flighty temperament paired with the confinement of stables and paddocks increases the likelihood of injury that they would not normally encounter in their natural environment. Furthermore, selective breeding for traits to excel at various disciplines, such as pulling, riding, or racing, resulted in a variety of breed phenotypes that diverge drastically from their wild pony-like ancestors[2–4], potentially at the expense of loss of fitness in other areas, such as wound healing[3,4]. Clinically, it has been long observed that ponies, despite being the same species as horses, have superior healing characteristics to horses, particularly in regards to wounds on the limb, and these observations have been confirmed with several seminal studies[4–8]. Whether the horse's propensity for impaired wound healing is because of selective breeding for desired traits at the expense of healing characteristics or is merely reflective of manmade enclosures that the horse is not evolved to be confined in is to be debated. Regardless, wounds and their related complications is the most common medical condition in horses, accounting for nearly 50% of all injuries in one report[9]. In addition to being a financial burden on the client, wounds can limit a horses functional career and be chronically painful or even life threatening in some circumstances[10]. Therefore, investigating methods to accelerate and improve wound healing is advantageous to the client and horse alike.

Regenerative medicine including multipotent mesenchymal stromal cells (MSCs) have become mainstream ancillary therapies for a variety of injuries such as tendon and ligament injuries, osteoarthritis, and wounds[11–14]. It was previously believed that MSCs improved healing outcomes by differentiating into supportive cells and integrating into the repair, but the current understanding is that MSCs impart their positive effects primarily

through signaling the release of inflammatory mediators to direct the normal sequence of healing[12–17]. However, despite their mainstream use in equine medicine, evidence of their efficacy compared to conventional therapies is still being elucidated[13,14,18]. In particular, the use of MSCs for improving cutaneous wound healing is especially lacking, with most related published literature involving case reports[12,19] and a single randomized control study[20]. Variables such as the source of MSCs, effective cell dose, route of administration, timing of administration, and number or treatments required for a measurable clinical effect are still not clear[13,14,18]. Additionally, although MSC therapy is generally considered to be safe, complications associated with administration are still possible and need to be further investigated[14,21].

2. LITERATURE REVIEW

2.1 The Physiology of Wound Healing

In all mammals, the skin acts as a protective barrier to prevent contamination and infection of the underlying tissues with environmental debris and pathogens. In the event that the skin is wounded, such as with disease or injury, the body must repair it both efficiently and effectively or risk infection that could compromise the entire organism and potentially lead to death of the individual.

Regardless of species, the basic principles of full-thickness cutaneous wound healing are the same amongst mammals. If a wound is sutured closed shortly after injury, it heals with little granulation tissue formation and epithelialization between the sutured edges and forms a minimal scar. Conversely, during second intention healing, the wound edges are not apposed and the wound fills first with granulation tissue followed by contraction of the skin edges and migration of keratinocytes from the outside borders to the center of the wound over the surface of the granulation bed[10,22]. Second intention healing is considered less desirable compared to primary intention healing as the healing process is prolonged, and has a larger scar that lacks sebaceous and apocrine glands in the epithelialized portion, causing the dermis to be functionally and cosmetically inferior. Furthermore, complications are more likely to occur which has both humane and financial implications for the horse and client[10,23,24].

Cutaneous wound healing is a complex integrative process with several phases that are not independent from one another, but rather happen concurrently in a sequential fashion. These phases are hemostasis, inflammation, cellular proliferation, and remodelling[10,15,22]. It is important that these phases are initiated and concluded at the correct time points during the healing process or the effectiveness of the repair can be compromised. The release of various inflammatory mediators at the correct time and amounts is critical to ensuring the progression of healing without complication (Table 2.1)[10,15,22].

Table 2.1 – Inflammatory Mediators Involved in Cutaneous Wound Healing[10,22,25–27]

Name	Overall effect	Functions
TNFα	Proinflammatory	• Leukocyte chemotaxis • Activate other mediators
CXCL8/CXCR2	Proinflammatory	• Neutrophil chemotaxis • Angiogenesis • Epithelialization
IL-10	Anti-inflammatory	• Suppress release of proinflammatory mediators
IL-4	Anti-inflammatory	• Suppress release of proinflammatory mediators • M2 phenotype differentiation • Downregulate CXCR2 on neutrophils • CXCL8 release for angiogenesis and epithelialization
CXCL10/CXCR3	Inflammation resolving	• Angiostatic • Monocyte and T lymphocyte chemotaxis
IFNγ	Inflammation resolving	• CXCL10 release • M1 phenotype differentiation
TGFβ1/TGFβ2	Profibrotic	• Induce Type III collagen deposition • Upregulate TIMPs
TGFβ3	Antifibrotic	• Decrease Type III collagen deposition • Promote Type I collagen formation and cross-linking • Upregulate MMPs
βarr2	Inflammation regulation	• Downregulate expression of CXCR2 and CXCR3

2.1.1 Hemostasis

When a cutaneous wound is created, vessels are disrupted and begin to haemorrhage. Arachidonic acid is released from the phospholipids of traumatized endothelial cells, which triggers the release of mediators that induces vasospasm and activates the endothelial surface to promote aggregation of platelets. Collectively, this initial vascular phase causes arteries and arterioles to constrict and a platelet plug to form, stymying blood loss. Shortly afterwards, smaller arterioles and venules vasodilate by relaxing smooth muscle and opening gap junctions between endothelial cells in response to mediators released from the damaged endothelium and platelet granules. This allows growth factors, cytokines, platelets, and inflammatory cells to infiltrate the wound site and initiate the proper cascade of events for healing. A blood clot forms as a result of both the intrinsic and extrinsic clotting cascades. The blood clot is referred to as the provisional extracellular matrix, which serves as a scaffold for cell migration and stromal components during subsequent healing phases. Over time, as the clot desiccates, an eschar or "scab" is formed which acts as a biological bandage that will slough as healing continues beneath[10,22].

2.1.2 Inflammation

Inflammation is a critical phase of healing as it debrides and prepares the wound bed for subsequent phases while continuing to release inflammatory mediators crucial for progression and resolution of healing. Platelet degranulation plays a pivotal role initiating this process by releasing a myriad of mediators, the most notable in regards to this review being tumour necrosis factor-alpha (TNFα) and those of the transforming growth factor-beta (TGFβ) class, which trigger release of other chemotactic proinflammatory mediators from adjacent activated cells[10,15,22]. Resident tissue macrophages, circulating neutrophils and monocytes, and adjacent fibroblasts are drawn to the wound site. The early inflammatory phase is characterized by the appearance of neutrophils at the wound site, followed by the late inflammatory phase that is characterized by the recruitment of both local resident macrophages and circulating monocytes[10,15].

TNFα triggers the release of chemokine (C-X-C motif) ligand (CXCL) 8 from surrounding activated cells. CXCL8 is a potent chemoattractant of neutrophils; it upregulates expression of its receptor chemokine (C-X-C motif) receptor (CXCR) 2 on the neutrophil surface while

also inducing further CXCL8 release from neutrophils themselves[25]. During the early inflammatory phase, neutrophils infiltrate the wound immediately following injury and peak at approximately forty-eight hours post injury. Neutrophils eliminate the wound of debris and bacteria through phagocytosis. Once the wound is cleared of contamination, recruited neutrophils apoptose and are either phagocytized by macrophages or become apart of the eschar that will later be shed during epithelialization[10,15,22]. Although powerful warriors in the war against infection, neutrophils are not vital to healing of non-infected wounds[28,29]. However, CXCL8 is vital for initiating angiogenesis and epithelialization and reduced neutrophil recruitment contributes to impaired healing[29,30].

Macrophages follow neutrophil infiltration and peak within seventy-two hours[15,22]. Macrophages are orchestral conductors in the remainder of the healing process and their roles change and adapt as healing progresses. There are three recognized phenotypes of macrophages: the classic M1 phenotype that primarily promotes inflammation through release of proinflammatory mediators and antigen presentation to lymphocytes; the "resolving" phenotype that releases resolving and anti-inflammatory mediators while continuing to debride the site; and the interleukin (IL)-4 activated M2 phenotype that stimulates and drives the proliferative phase of healing[10,31]. Macrophages are derived from two distinct and separate classes of cells - the bone marrow-derived monocyte, or the embryonic-derived resident tissue macrophage[32]. Resident tissue macrophages are self-replenishing and play an important role first by recruiting circulating monocytes at the time of injury then returning the wound to normal homeostasis after the initial inflammatory stage is complete. The resident tissue macrophage is also vital for initiating and maintaining the correct sequence of events and minimizing scar formation[31]. Interestingly, the dermis is nearly devoid of embryonic-derived resident tissue macrophages and requires constant recruitment from circulating monocytes, who cannot replenish themselves once inflammation resolves[33]. This physiologic phenomenon may partly explain why the dermis is prone to excessive fibrosis and scarring compared to other tissues such as bone[34]. Because macrophages simultaneously eliminate redundant neutrophils and stimulate repair, their presence and action serves as an important transition between the inflammation and proliferation phases.

2.1.3 Proliferation

Three distinct yet interconnected processes define proliferation: fibroplasia, angiogenesis, and epithelialization. These processes occur simultaneously and each is dependent on the other to be executed effectively[10,22].

Fibroplasia is characterized by infiltration of fibroblasts into the provisional matrix. Immediately at the time of injury, fibroblasts migrate through self-created proteolytic pathways into the provisional matrix and proliferate in response to several different mediators including the TGF class[22] and IL-4[25]. Approximately three days after injury, these fibroblasts produce constituents of the extracellular matrix to form immature type III collagen. As collagen is produced, the wound becomes exponentially stronger over the following seven to fourteen days. Replacement of the provisional matrix with the collagen rich extracellular matrix creates granulation tissue, a distinguishing characteristic of proliferation. Granulation tissue acts as a physical barrier to infection and a robust scaffold into which various inflammatory and regenerative cells can migrate. Once enough collagen is created, fibroblasts stop producing and either convert into contractile myofibroblasts or apoptose[10,22].

Angiogenesis is the creation of new vessels from the previously injured vessels to provide nutrients and oxygen to the collagen producing fibroblasts. At the immediate time of injury, endothelial cells upregulate CXCR2 and initiate neovascularization in response to several mediators including the TGFβ class[22] and CXCL8[25] while in the absence of neighbouring endothelial cells[10]. At approximately day three, hypoxia and lactate further stimulates angiogenesis and vascular buds will follow the proteolytic paths created by the migrating fibroblasts into the wound bed. These new vessels are what gives granulation tissue its characteristic pink color[10,15,22]. Mature granulation tissue becomes pale in color when vessels regress as hypoxia and lactate tension resolves and CXCR3 upregulates in response to interferon-gamma (INFγ) inducible angiostatic mediators including CXCL10[10,25].

Epithelialization is an integral component of wound closure. Keratinocytes proliferate, regenerate, and begin migration within hours of injury. Hemidesmosomes that bind keratinocytes to one another and the underlying dermis disintegrate to allow the cells to

migrate across the granulation bed as it forms[10]. Similar to endothelial cells, keratinocytes proliferate, upregulate CXCR2, and migrate to the center of the wound in the absence of neighbouring cells and in response to several different mediators also including CXCL8[10,25]. As the keratinocytes migrate centripetally, they dissect between the eschar and the granulating provisional matrix by secreting proteinases, causing the eschar to eventually slough. Once the keratinocytes meet in the center of the wound, proliferation stops and the cells express laminin, a protein that binds the new epithelial layer to the underlying granulation tissue[10].

2.1.4 Remodelling

Remodelling is the final stage of healing and can take up to two years to complete[10,15]. Contraction of the wound edges and structural changes to the extracellular matrix occur simultaneously to create a strong and functional repair. It is during remodelling that the final strength of the repair is achieved although is never greater than 80% of the original strength of unwounded skin[10,22].

As with epithelialization, contraction is important for facilitating wound closure. Contraction is the centripetal movement of full thickness skin toward the centre of the wound, effectively reducing the total area requiring epithelialization to complete the closure[10]. As fibroblasts migrate into the wound, many will change their phenotype to become myofibroblasts in response to many mediators including TGFβ1. These cells are primarily responsible for contraction[10,22]. Contraction of the wound is important, as it is stronger and cosmetically superior to a wound that heals primarily through epithelialization because it maintains apocrine and sebaceous glands. For the first two weeks while the myofibroblasts are transforming and infiltrating the wound bed, the wound edges will first retract and the surface area of the wound becomes larger; this is known as the lag phase. Following the lag phase, there is a rapid contraction phase where the wound gets noticeably smaller from day to day as the myofibroblasts contract, then a slow contraction phase where closure of the wound is less apparent. Contraction will cease when one of the following three criteria are met: keratinocytes meet at the center of the wound; tension of the tissue surrounding the wound is equivalent to or exceeds the contractile forces of the myofibroblasts; the number of myofibroblasts in the wound bed

are insufficient to fully contract the wound despite the surrounding skin not having excessive tension, as is the case with chronic wounds. Once contraction is complete, myofibroblasts will either change their phenotype back to fibroblasts or apoptose[10].

Remodelling is a fine balance between synthesis and degradation of the matrix. The M2 and resolving resident tissue macrophage phenotypes are integral to this process. M2 macrophages are activated by IL-4 released from T helper cells that causes the M2 macrophages to release remodelling mediators including those of the TGFβ family[30,31]. IL-4 also causes release of CXCL8 to further stimulate angiogenesis and epithelialization while downregulating CXCR2 on the neutrophil surface through intracellular β-arrestin-2 (βarr2) mediated endocytosis, preventing further neutrophil migration[25,27,35]. Resolving macrophages are activated by phagocytizing apoptotic neutrophils and release anti-inflammatory mediators such as IL-10 as well as more TGFβ[30,31]. There are three primary cytokines of the TGFβ family. TGFβ1 and TGFβ2 are primarily profibrotic and induce fibrosis, while TGFβ3 is antifibrotic and is associated with a scarless repair[36]. The correct equilibrium of these profibrotic and antifibrotic cytokines is necessary to promote a repair that rapidly gains strength without having excessive scarring. Over time through the action of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), collagen is converted from the haphazardly deposited immature type III to the uniformly linear mature type I with increased cross-linking between fibres. Eventually, the parenchyma will take on a more normal histologic appearance and an acellular scar will form[10,15].

2.2 Wound Healing of the Distal Limb Compared to the Body

Location of a wound can drastically impact the progression of healing as well as the overall outcome in the horse[24]. Limb wounds are much more likely to develop complications and have impaired healing characteristics compared to wounds on the body[24]. Contraction and epithelialization contribute to wound closure on the limb by approximately 30% and 70%, respectively, whereas the inverse is observed on the body[6]. Additionally, epithelialization is slower in limb wounds than body wounds, with limb wounds producing epithelium at only approximately 75% of the rate of body wounds[10]. Remodelling and maturation is also dysregulated as the final repair only gains 60% of the strength of non-wounded skin[37]. Limb

wounds also have a much higher propensity to develop exuberant granulation tissue (EGT), a fibroproliferative disorder where granulation tissue expands above and beyond the borders of the wound[24]. Why limbs wounds have delayed healing and are more likely to develop EGT is likely multifactorial with anatomic, physiologic, and genetic components.

Anatomically, the distal limb has many high motion joints whose movement counters the action of myofibroblasts within a wound, and limb wounds are more likely to be contaminated with debris and fecal material than body wounds because of their proximity to the ground. This induces chronic inflammation and impairs healing[38]. Recent studies have determined that limb wounds have different microbiota compositions than body wounds - specifically, higher levels of *Fusobacterium* and *Actinobacillus spp*.,[39] - and consistently develop biofilm whereas body wounds appear much less prone[40]. Although bacterial colonization likely hinders healing, other features unique to the limb also contribute to slower healing.

Histologically, the epidermis on limbs is nearly a third thicker than on the body and has an accessory-cordovan layer comprised of thickened collagen bundles that is absent in the body[41]. This may cause the skin on the limb to be less elastic than that on the body and resist contraction[41]. Myofibroblasts in limb wounds are haphazardly organized rather than in ordered linear patterns as in body wounds and contraction is consequently less effective[5,7,24]. Furthermore, oxygen saturation is consistently lower in wounds on the limb than on the body, despite increased neovascularization. Hypoxia induced angiogenesis is a normal expected physiologic response, but angiogenesis is abnormal in EGT as the microvessels are often occluded secondary to endothelial hypertrophy. Because occluded microvessels are unable to maintain aerobic metabolism, hypoxia persists which paradoxically exacerbates dysfunctional angiogenesis. Hypoxia is also a potent upregulator of TGFβ1, which stimulates fibrosis and further occludes microvessels, triggering a self-perpetuating cycle[24,42,43]. Prolonged elevations of TGFβ1 is a defining trait in limb wounds[44] and is even more pronounced in EGT[45], a phenomenon that is likely driven by hypoxia.

Acute inflammation is less pronounced and more prolonged in limb wounds. This is partly due to the local hypoxic environment that restricts the oxidative burst within the neutrophil

phagolysosome required for bacterial killing. Inflammation is then perpetuated by the resulting biofilm formation and increased oxygen consumption by the biofilm itself [24,40]. However, impairment of the initial inflammatory response to injury and resolution of inflammation is also largely driven by the inherently inferior immune response in the horse that is not seen in ponies[4,24].

Genetically, ponies and horses are the same species and yet ponies have superior healing characteristics[4]. Ponies are technically less than 14.2 hands (58 inches; 147 centimeters) tall at the withers, but improved wound healing appears to be unique in stocky phenotypes rather than simply small horses, suggesting that the horse's healing deficiency has been inadvertently enhanced by selective breeding for other desired traits[3,4]. In ponies, limb wounds have delayed healing compared to body wounds, but not to the same extent as horses, with limb wounds healing with approximately 50% contraction and 50% epithelialization[4,6]. Both ponies and horses alike develop EGT when limb wounds are bandaged, highlighting the importance of hypoxia in contributing to EGT formation. However, EGT resolves and wounds continue to heal normally when the wounds are uncovered in ponies, whereas horse limb wounds often require debridement and advanced wound management to resolve the EGT and promote healing[24].

An inadequate acute inflammatory response and delayed resolution of inflammation predisposes horses to EGT formation. Polymorphonuclear cells (PMNs) isolated from ponies have a more pronounced release of the proinflammatory mediators TNFα and IL-1 and higher production of reactive oxygen species compared to horse PMNs[8]. An inadequate initial proinflammatory response delays the release of TGFβ1, which in turn delays differentiation of fibroblasts into myofibroblasts and inhibits epithelialization, which is further inhibited by continued release of TGFβ1 by fibroblasts[24]. In addition, weak recruitment of neutrophils leads to fewer apoptotic neutrophils available to trigger differentiation of macrophages into resolving and M2 anti-inflammatory phenotypes and release of antifibrotic TGFβ3, allowing for prolonged low-grade profibrotic inflammation [30,31]. Indeed, this is observed in limb wounds that have lower levels of TGFβ3 than body wounds[44].

Also interesting to note is the reduced rate of fibroblast apoptosis in limb EGT compared to granulation tissue on the body. Ordinarily when cells become acutely hypoxic, they apoptose to prevent necrosis within the wound. This is facilitated by *p53*, a tumour suppressor protein that downregulates expression of the gene *bcl-2*, an inhibitor of apoptosis. However, if hypoxia is introduced gradually, such as during dysfunctional angiogenesis in EGT, *bcl-2* will instead become upregulated and inhibit apoptosis. Furthermore, mutated *p53* produced by keratinocytes no longer inhibits *bcl-2*, thus apoptosis does not take place at ordinary rates and EGT proliferates unabated[43].

2.3 Exuberant Granulation Tissue in Horses and Keloids in Humans

Horses have been proposed as animal models for studying keloids in humans as horses and people are the only two known species that develop spontaneous fibroproliferative disorders[46]. Contrary to EGT in horses, keloids are epithelialized and have characteristic thickened hyalinised collagen, known as keloidal collagen, that is absent in EGT[46,47]. However, EGT shares many similarities with keloids and it is important to understand the etiology of keloid formation as it may provide insights to why EGT develops in horses.

Keloids are highly proliferative fibroblastic growths that occur after trauma and, similar to EGT, expand beyond the border of the original wound[48]. Healing limb wounds share similar profibrotic and antifibrotic cytokine profiles, specifically elevated TGFβ1 and decreased TGFβ3 expression[44,45]. Both keloids and EGT have a genetic component; horses are much more likely to develop EGT than ponies[4,24] and keloids develop nearly solely in dark skinned individuals with familial inheritance patterns[49]. In addition, both keloids and EGT tend to form in areas of high skin tension, such as on the chest and back of humans[48] and the distal limbs of horses[24].

However, one of the most common areas of keloid formation is the earlobe, an area with arguably little tension[48]. The earlobe, chest, and back contain many sebaceous glands and theories suggest that autoimmune disease or delayed hypersensitivity response to sebum and/or sebaceous glands may also be a component in keloid formation[48,50]. As far as the author is aware, the possibility of horse EGT formation having a similar autoimmune mediated pathogenesis has not yet been investigated and exploring this avenue may

provide further clues as to say why horses develop EGT as well as provide avenues for further treatment options such as anti-allergic pharmaceuticals.

2.4 Multipotent Mesenchymal Stromal Cells and Cutaneous Wound Healing

Currently EGT is treated with serial debridement to lessen the bacterial load, topical corticosteroids to tamper the protracted chronic inflammatory response, and even skin grafting in stagnant cases[24]. These treatment protocols are typically eventually successful, but are expensive and labour intensive and not without harm and discomfort to the patient. Therefore, therapies that may accelerate wound healing and prevent EGT formation are warranted.

Multipotent mesenchymal stromal cells (MSCs) are being investigated as adjunct regenerative therapy to accelerate and/or improve the quality of cutaneous wound healing[15,51] and for treating keloids[52,53] in humans with promising preliminary results. In equine regenerative medicine, MSCs have been used primarily in tendon and ligament injuries[11], but research examining their effects in equine cutaneous wound healing is still in its infancy. *In vitro*, equine MSCs promote healing in wound scratch assays[54] and block the fibrogenic response of fibroblasts to TGFβ1[55]. Furthermore, MSCs secrete antimicrobial peptides that have direct antibacterial effects against common wound pathogenic contaminants[56] which may assist in healing of contaminated limb wounds. *In vivo*, however, only a experimental study has been performed examining the effects of topical and subcutaneous MSCs in cutaneous limb wounds in horses[20]. In that study, treated wounds had improved histologic healing scores and healing times than control wounds. These results are promising, but more *in vivo* studies are needed to fully determine and understand their effects.

2.4.1 Definition and Characterization

MSCs are derived from the mesoderm and can be isolated from adult or embryonic sources. According to the International Society for Cellular Therapy (ISCT), human MSCs are plastic adherent, express the surface markers cluster of differentiation (CD)73, CD90, and CD105, do not express CD11b/CD14, CD19/CD79a, CD34, CD45, and major histocompatibility complex (MHC)-II, and can differentiate into osteogenic, lipogenic, and chondrogenic

lineages under appropriate culture conditions[12,57,58]. The term "stem cell" is reserved for those cells that meet the above criteria, but also have demonstrated characteristics of true "stemness" *in vivo*; specifically, long term survival, self-renewal, and spontaneous differentiation[57,58]. The ISCT's position statement can be broadly applied to the horse although equine MSCs variably express other surface makers (CD29, CD90, CD44, CD166, CD13, CD146, CD117, CD40, CD80, CD73, CD105)[12,59,60] which is thought to be related to species variation and differences in antibody cross-reactivity[12,57] (Table 2.2).

2.4.2 Sources

Conventionally, MSCs are isolated in the horse from bone marrow or adipose tissue, although MSCs have also been isolated from synovium, muscle, gingiva, dental pulp, periodontal ligament, endometrium, hair follicles, peripheral blood, and the umbilical cord[12]. Umbilical cord blood is an attractive source as collection is non-invasive[59,61], cells are potentially more pluripotent and proliferative than other adult sources[59,61], have immunomodulatory effects *in vitro*[59,61,62] and *in vivo*[63,64], and can be cryopreserved and stored for future use[62]. Equine umbilical cord blood-derived MSCs (CB-MSCs) have trilineage differentiation capabilities[65,66] and consistently express the surface markers CD29, CD44, CD90, and occasionally CD105, and lack expression of CD4, CD8, CD11a/18, CD73 and MHC-I and MHC-II[59,67,68]. CB-MSCs have already shown promising effects in *in vivo* cutaneous wound healing[20] and synovitis[63] studies.

2.4.3 Autologous versus Allogeneic

Autologous MSCs (auto-MSCs) are collected and isolated from the same animal to which they are intended to be administered whereas allogeneic MSCs (allo-MSCs) are sourced from a different animal of the same species[69]. When characterized *in vitro*, both auto- and allo-MSCs do not demonstrate MHC-I and MHC-II surface markers, suggesting that allo-MSCs are immunoprivileged. However, allo-MSCs consistently induce a detectable immune response in the horse[12,70,71] and, in laboratory animal cardiac infarct models, engraft at lower rates and persist in the recipient for less time than syngeneic auto-MSCs[72,73]. It is believed that the inflammatory impetus triggers rejection following allo-MSC therapy because MHC-I and MHC-II are expressed in successfully engrafted cells that begin to differentiate[72,73] or become activated in direct contact with endogenous mononuclear

Table 2.2: Summary of Defining Characteristics of Human and Equine MSCs[12,57,58,67]

Criteria		Human	Horse
1) Plastic adherent ○ Differentiate from hematopoietic cells (not plastic adherent)		+ *	+
2) Surface markers	CD73 (5' nucleotidase) • Nucleoside hydrolysis	+ *	±
	CD90 (Thy1) • Cell to cell interactions	+ *	+
	CD105 (Endoglin) • TGFβ receptor complex	+ *	±
	MHC-II • Antigen presentation – B lymphocyte, macrophage	- *	-
	MHC-I • Antigen presentation - All nucleated cells	±	±
	CD11b/CD14 • Bind LPS – mononuclear cells	- *	-
	CD19/CD79α • B lymphocytes	- *	-
	CD34 • Primitive hematopoietic and endothelial cells	- *	±
	CD45 • All mature leukocytes	- *	-
	CD29 (β1-integrin) • Cell adhesion	+	+
	CD44 • Cell adhesion	+	+
	CD4 • Helper T lymphocyte	-	-
	CD8 • Cytotoxic T lymphocyte	-	-
3) Trilineage differentiation ○ Osteoblast ○ Chondroblast ○ Adipocyte		+ *	+

(*) = minimum criteria for human MSCs established by the ISCT[58]

cells[71,74]; therefore, allo-MSCs are considered to be temporarily immune evasive, rather than truly immunoprivileged[75]. Despite this, allo-MSC therapy has repeatedly shown beneficial therapeutic effects that is nearly equivalent to autologous or syngeneic cells[73,75]. In the horse, allo-MSCs have anti-inflammatory effects in experimentally created synovitis models [63,76] and improved lameness grading scales in naturally occurring arthritis[77,78] compared to controls. Furthermore, allo-MSCs improved cutaneous wound healing times and histologic scores compared to control wounds[20], suggesting that this beneficial effect is transferrable wound healing in horses.

In addition to autologous collection being invasive, isolation and expansion is a prolonged process[18] during which the ideal time of administration to best ameliorate the immune response may be missed. In spinal contusion[79] and colitis[80] models using mice, the best clinical outcomes were when MSCs were administered during the acute inflammatory phase. Allogeneic sources are attractive as collection is non-invasive, they can be expanded, characterized, and immune matched prior to storage, and can be administered "off-the-shelf" shortly following an injury during the acute inflammatory phase[18,62,78], which may result in accelerated and improved wound healing and decrease the risk of development of EGT, in spite of allogeneic induced immunogenicity.

2.4.4 Administration

MSC therapy for cutaneous wounds may be administered either locally, via topical[20,81] or intradermal means[20,82,83], and systemically[16,84,85], and the method of administration may affect the efficacy of homing and engraftment of the cells. Briefly, homing is transendothelial migration of cells to the site of injury, and engraftment is survival of the administered cells at the site[86,87]- more detail is provided in 2.4.6. Local administration is intuitive, but can be technically challenging and may cause further tissue damage iatrogenically secondary to the needle tract and a local micro-compartmentalization effect[16,88,89]. Local administration also does not necessarily improve engraftment rates compared to systemic administration, and exhibits its effects only locally rather than systemically[16].

Intravenous (IV) systemic administration is technically easy to perform, does not cause further trauma at the wound site, and administered cells have been shown to migrate, home, and engraft at the wound site[16,17,84]. However, complications following intravascular administration have been reported including fatal pulmonary embolism[21], cerebral embolism[90], and pulmonary edema and haemorrhage[91]. In addition, following IV administration, the majority of cells become entrapped in the lungs, known as the "first-pass-effect"[16,79]. Regardless, IV administration in laboratory animals and humans has repeatedly been shown to be well tolerated[16,79,80,84,85,92,93], and even if MSCs are not detected in the target tissue, wound healing is still improved, which is attributed to modulation of the systemic immune response[16,79,80,85]. Furthermore, IV MSCs may be less immunogenic than those administered locally[92]. Preliminary studies of jugular and palmar digital IV administration in horses show they are well tolerated[12,64,71,89,94–96], but no studies have yet been performed to assess their effects on wounds in horses.

2.4.5 Effective Cell Dose

The effective cell dose of IV MSCs required to influence wound healing in horses is currently unknown. To date, 5.0×10^7 is the highest cell dose administered IV to an equid[71]. Although the study was not examining cutaneous wound healing, there was evidence of lymphocyte allogeneic stimulation, and whether the detected immunomodulation would be enough to influence healing is impossible to say. In mice, 1×10^6 IV MSCs are typically administered in wound healing models[16]. This is equivalent to 33×10^6 cells per kilogram or over 14 billion for the average 450-kilogram horse. Clearly, administering this many cells to a horse would be challenging to produce and cost prohibitive, but a positive effect cannot be expected unless doses higher than those currently reported in horses are explored.

2.4.6 Homing and Engraftment

Homing is transendothelial migration of cells following arrest within the vasculature. Leukocytes are most commonly referred to in regards to this process, but other cells including MSCs are also capable. Following injury, both administered exogenous and native endogenous MSCs respond to inflammatory chemokine release at the injury site and actively home systemically by transmigrating through the endothelium following rolling

and diapedesis similar to leukocytes, albeit less efficiently[86]. Following transendothelial migration, administered MSCs that survive and integrate into the tissue are considered to be "engrafted"[87].

Although it was previously believed that MSCs contributed to healing by differentiating at the site of injury and integrating into the repair, current theories suggest that MSCs mainly modulate the inflammatory environment to optimize tissue healing, both locally and systemically, by conducting the release of anti-inflammatory and pro-angiogenic cytokines and growth factors[12–17]. Although successful engraftment does not seem to be critical to enhance healing[79,80,85], engraftment of even low numbers of exogenous MSCs has prolonged beneficial effects compared to MSCs that did not engraft and had a single effect at the time of administration[16,87]. Local administration does not necessarily improve engraftment, and does not utilize the additional benefit of systemic immunomodulation by systemic administration[16]. The state of inflammation also plays an important role in this process with studies showing that MSC engraftment rates are optimized when administered during the acute inflammatory phase rather than the later chronic inflammatory phase[73,79,80]. Therefore, timing of administration is an important variable that should be considered to maximize the benefits of MSC therapy.

2.5 Rationale

Cutaneous wounds are one of the most common reported injuries in horses and are a significant welfare concern[9,10]. Limb wounds in horses have different healing characteristics than wounds on the body related to anatomic, physiologic, and inflammatory differences[4,24]. These differences predispose limb wounds to complications such as infection, EGT, and delayed healing[4,24,40]. MSCs are immunomodulatory and are a promising therapy for promoting healing of limb wounds in horses, but clinical studies examining their efficacy and safety are lacking[12–14]. Ideal timing of administration in relation to the occurrence of the wound is unknown, but studies in other species have determined that administration during the acute inflammatory stage is ideal for promoting healing in a variety of injury models[73,79,80], which in the case of an unanticipated injury can only be achieved by administrating allogeneic sources. Although allogeneic sources do not have surface expression of MHC-I and MHC-II *in vitro*, allo-MSCs express cell surface

markers following engraftment and are rapidly rejected by the host[72–74]. Regardless, allo-MSCs still positively influence healing nearly as well as auto-MSCs through modulation of the immune response[16,73,75], and are generally well tolerated by the recipient. There are many different proposed routes of delivery of MSCs, but IV is attractive primarily for its ease of administration compared to other methods[16,17,84], and may be just as effective as other delivery methods despite low engraftment rates and entrapment of the cells in the lungs[16,79,80,85]. However, homing and engraftment to cutaneous wounds following jugular IV administration has never been documented in horses, and may be different compared to other species. Pulmonary injury following IV administration is an unlikely but very serious complication[21,91], but preliminary studies in horses have shown that this administration method is well tolerated[12,64,71,89,94–96] although they do stimulate a cellular immune response in equine recipients but without a clinical adverse response[71]. Furthermore, the effective IV cell dose to promote healing is unknown, but current published doses[71,95] are far below the cells administered per gram to that of laboratory animals[16], and investigation into the tolerance and efficacy of higher doses are needed. Compared to other sources such as adipose-derived and bone marrow-derived MSCs, allo-CB-MSCs are promising as collection is non-invasive[59,61], they have immunomodulatory properties *in vitro* and *in vivo*[59,61–64,67], they are well tolerated following IV administration[71], and they can be frozen and stored for later administration shortly following an injury[62].

3. OVERARCHING HYPOTHESES AND PRIMARY OBJECTIVES

Our overarching hypotheses are that following IV administration of >1.00 x 10^8 allo-CB-MSCs:

1) cells home and engraft to surgically created wounds.
2) produce no adverse clinical reactions.
3) promote macroscopic and histologic healing of limb wounds.
4) alter the immunomodulatory cytokine profile within the limb wound environment.

Our primary objectives are:

1) determine whether IV administration of >1.00 x 10^8 allo-CB-MSCs causes clinically detectable adverse reactions.
2) homing and engraftment of allo-CB-MSCs to surgically created limb and thoracic wounds over time following IV administration.
3) determine if IV allo-CB-MSC therapy accelerates closure of limb wounds.
4) determine if IV allo-CB-MSC therapy improves histologic healing scores of limb wounds.

Our secondary objectives are:

1) determine whether preliminary observations of mRNA expression of βarr2, CXCL8/CXCR2, CXCL10/CXCR3 differ between limb and thoracic wounds over time.
2) determine whether IV allo-CB-MSC therapy alters the mRNA expression of proinflammatory, anti-inflammatory, inflammation resolving, profibrotic, and antifibrotic cytokines in limb wounds over time.

4. HOMING AND ENGRAFTMENT OF INTRAVENOUSLY ADMINISTERED EQUINE CORD BLOOD-DERIVED MULTIPOTENT MESENCHYMAL STROMAL CELLS TO SURGICALLY CREATED CUTANEOUS WOUNDS IN HORSES: A PILOT PROJECT

Transition Statement:

This chapter presents the preliminary observations of homing and engraftment to surgically created wounds following IV administration of allo-CB-MSCs to two horses. The intents of this pilot project was to determine the feasibility of shipment and fluorescently labelling large quantities of CB-MSCs and its effect on viability of the cells prior to commencing with the major project, and also determine that administering large quantities of allo-CB-MSCs would not negatively affect the recipients. In addition, this pilot project intended to describe homing and engraftment of fluorescently labelled cells following IV administration, if present, to surgically created wounds on the limb and thorax to provide further insights into the mechanism of MSC influence on wound healing in horses.

This project determined that receiving and fluorescently labelling large amounts of CB-MSCs using the described techniques had minimal effect on viability and was technically feasible, and the recipients had no clinically adverse responses. In addition, CB-MSCs were detected in biopsied wound tissue and important initial observations suggest that there are differences in homing and engraftment between limb and thoracic wounds.

4.1 Introduction

Cutaneous wounds are common in horses and often must heal by second intention. Compared to body wounds, limb wounds have prolonged low-grade inflammation which causes slower healing and less contraction, and this in turn promotes development of exuberant granulation tissue (EGT) and a less durable and less cosmetic repair[4,24]. Keloid formation in humans is similar to EGT formation on the limbs of horses and, interestingly, humans and horses are the only two species that spontaneously develop these

fibroproliferative disorders, making the horse a good animal model for studying keloids and hypertrophic scars in humans[38].

Multipotent mesenchymal stromal cells (MSCs) have been investigated as ancillary therapy for complicated cutaneous healing and keloid treatment in humans[15,51–53]. MSCs improve wound healing by modulating the immune response both systemically and locally[16,17,97] and these positive effects are further enhanced when MSCs successfully home to and engraft within the wound[87]. However, experimental studies examining the effects of multipotent cells on equine cutaneous wounds are limited[20,98,99].

In horses, autologous MSCs are most commonly used for tendon healing and a lag time is required for isolation and expansion of MSCs before administration[11,18]. While the most appropriate time for administration of MSCs to enhance healing of cutaneous wounds is currently unknown, spinal cord contusion[79] and colitis[80] models using laboratory animals have shown that MSC therapy during the acute inflammatory phase is crucial to best ameliorate the inflammatory response. Thus delaying therapy to culture autologous MSCs may miss the "window of opportunity" for a good outcome. Allogeneic MSCs (allo-MSCs) can be isolated and expanded prior to injury for timely administration[18], and intravenous (IV) allo-MSCs have been shown to positively influence cutaneous wound healing in several species[15,17] but this effect has not yet been demonstrated in horses.

In human regenerative medicine, there is great interest in umbilical cord blood-derived MSCs (CB-MSCs) as collection is non-invasive, they can be stored long-term for future autologous or allogeneic use, are immunosuppressive and immunomodulatory and have higher expression of pluripotency markers as well as higher isolation and proliferation rates compared to bone marrow-derived MSCs[100]. Equine CB-MSCs are a very promising source of allo-MSCs because they can be similarly collected, stored, and expanded[66] and have been shown to have immunomodulatory properties *in vitro*[59,63].

While systemic administration of MSCs is minimally invasive and convenient, complications such as pulmonary injury[21,91], cerebral embolism[90] and hypersensitivity responses[101] are possible. Safety studies of relatively small amounts of IV administered MSCs (0.2 - 50 x 10^6)[64,71,95,102] have been performed in horses but to our knowledge no equine studies have

investigated whether there are complications associated with IV administration of >1.0 x 10^8 MSCs, or whether IV administered MSCs can home and engraft into equine cutaneous wounds.

The objectives of the this proof-of-principle pilot study were to (1) determine if subject horses developed adverse reactions during or for 6 weeks post-IV administration of >1.0 x 10^8 fluorescently prepared allo-CB-MSCs, (2) describe the presence and patterns of homing and engraftment of fluorescently prepared allo-CB-MSCs in biopsies from wounded and non-wounded cutaneous tissue of the limb and thorax during healing, and (3) determine whether preliminary results suggest limb wounds have different patterns of CB-MSC homing and engraftment than thoracic wounds over time.

4.2 Materials and Methods

The experimental protocols (AUP #20140096) involving animals were approved by the University Animal Care Committee and Animal Research Ethics Board of the University of Saskatchewan.

4.2.1 Overview of Study Design

Standardized cutaneous wounds were created on the left lateral third metacarpus (MCIII) and left hemi-thorax. Twelve hours after wound creation, 1.02 x 10^8 live allogeneic CB-MSCs labeled with a fluorescent dye (PKH26) and transduced with an enhanced green fluorescent protein (eGFP) transgene were administered intravenously via the jugular vein. Red fluorescence of PKH26 allowed immediate identification of CB-MSCs in biopsy samples before green fluorescence of translated eGFP was consistently produced by viable CB-MSCs. Detection of red fluorescent signal of CB-MSCs in the early stages of wound healing was considered indicative of homing but not necessarily viability and engraftment, whereas detection of green and red fluorescent signal of CB-MSCs outside of the vasculature was considered indicative of homing, transendothelial migration and engraftment of viable CB-MSCs. Biopsies were taken of wounds on the limbs and thoraces and of the corresponding contralateral non-wounded control sides on days 0, 1, 2, 7, 14, and 33 and evaluated by confocal microscopy for administered prepared CB-MSCs. Horses were monitored for adverse clinical reactions during injection and for the duration of the study period of 6 wk.

4.2.2 Recipient Animals

Two sound 7-yr-old Thoroughbred mares with no history of injury to the forelimbs or thoracic wall that were healthy on physical exam with normal complete blood counts and serum chemistry were used. Horses were vaccinated (West Nile-Innovator+EWT®, Zoetis) and dewormed (Equimax®, Bimeda). Twelve hours prior to surgery, an intravenous catheter was aseptically placed in the left jugular vein and feed was restricted eight hours pre-operatively.

4.2.3 Transducing and Labeling Trial

Prior to commencement of the project, a trial sample of 1.0 x 10^6 CB-MSCs (eQcell Therapies Inc.; King City, Ontario, Canada) was acquired in order to determine logistics and timing of shipping and receiving, and to establish ideal transducing and labeling conditions. CB-MSCs were of the same source as the CB-MSCs used in the *in vivo* project. Optimal multiplicity of infection (MOI) and concentration of PKH26 was determined using the same AAV2-CMV-eGFP vector (AAV2.CMV.PI.eGFP.WPRE.bGH; Penn Vector Core, Gene Therapy Program, University of Pennsylvania) and PKH26 labeling kit (PKH26 Red Fluorescent Cell Linker; Sigma-Aldrich, Oakville, ON) as in the *in vivo* project. After transducing and labeling, CB-MSCs were cultured long term to determine fluorescent expression patterns of prepared CB-MSCs, establish label longevity, verify passage of fluorescence to daughter cells, and to confirm absence of cell toxicity. Using a similar protocol, we have previously shown that almost all testis stem cells exposed to AAV2 were successfully transduced with eGFP[103] and stably expressed the eGFP transgene for several years *in vivo*[104].

4.2.4 Source and Transportation of CB-MSCs

CB-MSCs originating from cord blood of five unrelated male donor foals were used (eQcell Therapies Inc.). This source of CB-MSCS has been previously demonstrated to produce a surface marker phenotype consistent with MSCs[59] and were isolated using procedures as previously described[66]. At the time of shipment, the CB-MSCs had been cryopreserved once, passaged 4-5 times, and cultured for a total of 35-45 days. Twenty-four hours prior to injection, they were harvested using trypsin and disodium ethylenediaminetetraacetic acid (EDTA), washed in phosphate buffered saline (PBS), pooled to obtain a mixed population of donor cells, re-suspended in a serum-free commercial cell preservation medium

(HypoThermosol® FRS [HTS-FRS]; BioLife Solutions, Bothell WA) and cooled. The cooled CB-MSCs were shipped overnight to our laboratory in a reusable temperature controlled shipping container (Greenbox 2-8°C thermal management system, ThermoSafe ®, Arlington Heights, IL). A representative sample was taken and cell count and viability determined in duplicate and averaged using a haemocytometer counting chamber and trypan blue exclusion assay. A total of 4.16 x 10^8 cooled live CB-MSCs were received.

4.2.5 Preparation of CB-MSCs

Donor CB-MSCs were transduced *in vitro* with an eGFP transgene via an AAV2-CMV-eGFP vector as described in Section 4.2.3. CB-MSCs were exposed to the AAV2 at MOI of 1.0 x 10^4 genome copies/cell. CB-MSCs were then incubated at 37ºC for 3 hr *in vitro*, washed twice by centrifugation at 500 x g for 5 min, and re-suspended in Dulbecco modified Eagle medium (DMEM; Mediatech, Manassas, VA).

After transduction, CB-MSCs were labeled by incubation with PKH26 (Sigma-Aldrich) according the manufacturer's instructions. Briefly, CB-MSCs were washed in DMEM, centrifuged at 400 x g for 5 min, and re-suspended in Diluent C. Immediately before staining, 2.0 x 10^{-5} molar of PKH26 dye was prepared using Diluent C, gently mixed with the CB-MSCs, and incubated at 25ºC for 6 min. Staining was stopped by the addition of fetal bovine serum (PAA Laboratories, Etobicoke, ON), and CB-MSCs were subsequently washed three times in DMEM. After the last wash, CB-MSCs were re-suspended in HTS-FRS for a total volume of 60 mL in a sterile syringe and kept cool until injection one hour later. After preparation, a representative sample of CB-MSCs was retained and cell count and viability was repeated using a haemocytometer counting chamber and trypan blue exclusion assay. Viability of CB-MSCs was 49% (2.04 x 10^8 CB-MSCs) and hence 1.02 x 10^8 CB-MSCs were administered to each horse.

Additionally, a sample of prepared CB-MSCs was cultured into 6-well plates to serve as an *in vitro* reference for timing and pattern of fluorescence of prepared CB-MSCs. The supernatant from the last cell wash was also added to a cell culture of a sample of non-prepared CB-MSCs to confirm no contamination of the supernatant with free PKH26. These cultures were maintained and observed for the duration of the study.

4.2.6 Wound Creation

On day 0, horses were anaesthetized, maintained to effect on a guaifenesin, ketamine and xylazine intravenous drip (1 L 5% guaifenesin + 1000 mg ketamine + 500 mg xylazine) and placed in right lateral recumbency. After aseptic preparation, seven standardized full thickness excisional skin wounds were created using a scalpel on the left lateral MCIII and hemi-thorax at the region of the tenth costochondral junction of each horse (Figure 4.1). Wounds measured 0.5 cm x 2.0 cm in a horizontal orientation and orientated in a vertically stacked arrangement 2.0 cm apart. The wounds were covered during recovery from anaesthesia and then were left unbandaged to heal by second intention. Excised skin was retained for evaluation of baseline background fluorescence. Anti-inflammatories and antimicrobials were not administered at any time to avoid modification of inflammation.

4.2.7 Prepared CB-MSC Administration and Monitoring

On day 1, twelve hours after wound creation, the prepared CB-MSCs were injected via the indwelling catheter (4 mL/min over 15 min). During the injection, vital parameters were monitored for adverse clinical reactions (*i.e.*, tachycardia, tachypnea, pyrexia, respiratory distress, colic, urticaria) every minute for the first 5 min followed by every 5 min until the suspension was administered. A physical exam was performed every 12 hr for the following 36 hr then once daily for the following 7 days, followed by distance exams of general health and demeanour until the conclusion of the study 6 wk after injection.

4.2.8 Biopsy Collection

The two most proximal wounds on the limb and thorax were not biopsied and observed for healing characteristics in preparation for future studies. Horses were sedated and biopsies were taken of the remaining surgically created wounds of the limbs and thoraces and of the corresponding contralateral control sides on days 1, 2, 7, 14, and 33 in a distal to proximal sequence (Figure 4.1). Biopsied tissue was cooled and immediately processed and evaluated by confocal microscopy.

4.2.9 Fluorescence Evaluation of Biopsies

Confocal microscopy was used to determine normal background fluorescence of resected skin collected on day 0 and to detect fluorescent signal in biopsy tissue from administered

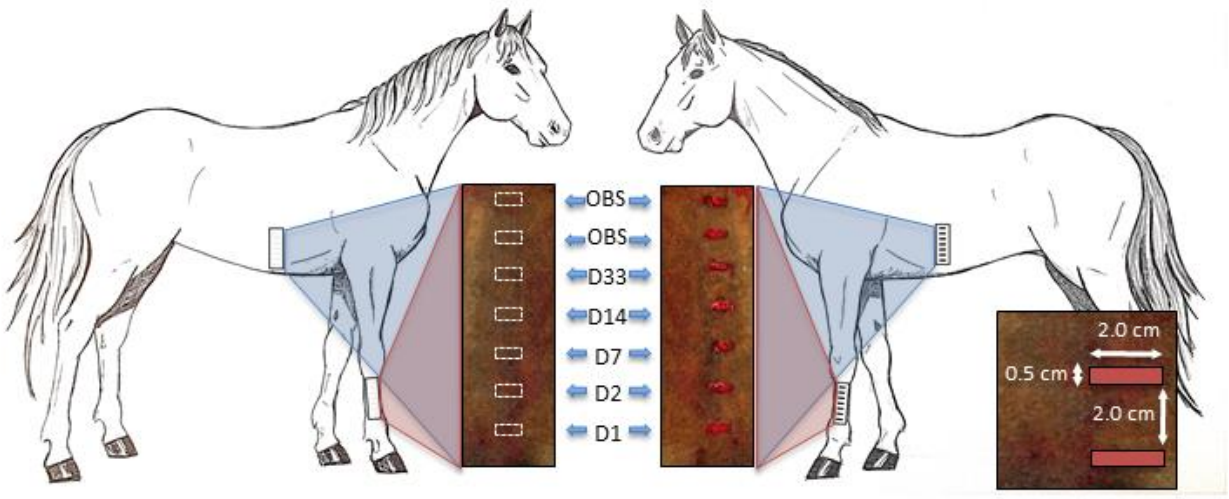

Figure 4.1: Basic schematic of wound creation and sequence of biopsy collection. On day 0, seven wounds were created on the left forelimb and hemi-thorax of each horse measuring 0.5 cm x 2.0 cm and placed 2 cm apart in a vertical orientation. Biopsies were collected on days (D) 1, 2, 7, 14, and 33 from the wound site and from the corresponding contralateral non-wounded site in a distal to proximal sequence. The top two wounds were left to heal by second intention and observed for healing characteristics. (OBS), observed.

prepared CB-MSCs. One mm slices of fresh cooled biopsies were prepared and examined under a Leica epifluorescence microscope for red and green fluorescent signal using filter sets for GFP (excitation 450-490 nm/emission LP515 nm) and rhodamine (excitation 515-560 nm/emission LP590 nm) with a 63 x oil objective and imaged with a Leica SP5 confocal microscope (Leica Microsystems; Watzlar, Germany) using the 488 nm line of an Argon laser (emission range of 500-535 nm for GFP) and the 543 nm line of a Helium Neon laser (emission range of 555-700 nm for PKH26). Biopsies from each collection period were evaluated by a blinded operator (E.K.). The operator subjectively evaluated the biopsies for presence of red and/or green signal and the intensity and distribution of the signal. Fluorescing cell-like structures were considered administered CB-MSCs if they had similar fluorescence patterns and were similar size to cultured CB-MSCs in our *in vitro* study. Fluorescent signal patterns were thoroughly described for each horse and then summarized. No difference in homing between the wound biopsies and the contralateral non-wounded biopsies was recorded as *=*, slightly more homing to wounds was recorded as *+1*, moderately more homing was recorded as *+2*, and markedly more homing was recorded as *+3*. Presence of red fluorescence was recorded as *R* and presence of green fluorescence

was recorded as *G*. Biopsies in which signal was absent or rarely detected was further recorded as *none* or *rare*.

4.3 Results

4.3.1 Adverse Reactions

Neither of the horses developed adverse clinical reactions during injection of the prepared CB-MSCs and all evaluated vital parameters remained within normal limits. One horse had symptoms of mild colic (abdominal pain) prior to injection of the CB-MSCs that was attributed to anaesthesia and because her vital signs remained normal the CB-MSCs were administered. After administration of the CB-MSCs, she responded well to minimal intervention (oral fluids, oral mineral oil, sedation [xylazine, 150 mg IV]) and did not experience any more colic symptoms throughout the duration of the study period. There was mild edema of the tissue adjacent to the wounds in both horses for a few days following wound creation and biopsy collection; however, lameness was not apparent at the walk and intervention was not needed. Both horses remained comfortable and did not develop any adverse reactions throughout the remainder of the study period.

4.3.2 Confocal Microscopy Observations

4.3.2.1 In vitro *culture*

On all days, CB-MSCs measured approximately 15-20 μm in length and 10-15 μm in width. On day 1, prepared CB-MSCs had dots of predominantly red fluorescence measuring on average 2 μm uniformly distributed throughout the cell on phospholipid membranes and very weak green signal was also detected as dots (Figure 4.2a). On day 7, dots of red fluorescence remained uniformly distributed and weak green signal was detected as small dots <1 μm in diameter and/or generalized green fluorescence of the cytoplasm with low amounts of colocalization of green and red signal (Figure 4.2b). On days 14 and 33, the distribution of green signal remained similar to day 7 but more vibrant and readily detectable (Figure 4.2c, d). Green signal was most intense on day 33 (Figure 4.2d). On day 14 and 33, amount of colocalization of green and red signal varied between individual cells but was typically low to moderate.

4.3.2.2 In vivo *tissue biopsies (Table 4.1):*

Day 0:

Collagen fibres demonstrated normal green and red auto-fluorescence consistently in limb and thoracic biopsies of both horses. There were no cell-like structures that had similar fluorescent patterns to cultured prepared CB-MSCs (Figure 4.3).

Day 1:

Red and green signal was typically colocalized and detected in all biopsies except in the non-wounded thoracic biopsy of horse 1. Signal was typically highly concentrated in the vasculature and both horses had slightly more signal in the wounded limb and thoracic biopsies (Figure 4.4a, c).

Day 2:

Colocalized red and green signal was detected in all biopsies. In biopsies of limb wounds of both horses, slightly more signal was detected compared to non-wounded limb biopsies. There was no difference in amount of signal or pattern of distribution in wounded and non-wounded thoracic biopsies of both horses. In all biopsies, signal was detected within the vasculature in clusters, but also was located along the endothelium of the vessels, possibly consistent with endothelial adhesion of CB-MSCs (Figure 4.5c, d). In wounded and non-wounded limb biopsies of horse 1, cell-like structures measuring ~8-10 μm were occasionally detected outside of the vasculature in the interstitium (Figure 4.5b).

Day 7:

Red and green colocalized signal was detected in all biopsy sites of both horses. In horse 1, there was no difference in amount of detected signal from wounded or non-wounded biopsies of the limb or thorax. Furthermore, in both wounded and non-wounded limb and thoracic sites, signal was detected primarily within and along the endothelium of the vasculature (Figure 4.6c, d). In wounded and non-wounded limb biopsies, occasional cell-like structures were detected in the interstitium measuring 8-10 μm (Figure 4.6a) and larger cell-like structures measuring 15-20 μm were detected within the vasculature along the endothelium (Figure 4.6b). In horse 2, there was no difference in detected signal in wounded and non-wounded limb biopsies. Occasional cell-like structures measuring 12-20

μm were detected within the vasculature of both wounded and non-wounded limb sites (Figure 4.6a). In the thorax, there was one area in the wounded site that contained several cell-like structures measuring 8-11 μm in the interstitium, but otherwise there was no difference in detected signal between wounded and non-wounded biopsies. Cell-like structures were occasionally detected within the vasculature of both wounded and non-wounded thoracic sites.

Day 14:

In both horses, there was no difference in pattern of distribution of red or green signal, or intensity of fluorescence in limb or thoracic wounded and non-wounded biopsies. Red and green signal was typically colocalized and located both within and out of the vasculature. In addition, signal that formed clumps of ~5 μm was often found along the endothelial surface of vessels, just adjacent to the vasculature or in the interstitium (Figure 4.7a). A single cell-like structure measuring 15 μm was detected in the interstitium of the non-wounded limb of horse 2 and wounded thorax of horse 1 (Figure 4.7b, c).

Day 33:

On day 33, red and green signal was more readily apparent in limb and thoracic wounds of both horses (Figure 4.8a, c). More specifically, signal was colocalized and predominately detected as cell-like structures between 10-20 μm. These cell-like structures were found in clusters and larger cell-like structures measuring 15-20 μm were often elongated. Both larger and smaller cell-like structures appeared to be integrated into the interstitium. In the non-wounded limb and thoracic biopsies, cell-like structures were rarely detected, rounded, located often within the vasculature, and measured between 5-12 μm, smaller than those typically detected in the wounded biopsies (Figure 4.8b).

Table 4.1: Summary of Labelled CB-MSC Fluorescent Signal Colour and Homing to Wounds

		Day 1		Day 2		Day 7		Day 14		Day 33	
Subject	**Biopsy Site**	**Color**	**Homing**	**Color**	**Homing**	**Color**	**Homing**	**Color**	**Homing**	**Color**	**Homing**
Horse 1	Limb Wounded	R, G	1+	R, G	1+	R, G	=	R, G	=	R, G	3+
	Limb Non-Wounded	R, G		R, G		R, G		R, G		R, G	
	Thorax Wounded	R, G	1+	R, G	=	R, G	=	R, G	=	R, G	3+
	Thorax Non-Wounded	none		R, G		R, G		R, G		R, G	
Horse 2	Limb Wounded	R, G	1+	R, G	1+	R, G	=	R, G	=	R, G	3+
	Limb Non-Wounded	R, G		R, G		R, G		R, G		R, G	
	Thorax Wounded	R, G	1+	R, G	=	R, G	=	R, G	=	R, G	3+
	Thorax Non-Wounded	R, G		R, G		R, G		R, G		R, G	

Homing was considered present if more red and/or green signal was detected in biopsies of wounds compared to the corresponding contralateral non-wounded site at the same time period, regardless of whether signal was detected intravascular or extravascular. Abbreviations: Color - *(R)*, presence of red signal; *(G)*, presence of green signal. Homing - *(=)*, no difference in homing; *(1+)*, slightly more homing; *(2+)*, moderately more homing; *(3+)* markedly more homing.

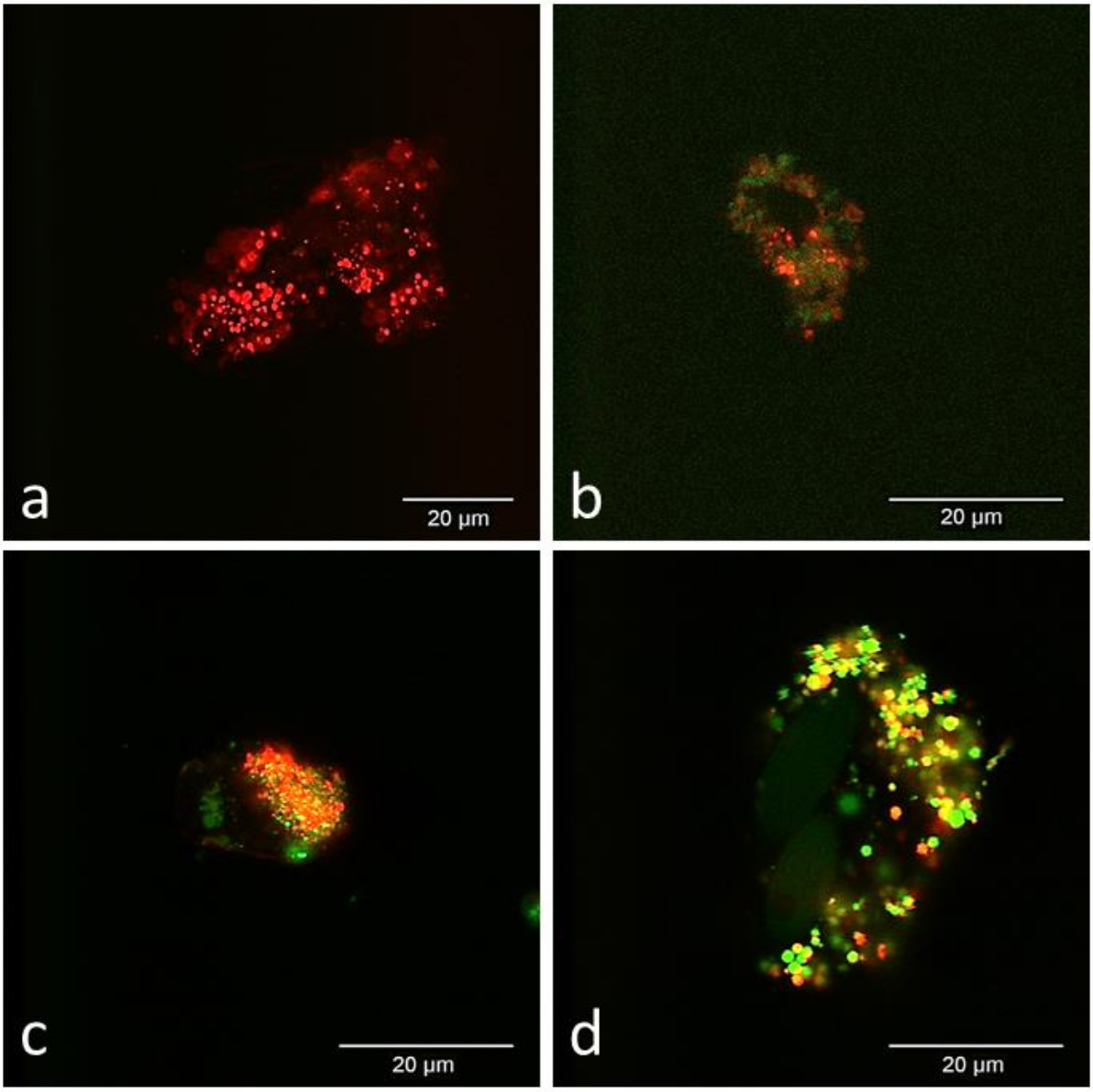

Figure 4.2: Representative confocal microscopy images of *in vitro* cultured prepared CB-MSCs. (a) Day 1, (b) day 7, (c) day 14, and (d) day 33. All images were captured using the same settings except for day 7 (b), which was captured with a higher sensitivity setting for green to demonstrate subtle green signal. CB-MSCs are clumped in day 1 (a) and day 33 (d). In all images, individual CB-MSCs measured between 781 15-20 µm lengthwise and 10-15 µm widthwise. On day 1 (a), many red fluorescent dots were uniformly distributed on phospholipid membranes with very weak green signal. On day 7 (b), CB-MSCs maintained red signal but also began to produce stronger green signal as dots or generalized throughout the cytoplasm. On day 14 (c) and day 33 (d), CB-MSCs remained similar to day 7 (b) but had brighter green signal of the cytoplasm and organelles. On day 33 (d) green signal was more intense than previous days. Colocalization of red and green signal varied between individual cells but was typically low to moderate.

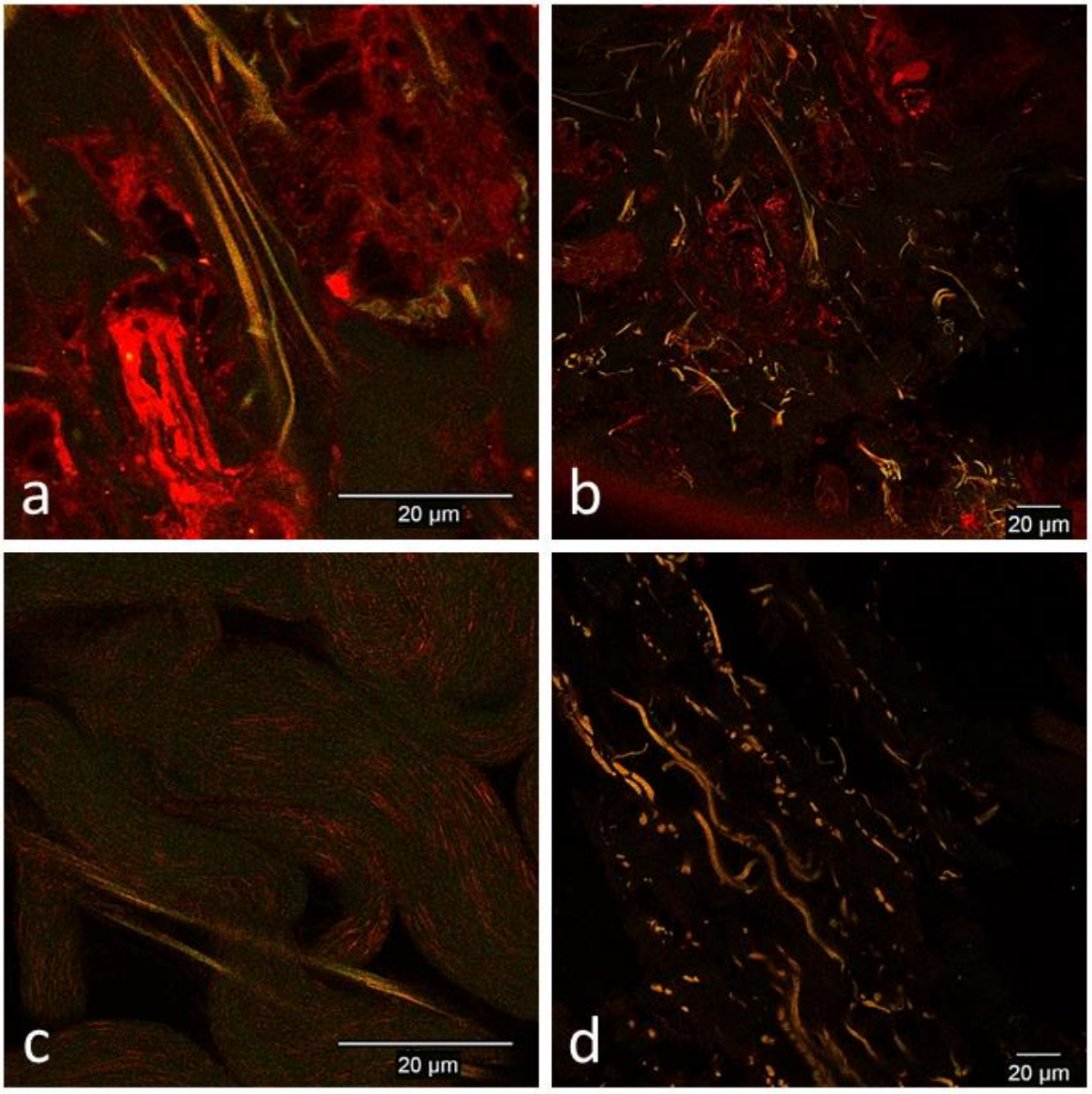

Figure 4.3: Representative confocal microscopy images of normal skin of the limb and thorax before surgical creation of wounds. a) Horse 2, thorax, b) horse 2, thorax, c) horse 1, thorax, and d) horse 1, limb. In both the limb and thorax, collagen fibers demonstrated normal green and red auto-fluorescence. No cell-like structures that demonstrated fluorescent patterns similar to cultured prepared CB-MSCs were detected.

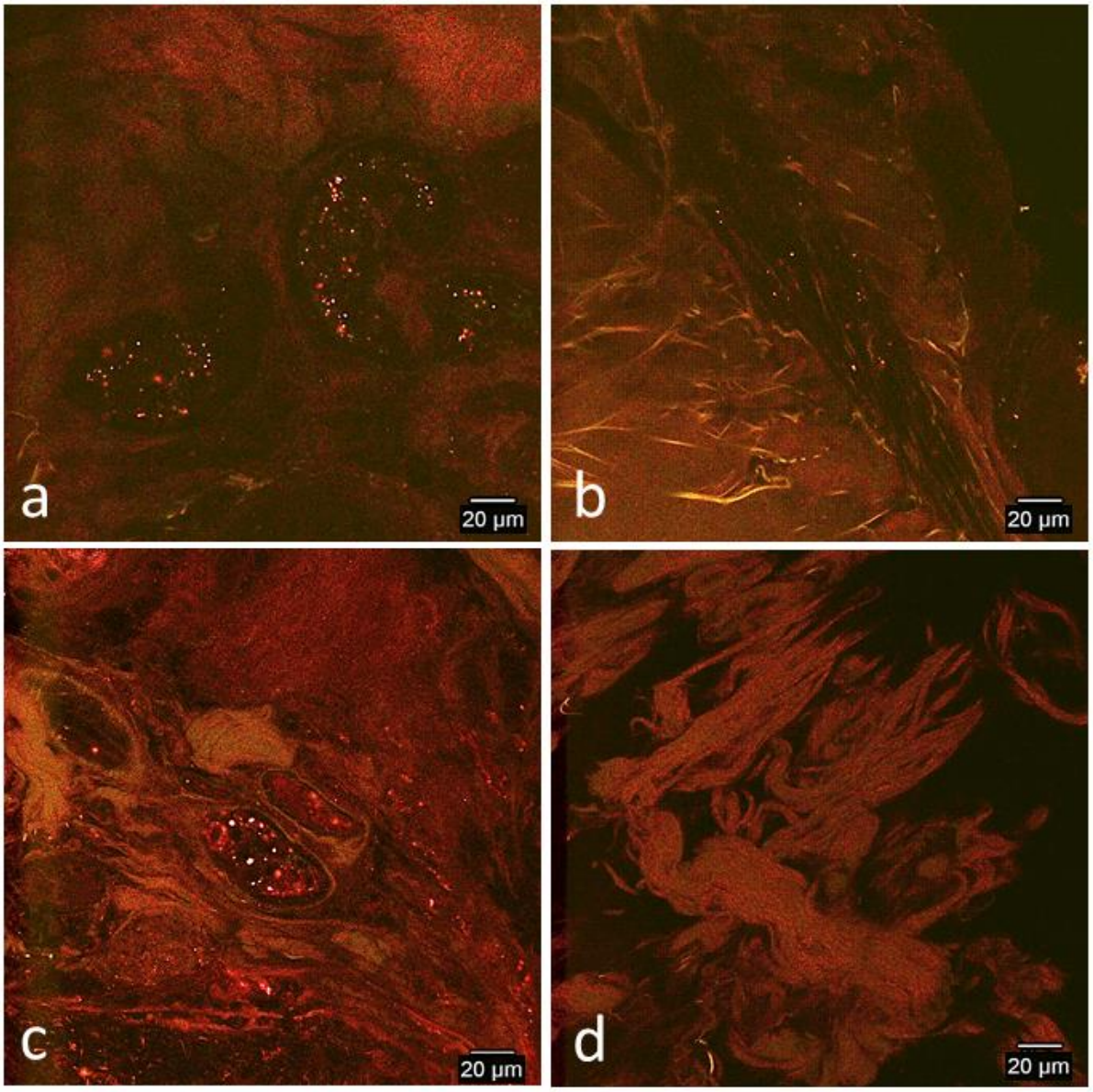

Figure 4.4: Representative confocal microscopy images of biopsies of limb and thoracic wounds and contralateral non-wounded skin on day 1. a) Wounded limb, b) non-wounded limb, c) wounded thorax, and d) non-wounded thorax. Images were selected from either horse based on best representation of patterns typical fluorescent signal patterns of both horses. Images have been selectively enhanced for purposes of demonstrating signal variation. There was slightly more homing to wounded limbs (a) and wounded thoraces (c) than the non-wounded sites (b, d). Red and green signal was highly concentrated in vasculature of wounded biopsies.

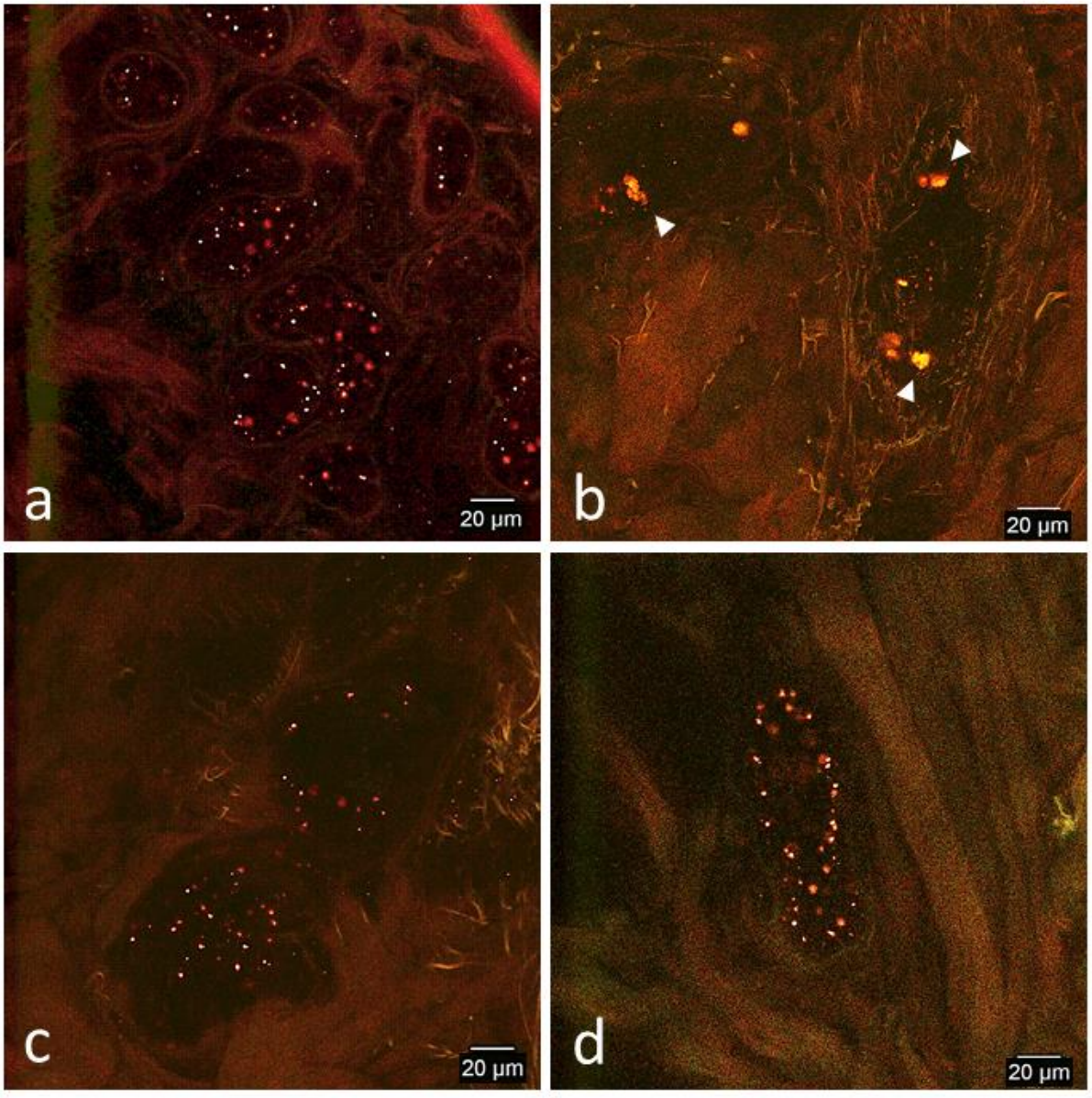

Figure 4.5: Representative confocal microscopy images of biopsies of limb and thoracic wounds and contralateral non-wounded skin on day 2. a) Wounded limb, b) non-wounded limb, c) wounded thorax, and d) non-wounded thorax. Images were selected from either horse based on best representation of patterns typical fluorescent signal patterns of both 803 horses. Images have been selectively enhanced for purposes of demonstrating signal variation. There was slightly more homing to limb wounds (a) than the non-wounded limb site (b). There was no difference in homing between the thoracic wounds (c) and non-wounded thoracic sites (d). Signal was red and green and located within the vasculature and occasionally along the endothelium (d) of both wounded and non-wounded biopsies. Cell-like structures measuring 8-10 um with colocalization of red and green signal were occasionally detected adjacent to the vasculature in wounded and non-wounded limb biopsies of horse 1 (arrowheads, b).

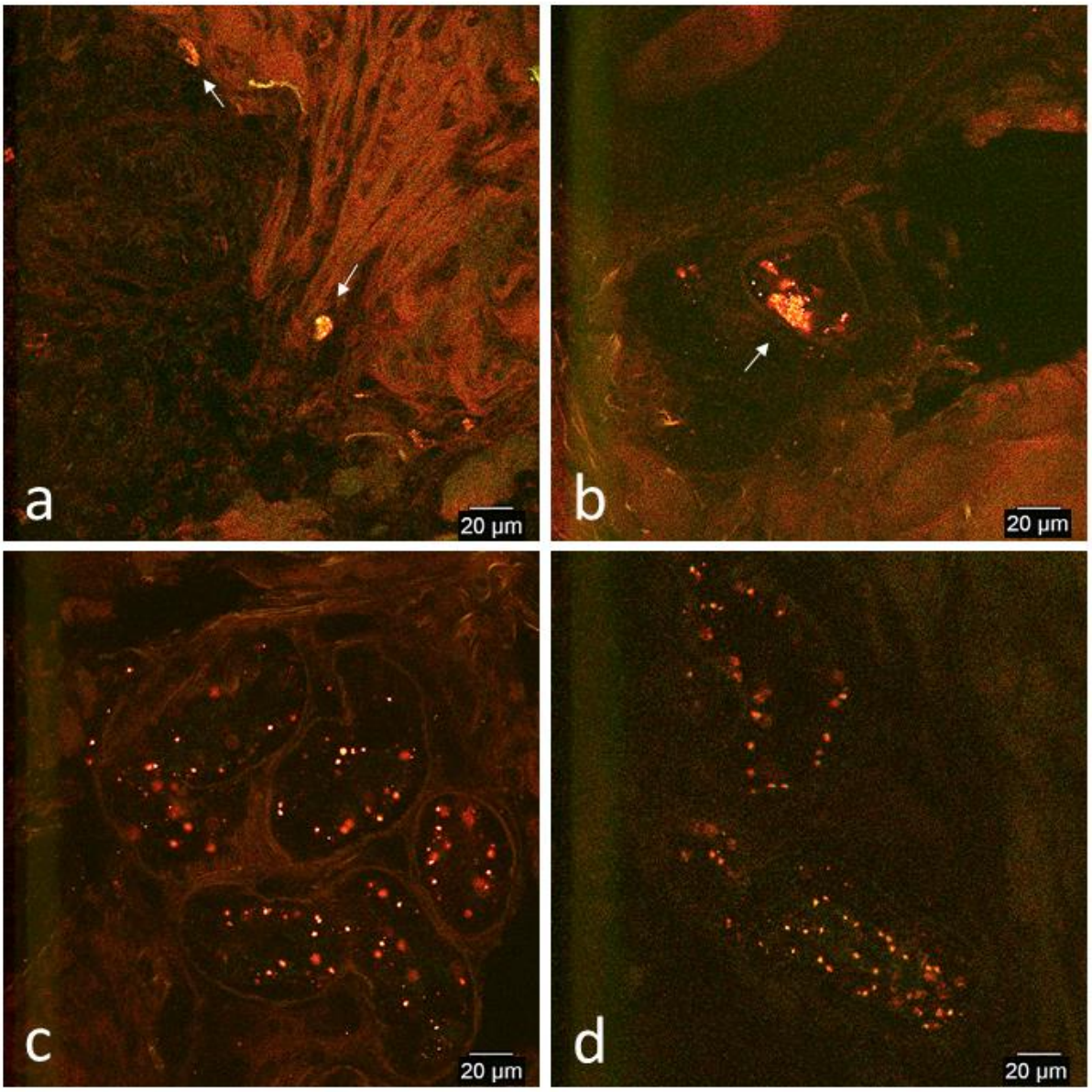

Figure 4.6: Representative confocal microscopy images of biopsies of limb and thoracic wounds and contralateral non-wounded skin on day 7. a) Wounded limb, b) non-wounded limb, c) wounded thorax, and d) non-wounded thorax. Images were selected from either horse based on best representation of patterns typical fluorescent signal patterns of both horses. Images have been selectively enhanced for purposes of demonstrating signal variation. Red and green signal was detected in all wounded and non-wounded biopsies. There was no difference in homing to wounds in either horse except for an area of the wounded thorax in horse 2 that had several cell-like structures. Signal was located within the vasculature along the endothelium in both horses (c, d) and cell-like structures consistent with *in vitro* CBMSCs were occasionally detected adjacent to or within the vasculature of all biopsies (arrow, b).

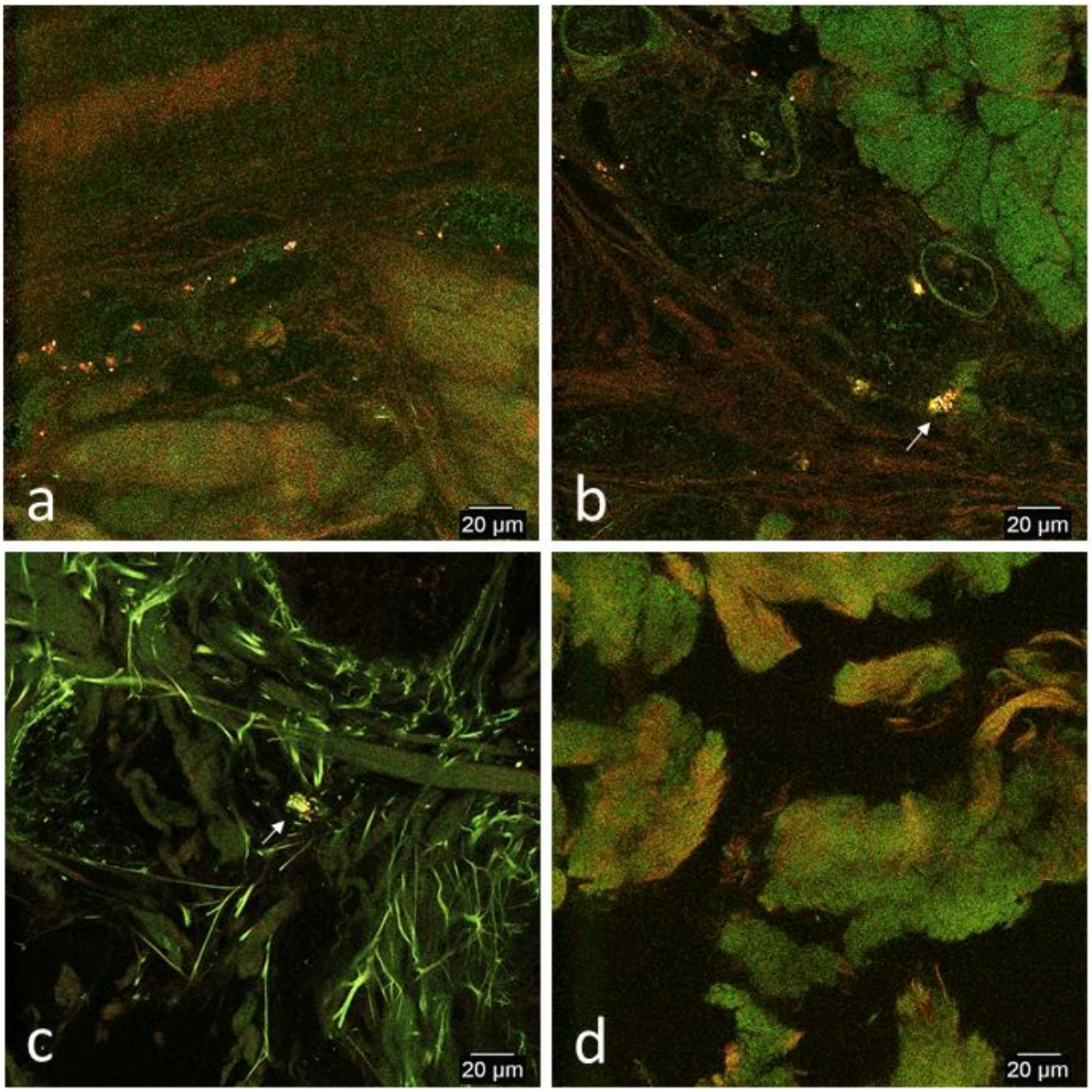

Figure 4.7: Representative confocal microscopy images of biopsies of limb and thoracic wounds and contralateral non-wounded skin on day 14. a) Wounded limb, b) non-wounded limb, c) wounded thorax, and d) non-wounded thorax. Images were selected from either horse based on best representation of patterns typical fluorescent signal patterns of both horses. Images have been selectively enhanced for purposes of demonstrating signal variation. In both horses, there was no difference in amount of distribution of red or green signal. Signal was often colocalized in small clumps and located within or just adjacent to the vasculature (a). Single cell-like structures consistent with *in vitro* CB-MSCs were detected in the interstitium of the non-wounded limb of horse 1 (arrow, b) and wounded thorax of horse 2 (arrow, c). Background auto-fluorescence is shown in (d).

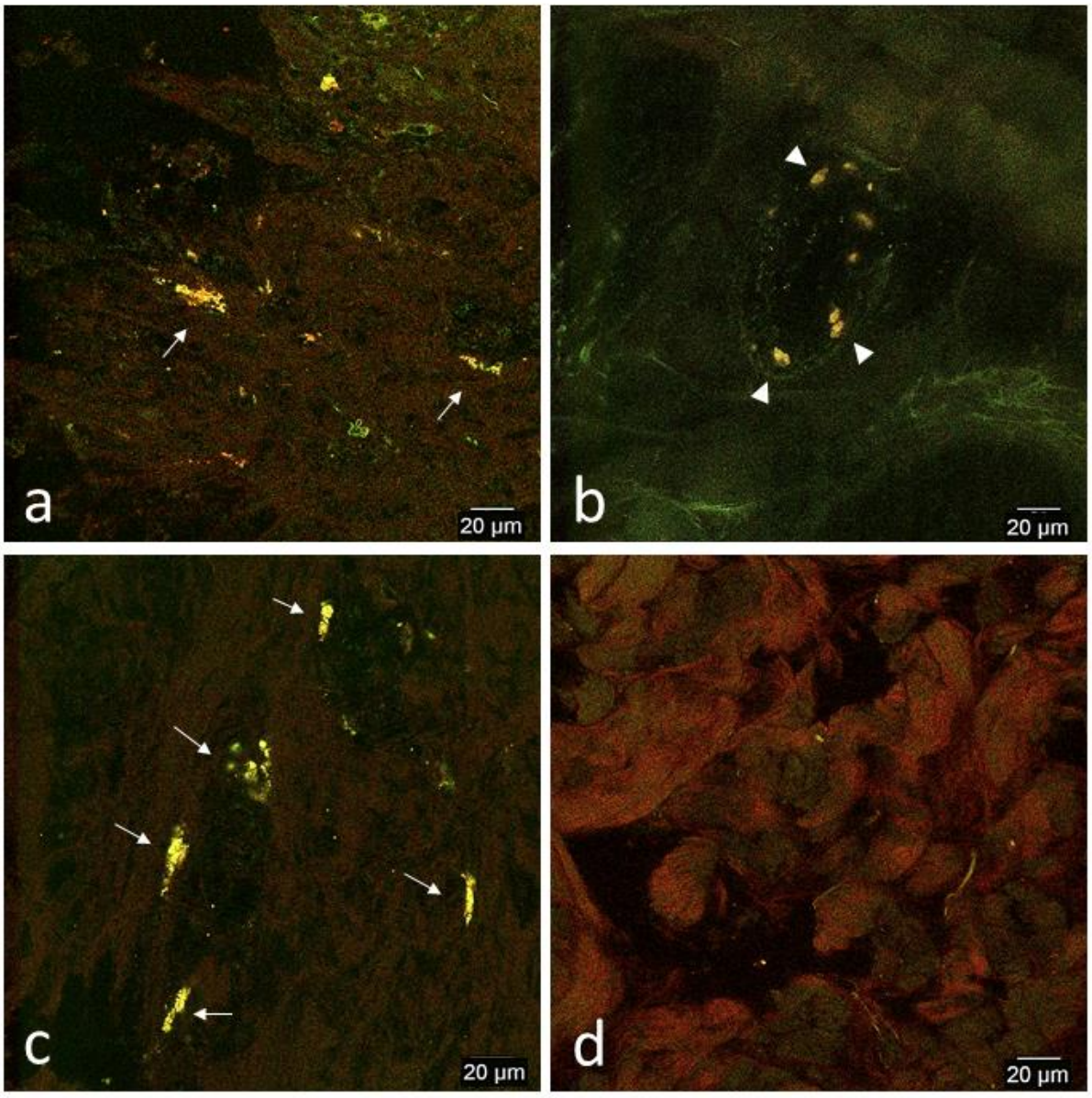

Figure 4.8: Representative confocal microscopy images of biopsies of limb and thoracic wounds and contralateral non-wounded skin on day 33. a) Wounded limb, b) non-wounded limb, c) wounded thorax, and d) non-wounded thorax. Images were selected from either horse based on best representation of patterns typical fluorescent signal patterns of both horses. Images have been selectively enhanced for purposes of demonstrating signal variation. In both horses, homing was marked in both limb and thoracic wounds and was characterized by cell-like structures consistent with *in vitro* CB-MSCs that appeared to be well integrated into the interstitium (arrows, a, c). In contrast, cell-like structures were only rarely detected in the non-wounded side and were rounded, located primarily in the vasculature, and were smaller than those typically detected in the wounded biopsies (arrowheads, b). Background auto-fluorescence is shown in (d).

4.4 Discussion

To the authors' knowledge, 1.02 x 10^8 is the largest amount of allo-MSCs that have been administered intravenously to horses, and we did not detect any clinical adverse reactions directly attributable to allo-MSC therapy. This is encouraging because despite evidence that equine MSCs have immunosuppressive properties[14,59,60,62,74,105–107], and intravenously administered MSCs are well tolerated[64,71,95,102], complications and reactions secondary to allo-MSC therapy and IV MSC administration can occur. Previously reported immune responses in horses to allo-MSCs include allo-antibody production[101,102,108], increased circulating lymphocyte population[64,71], wheal formation and local lymphocyte response after intradermal administration[98,101,109], and increased lameness and nucleated cell counts after intrasynovial injection[110]. Regardless, in these studies all local reactions were self-limiting and no horses developed systemic adverse reactions. Reported complications related to intravascular MSC administration in other species include fatal pulmonary embolism in mice[21], cerebral embolism in rats[90], pulmonary parenchymal edema and haemorrhage in dogs[91], and portal vein thrombosis in humans[111]. Despite these reports, complications are typically not encountered and IV administration of MSCs appears to be well-tolerated in most species[112,113]. However, laboratory animals used in IV MSC studies are often administered upwards of 1.6 x 10^4 MSCs per gram[16,92]. Clearly, administering similar amounts of MSCs per gram to a horse would be cost prohibitive. While the ideal dose of MSCs for a beneficial effect is unknown, neither positive clinical effects nor adverse reactions may be seen unless higher doses are used; therefore, we felt it important to evaluate the effects of a larger dose of IV MSCs than had been previously administered. Although we did not evaluate peripheral blood or tissues, the lack of clinical complications following injection of twice the amount of the past largest recorded dose of allo-MSCs[71] suggests it may be clinically safe to do so, but trials with larger numbers of subject horses must be repeated to assert clinical safety.

Cell-like structures detected *in vivo* were occasionally smaller than measured CB-MSCs *in vitro*. This was expected as confocal imaging wound have captured CB-MSCs in transverse, oblique, or sagittal planes as they interacted three-dimensionally with the surrounding niche, compared to the images of *in vitro* CB-MSCs that were flattened on the surface of the

culture dish. Also, detected cell-like structures *in vivo* had different colocalization patterns of red and green signal compared to *in vitro* CB-MSCs. *In vivo*, red and green signal was predominantly colocalized whereas *in vitro*, red and green signal was typically separate although colocalization of signal was occasionally seen in some cells. The difference in signal colocalization of *in vitro* and *in vivo* CB-MSCs is likely due to differences of interactions of the cells with their environment; more specifically, signal was more commonly colocalized in *in vivo* cells because of three-dimensional interaction with the local niche and ligand binding, stimulating increased packaging and transportation of eGFP in PKH26 labeled organelles compared to *in vitro* CB-MSCs[114,115]. Regardless, there was still striking similarity between cultured CB-MSCs and *in vivo* cell-like structures. Hence, we considered cell-like structures that had red and green signal similarly distributed as cultured CB-MSCS to be injected fluorescently prepared CB-MSCs, irrespective of colocalization of signal.

MSC homing is defined as "the arrest of a MSC within the vasculature followed by transmigration across the endothelium"[86] and MSC engraftment is defined as integration and survival of MSCs in the target tissue after transendothelial migration[87]. Inflammation is a very important factor for influencing both homing and engraftment rates. Although not as well understood as leukocyte homing, MSCs can home to target tissues actively by rolling, adhering and migrating through vasculature activated by the inflammatory cascade, and passively by migrating through the endothelium after becoming entrapped in non-activated capillaries[86]. Recently developed techniques to increase MSC homing have integrated principles of inflammation to either "prime" MSCs to increase efficiency of transendothelial migration or modify the target tissue to be more receptive to MSC engraftment[86,87,116,117]. For example, preconditioning MSCs in a hypoxic environment will cause upregulation of CXCR4, a chemokine receptor important for homing, on MSC surface membranes and improve homing rates[86,87,116,117]. Ischemic post-conditioning of the target tissue has also been shown to improve homing and engraftment rates by decreasing reperfusion injury and creating a less hostile environment for engrafting MSCs[87]. Determining the exact mechanism of CB-MSC homing and engraftment and the relationship of the CB-MSCs with the vasculature was beyond the scope of this project design, but regardless it is widely

agreed upon that inflammation plays a role in both active and passive mechanisms of MSC homing and engraftment. Therefore, we considered homing synonymous with engraftment and that homing to have occurred when red and/or green signal was detected more frequently in biopsies of wounds compared to non-wounded sites at each time period. Although only two horses were used in this pilot project and conclusions must be drawn cautiously, our results suggest CB-MSC homing and engraftment indeed occurred and temporal and regional inflammation likely influenced detection patterns.

Except for thoracic wounds on day 2, CB-MSCs preferentially homed to wounds in both horses on days 1 and 2 during the acute inflammation phase, and on day 33 during the early remodelling phase, where homing and engraftment was most obvious (Table 4.1; Figure 4.8). Furthermore, the engrafted CB-MSCs in the wounded sites on day 33 were elongated and occasionally found in clusters, possibly representative of differentiation and/or proliferation (Figure 4.8). This is in contrast to homing and engraftment patterns of intravenously administered MSCs in mice where homing is typically greatest during the acute inflammatory phase followed by a rapid decrease or absence of engrafted MSCs as inflammation resolves[17,82,83], suggesting that the majority of engrafted MSCs are eventually rejected. Why we saw a different homing pattern is unknown, but we can speculate based on findings from other research. In a rat knee osteoarthritis model, MSCs administered intra-articularly persisted in arthritic knees more than twice as long than in normal knees and began to proliferate[118]. The investigators hypothesized that a local inflammatory environment is favourable for MSC engraftment and supports MSC viability and proliferation. In addition, other researchers have shown that successfully engrafted MSCs can recruit more locally engrafted MSCs[119] as well as local and distant endogenous MSCs[120]. Based on these studies, it is possible on day 33 that ongoing and persistent chronic inflammation in the wounds produced an inflammatory environment that was favourable for CB-MSC engraftment and proliferation, and the engrafted CB-MSCs then recruited other locally engrafted CB-MSCs to the site as well as began to proliferate. In contrast, CB-MSCs were detected only rarely if at all in biopsies of the contralateral non-wounded site because local intermittent acute inflammation created during previous biopsy collections had resolved and any previously engrafted CB-MSCs did not persist or proliferate because of

lack of chronic inflammatory stimulus. Although dual Ki-67/eGFP immunohistochemistry was not performed to confirm differentiation and proliferation, we cannot disregard CB-MSC proliferation as a possible reason for increased CB-MSC detection on day 33. Regardless, the marked homing of CB-MSCs to the wounded sites and not the control sites suggests that CB-MSCs successfully homed and engrafted to the wounded site and possibly began to proliferate while continuing to recruit other engrafted CB-MSCs.

Because the original intents of this project was not to determine the relationship of the CB-MSCs with the vasculature, imaging of vessels was inconsistent and we cannot state whether the relationship of the CB-MSCs with the vasculature was consistent between biopsy sites or throughout time. However, in hindsight, the relationship of the CB-MSCs with the vasculature and apparent engraftment seemed to be related to the phases of healing and are important to note in our observations. During the inflammatory phase, CB-MSCs were detected in close relationship with the vessels, either intravascularly along the endothelium, or adjacent to the vessels within the interstitium (Figures 4.4, 4.5), but during the early remodelling phase CB-MSCs were typically detected engrafted in the interstitium and not immediately adjacent to the vessels (Figure 4.8). These preliminary observations are consistent with the extravasation phase and interstitial migration phase of systemic MSC homing and engraftment[86] although further studies are needed to validate this observation.

Differences between degree of CB-MSC homing in the limb and thoracic biopsies were only apparent on day 2; more specifically, mild homing was apparent in limb wounds but there were no differences in homing in thoracic wounds (Table 4.1; Figure 4.4). This was unexpected as wounds on the body of horses have a more pronounced acute inflammatory response than limb wounds[4,24], which theoretically could cause greater MSC homing and recruitment. However, we did not observe this phenomenon. It is possible, that inflammation created at the control hemi-thorax sites from biopsy collection the day before (day 1) created an inflammatory response that caused CB-MSCs to home to the contralateral non-wounded biopsy site, negating any difference in CB-MSC homing to the thoracic wound sites on day 2. Because limb wounds have a more mild inflammatory response, it is possible that confounding inflammatory recruitment of CB-MSCs was not

apparent in the limbs. It is also possible that limbs have a unique feature that increases their ability to recruit MSCs compared to other areas of the body. It is widely accepted that equine limb wounds have a different temporal cytokine profile that affects wound healing compared to other areas of the body[4,24], and so it possible that limb wounds may also have unique cytokine profiles or regional anatomical features that are favourable to MSC recruitment, such as increased hypoxia-inducible factors, endothelial adhesions receptors, and signalling pathways[86,87,116]. However, because we did not detect any other differences of homing between limb and thoracic wounds at any of the other time points this theory is less likely and purely speculative although further studies investigating this theory would be interesting.

After intravenous MSC administration, the majority of MSCs are entrapped in the pulmonary vasculature, which is known as the "first pass effect"[79]. Despite this, positive effects of intravenous MSC therapy are still seen due to a rapid release of immunomodulatory factors from entrapped MSCs that systemically influence healing[79]. In addition, even low engraftment rates improve wound healing through secretion of factors that recruit endogenous MSCs and promote angiogenesis[16,120]. Even though most of the administered CB-MSCs likely became entrapped in the lungs, we still detected CB-MSCs in the biopsies, indicating that CB-MSCs can home to and engraft in cutaneous wounds after passing through the lung vasculature in horses. As far as we are aware, we are the first group to detect CB-MSCs in equine cutaneous wounds after intravenous administration.

There are some limitations to the present study. First, this was a proof-of-principle pilot project with only two treatment horses. However, except for day 7 where horse 2 had increased homing to wounds and horse 1 did not, both horses had similar homing patterns for all time periods, and we feel our results are accurate and collectively our observations can be viewed as visual evidence of MSC homing. Second, the total fluorescence of the biopsied tissues was not quantified by software or graded. However, software evaluation can introduce further bias as subjective evaluation is required to determine background versus cellular fluorescence, while a grading system would have had limited application with only two horses. Third, the donor and the recipient horses were not cross-matched so it is possible that no adverse reactions were seen because the donors and recipients had

matching haplotypes. However, domestic horses have upwards of 29 different haplotypes [121], and the CB-MSCs were pooled from five different individual donors, therefore it is very unlikely that all the donors and the recipients had matching haplotypes. Fourth, tissues and blood samples were not collected to determine if there were immunologic or physiologic reactions. However, because the horses did not experience clinically detectable adverse reactions, humoral and cellular immune responses are likely inconsequential. And finally, biopsy collection at the control sites created inflammation that may have influenced homing to the sequential control sites, although the amount of inflammation was far less than the wounded sites. This could have been controlled for by including more horses and collecting a biopsy from each horse only once at a single time period; however, this would be cost prohibitive and the benefit of using the same animal as its own control would be lost.

4.5 Conclusions

In the present proof-of-principle pilot project, we are able to report that after intravenous administration of the largest recorded dose of CB-MSCs (1) our study horses did not have any clinical adverse effects, (2) CB-MSCs preferentially homed and engrafted to wounds during the acute and early remodelling phases of cutaneous wound healing, and (3) there was no difference in homing between limb and thoracic wounds except on day 2 where CB-MSCs homed to limb wounds, but not thoracic wounds. The lack of adverse effects and demonstration of visual validation of homing and engraftment after intravenous administration is new to the equine literature and it is important information for further MSC research in horses. Moreover, these results further promote the horse as a good animal model for studying keloid MSC therapy in people. Although preliminary, the initial results are promising and future studies are warranted.

5. IDENTIFICATION OF mRNA OF THE INFLAMMATION-ASSOCIATED PROTEINS CXCL8, CXCR2, CXCL10, CXCR3 AND β-ARRESTIN-2 IN EQUINE WOUNDED CUTANEOUS TISSUE: A PRELIMINARY STUDY

Transition Statement:

This chapter presents the findings of the gene expression of the inflammation-associated proteins CXCL8/CXCR2, CXCL10/CXCR3 and βarr2 in equine limb and thoracic wound tissue and describes their expression throughout wound healing. The intents of this project was to determine the feasibility of processing wound tissue biopsy tissue for mRNA multiplex assay as well as confirm that the primers of these proteins would have good detection in equine cutaneous tissue before commencing with the main project.

This study confirmed that using primers of the aforementioned inflammation-associated proteins were well detected using mRNA multiplex assay, and initial observations suggest that there are differences in expression of CXCL8/CXCR2 and βarr2 between limb and thoracic wounds. This study contributes important preliminary information to the equine literature about the expression of these proteins in wound tissue that has not been described before, and variations in their expression provides further insights into the variation of limb and thoracic wound healing in horses.

Full citation: Suzanne J.K. Mund, Charlotte Corbett, Daniel J. MacPhee, John Campbell, Ali Honaramooz, Bruce Wobeser, Spencer M. Barber. 2018. Journal of Equine Veterinary Science. Vol. 68. Pages 51-54.

Author contributions: Mund, MacPhee, Campbell, Honaramooz, Wobeser, and Barber were responsible for experimental design. Corbett performed the tissue processing and mRNA multiplex assay. Mund wrote the manuscript with editorial input from MacPhee, Honaramooz, Wobeser, and Barber.

5.1 Introduction

Cutaneous wounds are common in horses and often lead to prolonged and complicated healing. Wound healing is a complex process finely orchestrated by cytokines and growth factors that influence all phases of healing and deviations in the amount or order of cytokine release contributes to abnormal healing[26]. Limb wounds heal slower and have a propensity to develop exuberant granulation tissue (EGT) compared to body wounds[6,42,44,45,122]. Past studies have identified that chronic low-grade inflammation in limb wounds causes dysregulation of cytokine profiles, promoting EGT formation[6–8,42,44,45,122,123]. Similarly, humans also develop hypertrophic scars and keloids when cytokine release is dysregulated[38,124,125]. The development of fibroproliferative disorders in both horses and humans can severely affect quality of life and is a major financial burden[38]. EGT formation on the limbs of horses shares similar qualities with fibroproliferative disorders in people, including a similar contraction to epithelialization ratio, heritability of the disorder, dysregulated cytokine profiles, and haphazard collagen deposition[38,46]. Interestingly, horses and humans are the only species that spontaneously develop these fibroproliferative disorders; thus, horses may be good models for studying aberrant wound healing in humans[38]. Consequently, recording cytokine profiles during wound healing in the horse is important to identify possible time points for intervention to avoid aberrant healing and for establishing its usefulness as a model of wound healing in humans.

Chemokines are small proteins that initiate chemotaxis of leukocytes to the site of inflammation through binding of G-protein coupled receptors (GPCRs). Chemokines are classified based on their conserved cysteine (C) residues of which there are 4 groups: CXC, CC, C, and CX3C. The CXC group is further divided into subtypes according to the glutamate-leucine-arginine (ELR) motif located immediately adjacent to the CXC motif. ELR positive

CXC chemokines are strong neutrophil attractants and promote angiogenesis, and are likewise considered pro-inflammatory mediators. In contrast, ELR negative CXC chemokines are poor neutrophil activators but strong lymphocyte and monocyte attractants and are angiostatic; hence, they are considered inflammation-resolving mediators[126,127].

CXC ligand 8 (CXCL8) is an ELR positive CXC chemokine that is released by damaged cells at the site of injury. As such, it is a potent chemoattractant for neutrophils and triggers angiogenesis. The GPCR for CXCL8 is CXC receptor 2 (CXCR2) and is primarily expressed on the surface of neutrophils and endothelial cells[126,127]. The CXCL8/CXCR2 axis is associated with acute inflammation and human wound-healing models have identified a direct temporal and spatial relationship between expression of CXCL8 and infiltration of neutrophils into the wound[128,129]. The expression of CXCR2 is tightly regulated to prevent excessive inflammation. Within five minutes of binding with CXCL8, CXCR2 internalizes within neutrophils, then is later recycled to the surface[130]. This process is regulated primarily through β-arrestin-2 (βarr2), a ubiquitously expressed intracellular protein that negatively regulates inflammation through internalization of GPCRs, including CXCR2[131]. βarr2 null mice have improved wound healing secondary to increased expression of CXCR2 by neutrophils which allows continuous binding with CXCL8 and therefore more pronounced neutrophil chemotaxis to the wound, initiating the inflammatory cascade[132]. However, βarr2 null mice also have accelerated tumour growth through continued CXCL8/CXCR2 binding on endothelial cells, promoting angiogenesis[133]. This emphasizes the importance of tight regulation of cytokine production.

In contrast to the effects of CXCL8, CXC ligand 10 (CXCL10) is an ELR negative CXC chemokine and is released during the proliferative and maturation phases of wound healing. It is angiostatic and it attracts lymphocytes and monocytes, which are involved in resolving inflammation and promoting scar formation[126,134]. The GPCR for CXCL10 is CXC receptor 3 (CXCR3) which is expressed on the surface of T-lymphocytes, monocytes, fibroblasts, endothelial cells, and keratinocytes[134].

The importance of the resolution phase of wound healing is well demonstrated in CXCR3 null murine models[135]. These mice have an adequate acute inflammatory response but delayed re-epithelialization and hyperkeratotic scar formation characterized by haphazard fibroplasia and decreased scar strength. Similar to the relationship of CXCL8 and CXCR2, the CXCL10/CXCR3 axis is also tightly regulated although βarr2 plays a less pivotal role. This tight regulation is especially important to prevent excessive inflammation secondary to T-lymphocyte activation - leukocytes that are also involved in autoimmune diseases. Shortly after ligand binding, CXCR3 becomes internalized by βarr2, but then is degraded rather than recycled as with CXCR2. More CXCR3 must then be translated from mRNA, delaying expression of CXCR3 and preventing further T-lymphocyte activation. In addition to βarr2 mediated internalization, the responsiveness of CXCR3 can also be decreased through binding of intracellular regulatory $G\alpha_{i2}$ subunits to CXCR3, another checkpoint to prevent excessive inflammation by T-lymphocytes[136].

In normal circumstances, the local cytokine environment triggers appropriate phases of acute inflammation, inflammation resolution, and maturation of the wound. However, if appropriate cytokine release is interrupted at any of these phases, delayed healing and dystrophic scarring can occur[26].

To the authors' knowledge, gene expression of these inflammation-associated proteins have not been identified in equine cutaneous tissue. Identification of these proteins is an important first step to understanding the roles they play in equine wound healing. Variations in temporal profiles may further explain why limb wounds heal differently than body wounds in horses. In addition, documentation of temporal cytokine patterns is important to add to our understanding of equine wound healing and strengthens the usefulness of the horse as an animal model for studying fibroproliferative disorders in humans. Furthermore, understanding the role of these proteins in wound healing may lead to novel ancillary treatments including CXC chemokine/GPCR axis manipulation[137,138].

Our primary objective was to determine if mRNA of CXCL8, CXCR2, CXCL10, CXCR3 and βarr2 can be detected in convenience-sampled frozen equine wounded cutaneous tissue at six different time points throughout wound healing using multiplex mRNA assay

technology. Our secondary objective was to graph the mean fluorescent intensity (MFI) of the mRNA of these proteins over time for limb and thoracic wounds to determine if there are temporal or site variations to guide future investigations.

5.2 Materials and Methods

The mRNA expression levels of CXCL8, CXCR2, CXCL10, CXCR3 and βarr2 were measured using the QuantiGene Plex assay method (Thermo Fisher Scientific, Santa Clara, CA, USA) on frozen equine tissue previously collected from another cutaneous wound healing study. Use of animals in the project was approved by University Animal Care Committee (UACC) and Animal Research Ethics Board (AREB) of the University of Saskatchewan. In that study, seven standardized surgical excisional cutaneous wounds were created under general anaesthesia on the left lateral metacarpus and left thorax of two seven-year-old Thoroughbred mares on day 0. Resected skin was retained for reference as normal non-traumatized tissue. Both horses received $1.02x10^8$ equine umbilical cord blood multipotent mesenchymal stromal cells (MSCs) (eQcell Therapies Inc., King City, Ontario, Canada) injected into the left jugular vein twelve hours after wound creation. Biopsies were collected from the wounds sequentially starting from the most distal and ventral site on the metacarpus and thorax, respectively, on days 1, 2, 7, 14 and 33. All biopsy tissue was immediately snap-frozen and stored at -80°C. mRNA was isolated from frozen tissue samples using a QuantiGene sample processing kit for fresh tissue (Thermo Fisher Scientific). The assay was performed in duplicate using equine specific primers according to the manufacturer's protocol. Samples were analyzed using Luminex Bio-Plex 200 instrumentation (Bio-Rad Laboratories Ltd., Mississauga, Ontario). Results were normalized using expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT). Data for both horses was averaged and graphed over time. Results are expressed as MFI for the mRNA of each protein.

5.3 Results and Discussion

Multiplex mRNA assay technology detected mRNA of all target proteins at all of the time points (Figure 5.1), meeting our primary objective. To our knowledge, this is the first time gene expression of these inflammation-associated proteins have been detected in equine cutaneous tissue. The multiplex mRNA assay technology using the QuantiGene Plex assay

method was simple and effective and eliminated the need to isolate mRNA for each protein from each tissue biopsy, which increased processing speed and decreased chance for error. Multiplex mRNA assay technology is capable of identifying up to 36 different assays in a single sample and has been proven to be a time-efficient and cost-effective alternative to real-time PCR[139,140]. Multiplex mRNA assay technology is a promising tool for wound studies as many cytokines and growth factors involved in healing can quickly be profiled and their complex relationships compared.

The MFI of gene expression of each of the proteins was graphed over time (Figure 5.1). Although the initial data is preliminary, it is interesting and warrants further thought and investigation. The CXCL8/CXCR2 axis demonstrated a pattern of expression that was distinct for the limb and thorax. Specifically, thoracic wounds had a monophasic pattern of expression with a peak MFI for both CXCL8 and CXCR2 on day 7, whereas limb wounds had a biphasic pattern of expression with peak expression during the resolution phase of wound healing on day 33 for both CXCL8 and CXCR2 (Figures 5.1a, b). Interestingly, the expression pattern of CXCR2 was similar to expression of βarr2 (Figure 5.1e). After internalizing its respective GPCR, βarr2 is downregulated transcriptionally and translationally minutes to hours after ligand binding; as such, βarr2 expression patterns reflect its respective GPCR[141] so it would be expected that βarr2 expression would mirror that of CXCR2. The expression patterns of CXCR2 and βarr2 in the limb and thorax are similar to hemoglobin levels in a study by Celeste et al[42]. In that study, hemoglobin was measured over 4 weeks at various time points using near infrared spectroscopy and was considered an indicator of blood flow and angiogenesis. Hemoglobin levels were biphasic in the limb with peak levels during early and late stages of healing, whereas body wounds had peak hemoglobin levels on day 7 of healing. A major stimulus for angiogenesis is hypoxia through the action of CXCL8/CXCR2 binding on endothelial cells[26], and Celeste et al[42] hypothesized that early and late hemoglobin levels in the limbs were reflective of angiogenesis and vasodilation in response to early and late hypoxia in limb wounds. As CXCR2 is found on the surface of

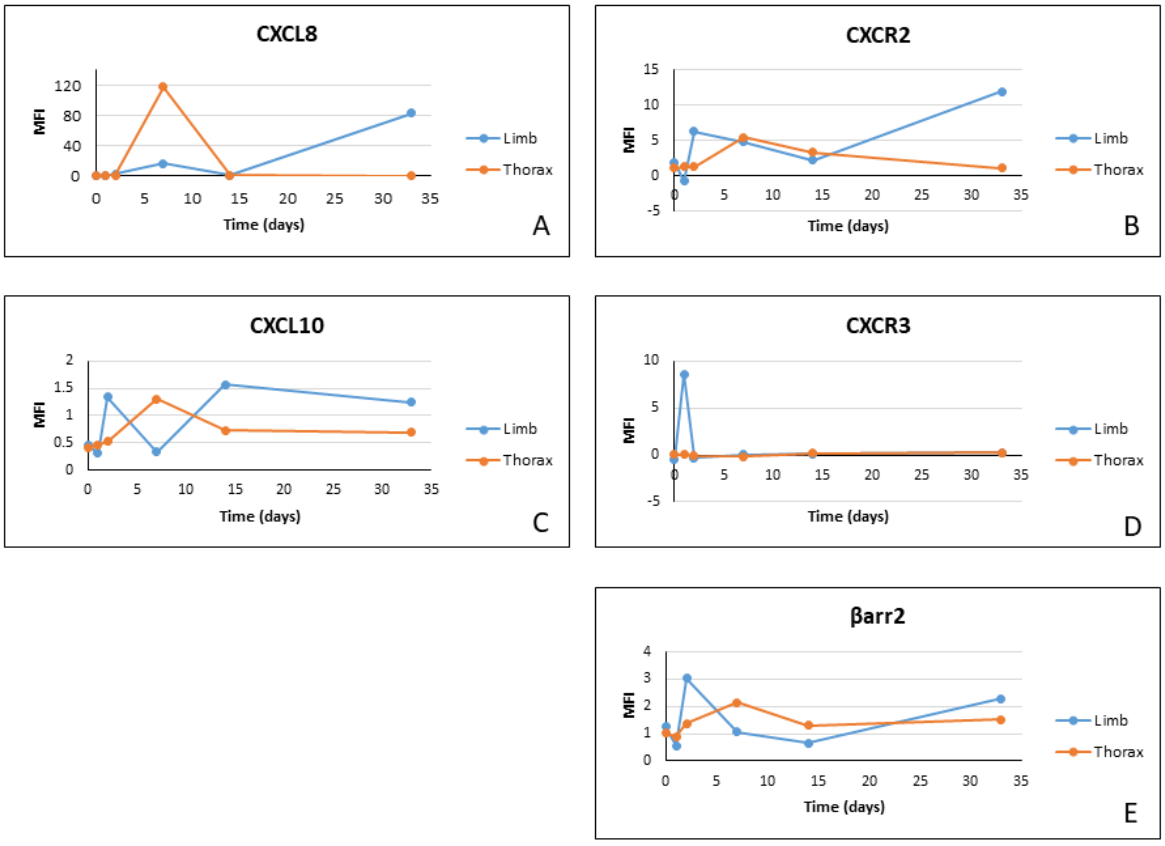

Figure 5.1. Gene expression of CXCL8 (A), CXCR2 (B), CXCL10 (C), CXCR3 (D) and βarr2 (E) measured in mean fluorescent intensity (MFI) in wounded equine cutaneous tissue over time (days). Thoracic wounds demonstrate monophasic expression patterns with peak MFI on day 7 in all proteins except CXCR3. Limb wounds demonstrate biphasic expression patterns with peak MFIs in acute and resolution phases of wound healing in all proteins except CXCR3. Changes in CXCR3 expression patterns were minimal except for peak MFI on day 1 in the limb.

endothelial cells as well as neutrophils, it is possible that the biphasic CXCL8/CXCR2/βarr2 gene expression patterns that we detected are reflective of early and late angiogenesis as well as chronic low-grade inflammation.

CXCL10 mRNA was also expressed biphasically in limb wounds and monophasically in the thorax, although the magnitude of peak MFI is less than the CXCL8/CXCR2 axis (Figure 5.1c). In addition, this pattern was not seen in expression of CXCR3 (Figure 5.1d). Other than a peak MFI on day 1 of limb wounds, CXCR3 gene expression remained near baseline. CXCR3 is very tightly regulated through several check-points including βarr2 mediated internalization, $G\alpha_{i2}$ binding, and de-novo production to prevent induction of auto-

inflammation[136]. It is possible slight variations of expression of the CXCL10/CXCR3 axis between limb and thoracic wounds may not have been apparent in our study due to tight regulation.

Overall, our preliminary results suggest that differences in gene expression patterns of CXCL8/CXCR2, and βarr2 between the limb and thorax are controlled locally rather than systemically as there are regional anatomic variations in expression. However, CXCL10/CXCR3 expression did not seem to vary greatly between limb and thoracic wounds. We are currently determining whether these patterns are significantly different between limb and thoracic wounds in a larger study.

It is imperative to note that these preliminary results must be interpreted cautiously as there are only two horses involved. Furthermore, mRNA levels reflect transcription of genes and do not necessarily represent translation of mRNA into functional proteins. In addition, the biopsies were collected from subject horses that received intravenous MSCs and MSCs have known immunomodulatory effects[142] and thus the detected mRNA may not be representative of normal gene expression for limb and thoracic wounds. Nevertheless, this is the first time that the mRNA of these inflammation-associated proteins have been detected in equine cutaneous tissue and we have confirmed that they can be detected at several time points throughout wound healing. Our results provide an important starting point for further investigations into the characterization of the effects of these proteins in equine wound healing.

5.4 Conclusions

In conclusion, we were successful in identifying gene expression of CXCL8/CXCR2, CXCL10/CXCR3 and βarr2 at all time points during healing using multiplex mRNA assay technology. Our preliminary results suggest that the temporal expression patterns for CXCL8/CXCR2 and βarr2 are different for limb and thoracic wounds in horses and are likely controlled locally as there are regional anatomical differences. These variations may further explain why limb wounds are more prone to developing EGT than body wounds. In addition, this study further supports the notion that horses may be suitable animal models for studying wound healing in humans.

6. THE MACROSCOPIC, HISTOLOGIC, AND IMMUNOMODULATORY RESPONSE OF EXPERIMENTALLY CREATED LIMB WOUNDS FOLLOWING INTRAVENOUS ALLOGENEIC CORD BLOOD-DERIVED MULTIPOTENT MESENCHYMAL STROMAL CELL THERAPY IN HORSES

Transition Statement:

This chapter presents the results of allo-CB-MSC IV therapy on equine limb wounds with specific regard to macroscopic wound closure, histologic response, and gene expression of wound associated cytokines, using techniques and methods previously verified in Chapters 4 and 5.

Results determined that minor transitory adverse responses may occur following administration, and treated horses had improved macroscopic wound closure characteristics. Furthermore, allo-CB-MSC IV therapy may have a generally anti-inflammatory effect as determined by the measured cytokine profiles. These results provide new important information to the equine literature in regards to wound healing, and that ancillary MSC therapy may improve wound healing characteristics and provide a better final outcome, hence improving the long-term welfare of the affected horse.

6.1 Introduction

Traumatic cutaneous wounds occur frequently in horses and often develop complications, particularly if the wound is on the limb. Compared to wounds on the body, limb wounds have less contraction and heal primarily through epithelialization, resulting in a repair that lacks hair and sebaceous glands and is cosmetically and functionally inferior and prone to reinjury[24]. Furthermore, limb wounds are more likely to develop exuberant granulation tissue (EGT), a fibroproliferative disorder similar to keloid formation in humans[24,38]. Anatomically, the limb lacks a *panniculus carnosus* muscle to assist wound contraction and high mobility of the distal limb puts repeated tension on the wound that slows contraction[24,38]. Furthermore, limb wounds are more likely to become contaminated and colonized with bacteria[24,38,40] whereas body wounds seem to be highly resistant to biofilm formation[40]. Also, limb wounds undergo abnormal angiogenesis during wound healing

compared to body wounds[24,43], leading to hypoxia and upregulation of transforming growth factor-beta (TGFβ)1, a potent stimulator of fibrosis and granulation tissue[44,45]. Furthermore, compared to horses, pony wounds heal faster regardless of their location and rarely develop EGT, even though ponies and horses are the same species[4,24], suggesting that horses may have inherent deficient healing characteristics.

Regenerative therapies, such as multipotent mesenchymal stromal cells (MSCs), have been investigated in recent years as ancillary treatments to improve wound healing in several species, including horses[19,20]. Intravenous (IV) administration of MSCs has been shown to accelerate and improve cutaneous healing in laboratory animal wound models[15,84,143]. It is believed that improved quality and acceleration of healing occurs by immunomodulation locally following MSC migration and engraftment at the site[16,17,84] as well as systemically in response to entrapment of MSCs in the lungs known as the "first-pass-effect"[16,79]. Allogeneic (allo)-MSCs are promising as they can be collected non-invasively, be characterized, expanded and stored long-term for future use, and be administered shortly after injury during the acute inflammatory phase when MSC therapy may be the most effective[18,62,78]. In the equine literature, several studies have been performed to determine the recipients' responses to IV MSC administration[12,64,71,89,94–96], but none have determined whether IV MSC therapy alters cutaneous wound healing or influences the local immune response.

The effective cell dose of IV MSCs required to influence wound healing in horses is unknown. Currently, the highest dose administered IV to an equid is 5×10^7 cells per animal[71] with no reported clinical adverse responses. In studies with laboratory mice, typically 1×10^6 cells are administered per mouse[16], equivalent to 33×10^6 cells per kilogram. Administering the same dose to a horse would be cost prohibitive and technically very challenging, but a clinically measurable response is more likely to be seen with a total IV dose beyond what is currently being administered to horses, provided there are no adverse reactions.

The primary objectives of this study were to determine whether $1.51 – 2.46 \times 10^8$ allo- cord blood-derived MSCs (CB-MSCs) administered intravenously following experimental

creation of limb wounds would (1) cause a clinically detectable adverse response, (2) influence epithelialization and contraction rates and hence wound closure, and (3) influence histologic healing characteristics. A secondary objective was to record gene expression levels of proinflammatory (chemokine [C-X-C motif] ligand 8 (CXCL8), tumour necrosis factor-alpha [TNFα]), anti-inflammatory (interleukin [IL]-4, IL-10), inflammation resolving (CXCL10, interferon-gamma [INFγ]), and fibrosis related (TGFβ1, TGFβ2, TGFβ3) cytokines over time and determine whether IV allo-CB-MSC therapy would significantly alter their pattern of expression. Our hypotheses were that IV allo-CB-MSC therapy would not cause a clinically adverse response, would accelerate the rate of wound closure, and would improve histologic healing characteristics.

6.2 Materials and Methods

6.2.1 Overview of Study Design

Twelve horses were randomized into treatment and control groups for a total of six horses in each group. To accommodate constraints of facility housing and anaesthesia personnel, and to allow time for expansion and shipment of allo-CB-MSCs, horses were further divided into three subgroups consisting of two treatment and two control horses (Subgroups 1, 2, and 3). Standardized cutaneous wounds were surgically created on the lateral left forelimb of all four horses in each subgroup on a single day. Twelve hours after wound creation, each treatment horse was administered half of the CB-MSCs procured from a third party via the jugular vein after quantifying viability and re-suspending the CB-MSCs in a cell preservation medium. At the same time, control horses received a placebo injection of 50% diluted cell preservation medium only (Figure 6.1). Full thickness biopsies were taken of the wounds on days 0, 1, 2, 7, 14, and 28. The biopsies were graded histologically for inflammation and repair and mean fold change (MFC) of messenger ribonucleic acid (mRNA) expression levels of proinflammatory, anti-inflammatory, inflammation resolving, profibrotic, and antifibrotic cytokines were measured using multiplex mRNA assays. The proximal most wound was never biopsied and was photographed on days 7, 14, 21, and 28 (Figure 6.2). Planimetric analysis of the digital images was used to determine the wound size and percentage of contraction and epithelialization contributing to wound closure

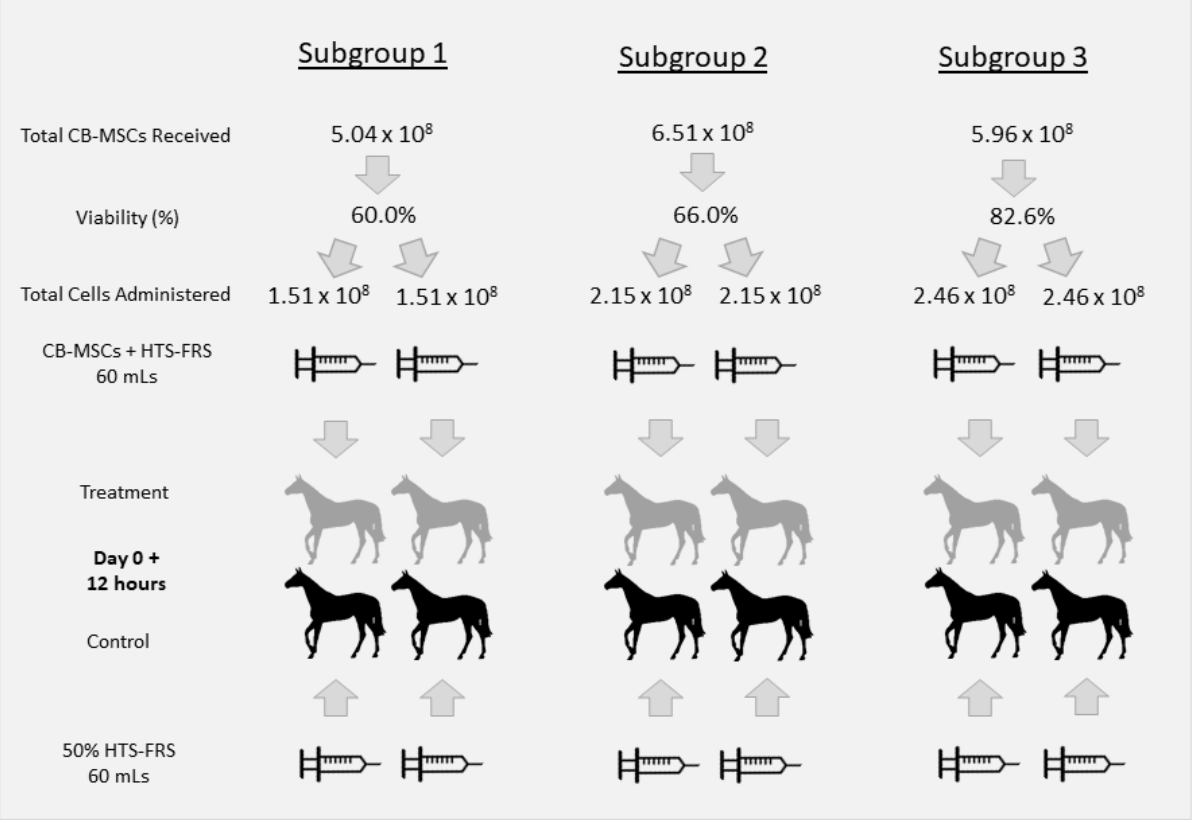

Figure 6.1: Basic schematic of division of horses into treatment and control subgroups and total number of allo-CB-MSCs administered. A total of twelve horses were randomly placed into a treatment and control group and then further divided into three subgroups. Following shipment of cells, a total cell count and assessment for viability using trypan blue staining and enumeration with a haemocytometer was performed. The total remaining live cells were then evenly divided and administered IV to the treatment horses and the control horses were administered a placebo IV injection of 50% diluted HTS-FRS.

(Figure 6.3). The day the wound was considered healed was recorded, which was when the epithelial borders met at the center of the wound. Horses were monitored for adverse clinical reactions during injection and for 4 weeks following administration.

6.2.2 Recipient Animals

Twelve female adult horses of Quarter Horse descent with no physical evidence of past injury to the limbs were acquired. Horses ranged in age from 3-16 years (mean +/- SD, 8.3 +/- 3.5 years) and weight from 425-547 kg (mean +/- SD, 486 +/- 39.2 kg). All horses were healthy on physical exam and had normal complete blood counts and serum chemistry at the time of surgery. Horses were vaccinated (West Nile-Innovator+EWT®, Zoetis) and

dewormed (Equimax®, Bimeda). An intravenous catheter was placed aseptically in the left jugular vein twelve hours prior to surgery and feed was withheld for eight hours prior to induction of anaesthesia. As the created wounds were objectively small and discomfort associated with the wounds would be relatively minor, no anti-inflammatory analgesics or antibiotics were administered throughout the study as to not alter the inflammatory response.

6.2.3 Wound Creation

On day 0, horses were anesthetised as previously described (see Chapter 4). After clipping and prepping the area aseptically, six full-thickness excisional skin wounds measuring 0.5 x 1.5 cm in a horizontal orientation were created on the lateral aspect of the left metacarpus in a vertically stacked arrangement 2.0 cm apart. Similarly, wounds were created on the left lateral thorax at the site of the 10th costochondral junction for purposes of studying wound healing characteristics of thoracic wounds in a separate study. Results of healing of the thoracic wounds are not reported in this manuscript. A portion of excised skin was placed in formalin for histologic evaluation and the remainder was snap frozen and stored at -80°C for later multiplex cytokine mRNA assays for baseline analysis of aforementioned cytokines (Figure 6.2). A bandage was placed on the limb to prevent blunt trauma during recovery from anaesthesia and removed afterwards to prevent influencing granulation tissue formation[24]. Wounds were left to heal by second intention with no intervention such as cleansing or bandaging. Horses were monitored daily for lameness and excessive swelling throughout the course of the study.

6.2.4 Source of CB-MSCs, Administration, and Monitoring

CB-MSCs originating from five unrelated male donor foals (eQcell Therapies Inc.; Kingston, Ontario, Canada) were characterized, prepared, processed, and shipped as previously described (see Chapter 4)[59,66]. Upon arrival of the shipment, viability was determined using a haemocytometer counting chamber and trypan blue exclusion assay and cells re-suspended in a serum-free commercial cell preservation medium (HypoThermosol® FRS

Figure 6.2: Basic schematic of wound creation and sequence of biopsy collection. On day 0, six wounds were created on the left forelimb of each horse measuring 0.5 cm x 1.5 cm and placed 2 cm apart in a vertical orientation. On days 1, 2, 7, 14, and 28 a biopsy was collected from both the dorsal and palmar aspect of the wound in a distal to proximal sequence. The dorsal biopsy was submitted for histology and the palmar biopsy was snap frozen for later multiplex mRNA assays. The top wound was left to heal by second intention imaged on days 7, 14, and 28 and assessed by planimetric analysis for rate of wound closure.

[HTS-FRS]; BioLife Solutions, Bothell WA) to a total volume of 60 mLs, immediately placed on ice and cooled to approximately 4°C. Twelve hours after wound creation, suspended CB-MSCs were administered to treatment horses, and control horses were administered a placebo injection of 60 mLs 50% diluted HTS-FRS, over 15 minutes (4 mL/minute) via the indwelling catheter. Vital parameters were measured prior to injection and then every minute for the first five minutes followed by every five minutes until administration of the suspension was complete. If a horse demonstrated signs of an adverse reaction (tachycardia, tachypnea, hyperthermia, colic, urticaria, fasciculations, agitation/anxiety), administration was suspended until symptoms abated and continued at a slower rate until the administration was complete. In subgroup 1, a total of 5.04×10^8 CB-MSCs with 60.0% viability were received and a total of 1.51×10^8 viable CB-MSCs were administered to each treatment horse. In subgroup 2, a total of 6.51×10^8 CB-MSCs with 66.0% viability were

received and a total of 2.15 x 10^8 viable CB-MSCs were administered to each treatment horse. In subgroup 3, 5.96 x 10^8 CB-MSCs with 82.6% viability were received and a total of 2.46 x 10^8 viable CB-MSCs were administered to each treatment horse (Table 6.1; Figure 6.1). A physical exam was performed every 12 hours for the first seven days followed by a distance exam daily until the conclusion of the study at 28 days.

Table 6.1 Summary of Total CB-MSCs Received and Administered per Horse in Each Subgroup following Viability Assessment

	Total Cells Received	**Viability**	**Total Cells Administered per horse**
Subgroup 1	5.04 x 10^8	60.0%	1.51 x 10^8
Subgroup 2	6.51 x 10^8	66.0%	2.15 x 10^8
Subgroup 3	5.96 x 10^8	82.6%	2.46 x 10^8

6.2.5 Biopsy Collection

Biopsies were collected during the per acute (day 1) and acute (day 2) inflammatory phase, during the mid (day 7) and late (day 14) proliferative phase, and the early (day 28) remodelling phase. At each time point, treatment and control horses were sedated (0.01 mg/kg detomidine + 0.01 mg/kg butorphanol, IV) and biopsies collected from one site at a time in a distal to proximal sequence after subcutaneous infiltration of local anaesthetic (lidocaine 2%). More specifically, on day 1, biopsies were collected from the most distal wound; on day 2, from the second most distal wound; on day 7 the third most distal wound; on day 14 the third most proximal wound; and on day 28 the second most proximal wound. Two biopsies were collected from each of the dorsal and palmar margins of the wound using a 6 mm punch biopsy instrument that included approximately half skin and half wounded tissue. The dorsal biopsy was preserved in formalin for histopathology and the palmar portion snap frozen and stored at -80°C for later multiplex cytokine mRNA assays (Figure 6.2).

6.2.6 Wound Closure Analysis

Photographs were taken of the proximal most wound with a metric ruler adjacent to the wound for calibration on days 7, 14, 21, and 28, and the day of its complete wound closure was recorded. Wound closure was considered complete when epithelialization covered the granulation tissue bed and met at the center of the wound. Images were randomized and the wound areas were measured in triplicate, calibrated to the ruler in the image by a blinded evaluator (S.M.). Using planimetric analysis, the percentage of closure by contraction and epithelialization was determined compared to the original wound size (Image-Pro®, Media Cybernetics, Inc., Rockville, MD., USA) (Figure 6.3). The following formulas were used:

- Percent epithelialization
  (Area of non-haired wound on day(x)^{mm2} – Area of open granulation tissue on day(x)^{mm2}) / Area of original created wound on day(0)^{mm2} x 100

- Percent contraction
  Area of non-haired wound on day(x)^{mm2} / Area of original created wound on day(0)^{mm2} x 100

6.2.7 Histologic Analysis

Biopsies were randomized by treatment and time and graded by a blinded board certified veterinary pathologist (B.W.). A grading scale modelled after other wound grading scales (Table 6.2)[20,144] was created based on progression of repair and inflammation on days 1, 2, 7, 14, and 28. More specifically, the total repair score was the sum of epithelialization, collagen and vascularization scores, and a separate inflammation score was given for the state of vascular and cellular inflammation. As healing progressed, theoretically the repair score should increase and the inflammation score should decrease.

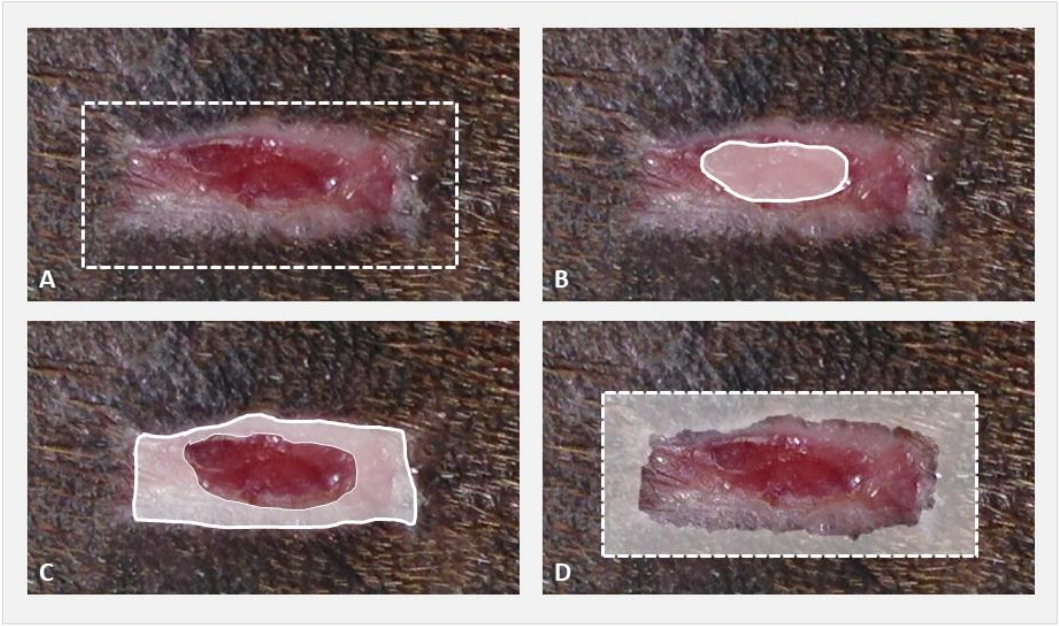

Figure 6.3: Representative diagram of measured areas of wound to determine proportion of wound closure by contraction and epithelialization by using calibrated planimetric analysis. A) Representative area of the original created wound (day 0) in relation to the current (day x) measured wound. B) Shaded area represents area of open granulation tissue. C) Epithelialization was determined by measuring the total area of non-haired wound area and then subtracting open granulation tissue previously measured in B. D) Total contraction was determined by subtracting both the previously measured open granulation tissue in B and epithelialized tissue in C from the original created wound size in A (day 0).

6.2.8 Multiplex Cytokine mRNA Assays

Messenger RNA of proinflammatory (CXCL8, TNFα), anti-inflammatory (IL-4, IL-10), inflammation resolving (CXCL10, IFNγ), profibrotic (TGFβ1, TGFβ2) and antifibrotic (TGFβ3) cytokines were isolated from the frozen biopsies using a QuantiGene Plex assay processing kit according to the manufacturer's protocol (Thermo Fisher Scientific, Santa Clara, CA, USA) for each of the biopsy collection days. The assay was performed in duplicate using equine specific primers including CXCL10 that was previously developed in a preliminary study (See Chapter 5)[35]. Samples were analyzed using Bioluminex processer (Bio-Rad Laboratories Ltd, Mississauga, Ontario) and results normalized to hypoxanthine guanine phosphoribosyltransferase and expressed as combined MFC for the proinflammatory, anti-inflammatory, inflammation resolving, profibrotic, and antifibrotic cytokines.

Table 6.2: Histologic Repair and Inflammation Grading Scale

Repair Grade						Inflammation Grade	
Score	**Epithelialization**	**Score**	**Collagen**	**Score**	**Vascularization**	**Score**	**Inflammatory Response**
0	No epithelium present	0	Absent	0	No activated endothelium	0	No inflammation present
1	<50% covering by epithelium	1	Minimal granulation tissue	1	Activated endothelium	1	Hemorrhage – no inflammatory cells
2	>50% covering by epithelium	2	Granulation tissue	2	Vascular proliferation with vertical orientation	2	Macrophage dominated
3	Bridging of excision	3	Fibroplasia with minimal collagen	3	Declining vascular proliferation	3	Admixed neutrophils/macro-phages
4	Keratinization	4	Return to normal dermal collagen	4	Return to normal vascular	4	Neutrophil dominated
Total = Repair Score						**Total = Inflammation Score**	

6.2.9 Statistical Analysis

Collected data was entered into a commercial spreadsheet (Microsoft Excel, Version 2016). Statistical analysis was performed with a commercial software package (Stata 15.1; Statacorp, College Station, Texas, USA). Data from all treatment and control subgroups were combined into a single treatment group and single control group as there was minimal subgroup variation. The outcome variable of days to complete healing was determined to have a normal distribution using the Shapiro-Wilk test and then treatment and control groups were compared using the two-sample t-test with significance set at $P \leq 0.05$. Histologic healing and inflammation scores for each day were evaluated using Wilcoxon-Rank Sum with significance set at $P \leq 0.05$. The MFC of cytokines, area of non-haired portion of the wounds, and percentage of contraction and epithelialization were analyzed with a generalized estimating equations (GEE) model utilizing Gaussian data distribution, exchangeable correlation structure, and robust standard errors. Variables for consideration in the GEE model were the treatment group (treatment vs. control) and the day of measurement. Interaction effects for treatment group and day were evaluated and where significant interaction existed an overall treatment effect is not reported. A limit of $P \leq 0.2$ for overall treatment effect of all measured days was set as the threshold to further consider effect of treatment at each individual day. Pairwise comparison was performed for individual days and a P value of ≤ 0.05 (adjusted for multiple comparisons over days) was considered significant. Outcomes from GEE are reported as linear predicted contrast (95% confidence interval) between the treatment and control groups. Differences in measures were estimated with the contrast of predicted margins.

6.3 Results

6.3.1 CB-MSC Administration and Monitoring

Three horses (50%) in the treatment group (two in subgroup 2, and one in subgroup 3) and one horse (17%) in the control group (subgroup 3) experienced transient infusion reactions (Table 6.3). All horses remained normothermic. In the treatment group, horses were mildly to moderately tachycardic (48-60 beats per minute), moderately to markedly

tachypneic (40-64 breaths per minute), were restless, and had mild colic symptoms (lip

Table 6.3 Summary of Adverse Clinical Responses Following IV allo-CB-MSC Administration

Horse	Group	Subgroup	Symptoms Summary	Response to Decreased Infusion Rate
8	Treatment	2	Reaction following 28 mLs of infusion. Normothermic, mild tachycardia (48 bpm), moderate tachypnea (64 bpm), mild restlessness	Discontinued infusion for five minutes. Vital parameters returned to normal. Continued infusion. No alterations in vital parameters.
12	Treatment	2	Normothermic, mild tachycardia (48 bpm), moderate tachypnea (42 bpm), mild restlessness, mild colic symptoms (lip curling)	Continued infusion at a slower rate. Symptoms resolved within fifteen minutes.
7	Treatment	3	Reaction following 28 mLs of infusion. Normothermic, moderate tachycardia (60 bpm), moderate tachypnea (40 bpm), moderate restlessness, moderate colic symptoms (stretching, looking at flank)	Discontinued infusion for ten minutes. Heart rate and respiratory rate returned to normal. Continued infusion slowly. Colic symptoms resolved 15 minutes after infusion completed.
11	Control	3	Reaction following 12 mLs of infusion. Normothermic, marked tachycardia (72 bpm), moderate tachypnea (40 bpm), marked restlessness, diffuse muscle fasciculations	Discontinued infusion for fifteen minutes. Muscle fasciculations resolved. Continued infusion slowly. Heart rate fluctuate between 48 and 60 bpm during infusion. Restlessness resolved shortly after completely infusion.

curling, stretching, flank watching). The control horse had marked tachycardia (72 beats per minutes) and moderate tachypnea (40 breaths per minute) as well as marked restlessness and muscle fasciculation, although no overt colic symptoms were noted. In all

horses, administration was suspended until symptoms abated (5-15 minutes) and then continued at a slower rate. All horses returned to normal vital parameters shortly after completion of administration and had normal physical exams for the remainder of the study.

6.3.1 Wound Closure Analysis

One of the treatment horses in subgroup 3 sustained a large degloving injury on the lateral aspect of the right neck on day 22. Because inflammation created by the neck wound may have influenced healing of the experimental limb wounds and confound results, the data collected from her was subsequently eliminated from analysis for the remainder of the study.

In the control group, wounds had complete closure on day 27 ± 3 and the treatment group had complete closure on day 26 ± 4. There was no significant difference between treatment and control groups based on the number of days recorded for complete

Table 6.4 Time to Limb Wound Closure following IV allo-CB-MSC administration

Horse	Group	Days to Closure	Mean Days to Closure ± SD	P value
2	Treatment	N/A	26 ± 4	0.702
3	Treatment	22		
6	Treatment	21		
7	Treatment	26		
8	Treatment	30		
12	Treatment	30		
1	Control	28	27 ± 3	
4	Control	28		
5	Control	28		
9	Control	30		
10	Control	24		
11	Control	22		

healing to occur (two sample t-test, 95% CI, -4.10 to 5.83; P = 0.702) (Table 6.4). Non-haired wound size became larger than the original created wound size, known as the lag phase[10], in both the treatment and control groups on day 7, and continued to get larger in the control group on day 14, but became smaller than the original wound size in the treatment group on day 14 (Figure 6.4). An overall treatment effect over all days was seen for non-haired wound size, where treated horses had smaller wounds, and hence potentially a smaller dermal scar, than control horses (GEE, 95% CI, -23.9 to 3.51; P = 0.145), but there was no significant interaction at individual days (Table 6.5; Figure 6.4). The rates of epithelialization and contraction were compared between groups overtime. CB-MSC treatment had an overall treatment effect on epithelialization over all days (GEE, 95% CI, -0.214 to -0.023; P = 0.015) with a significant treatment effect on day 14 (GEE, 95% CI -0.280 to -0.0189; P = 0.0173) (Table 6.5; Figure 6.5). CB-MSC therapy influenced an overall treatment effect on contraction over all days (GEE, 95% CI, -0.0462 to 0.313; P = 0.146) characterized by a trend for a less pronounced lag phase in the treatment group than the control group, although no significant interaction was identified at individual days (Table 6.5; Figure 6.6). By day 28, the treatment group had healed with approximately 75% contraction and 25% epithelialization (Figure 6.7b) whereas the control group had approximately 60% contraction and 40% epithelialization (Figure 6.7a).

6.3.3 Histologic Analysis

Histologic repair and inflammation scores between each group were virtually identical with very little variation at each of the days; thus, statistical analysis was not performed. In both treatment and control groups, the repair score increased and the inflammation score decreased throughout time as anticipated. The following repair and inflammation scores are reported sequentially biopsies collected on days 1, 2, 7, 14, and 28. In the treatment group, average repair scores were 3.00, 4.00, 8.12, 11.67, and 15.00; likewise, the control group had average repair scores of 3.00, 4.00, 8.00, 11.67, and 15.00. The treatment group had average inflammation scores of 5.00, 5.00, 4.00, 3.00, and 1.00, and similarly the control group had average inflammation scores of 5.00, 5.00, 4.00, 2.67, and 1.00. Descriptive data only is presented in Table 6.6.

Table 6.5 Rate of Limb Wound Closure by Epithelialization and Contraction following IV allo-CB-MSC Administration

	Wound Area (mm^2)			Epithelialization (%)			Contraction (%)		
Day	**Contrast**	**95% CI**	**P Value (Adjusted)**	**Contrast**	**95% CI**	**P Value (Adjusted)**	**Contrast**	**95% CI**	**P Value (Adjusted)**
7	-8.12	-34.9 to 18.7	0.909	-0.121	-0.419 to 0.176	0.774	0.0974	-0.224 to 0.419	0.909
14	-19.5	-43.1 to 4.02	0.147	-0.140	-0.280 to -0.0189	0.0173*	0.260	-0.0536 to 0.574	0.147
21	-6.03	-26.8 to 14.7	0.921	-0.0537	-0.282 to 0.175	0.962	0.0804	-0.196 to 0.357	0.921
28	-7.09	-23.6 to 9.41	0.782	-0.151	-0.342 to 0.0409	0.186	0.0946	-0.125 to 0.315	0.738

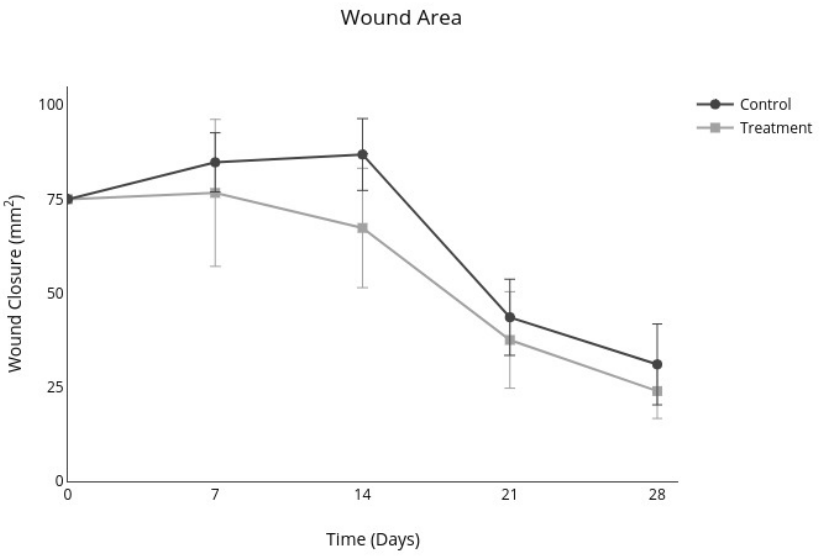

Figure 6.4: Total non-haired wound area of limb wounds following IV CB-MSC administration. There was an overall decrease in total non-haired wound area over all days but no significant treatment effect at individual days. Both treatment and control wounds had an increase in non-haired wound area during the lag phase but was less pronounced in the treatment group.

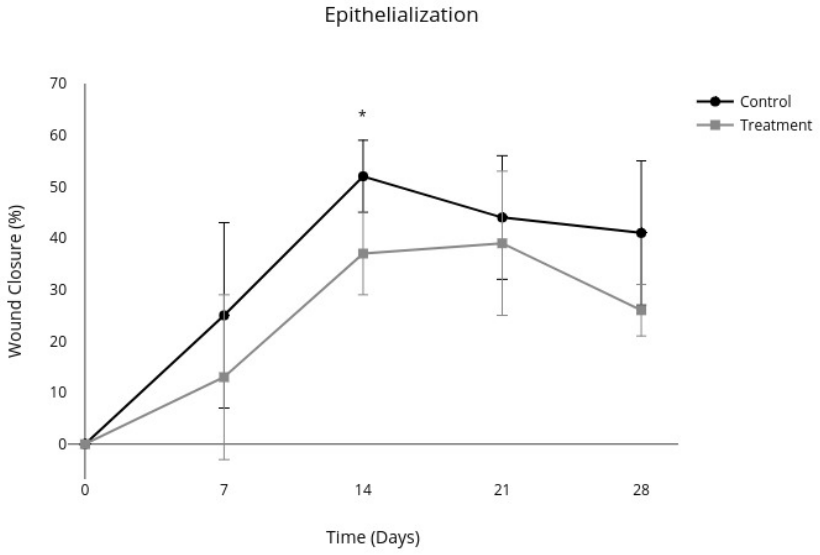

Figure 6.5: Total area of epithelialization (%) contributing to wound closure of limb wounds following IV CB-MSC administration. There was an overall decrease in total epithelialization over all days with significant treatment effect on day 14 (P = 0.0173).
(*) = significant difference

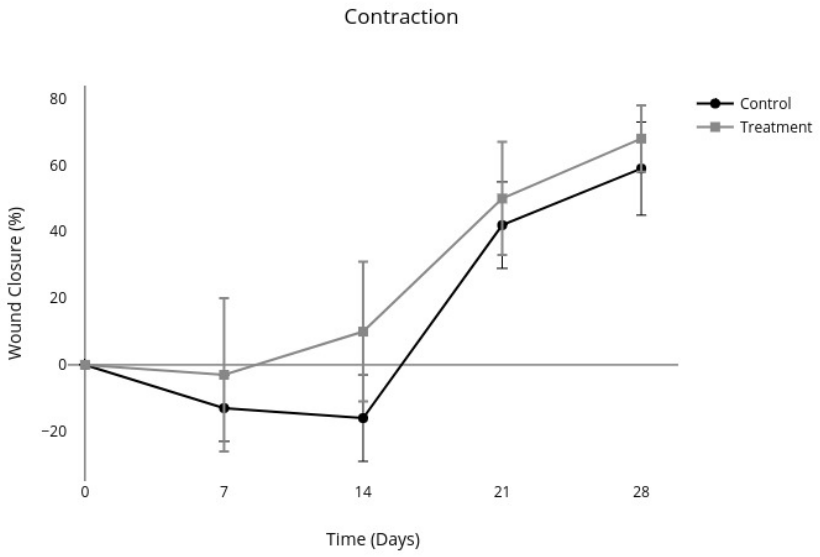

Figure 6.6: Total area of contraction (%) contributing to wound closure of limb wounds following IV CB-MSC administration. There was an overall increase in total contraction over all days with no significant treatment effect on individual days.

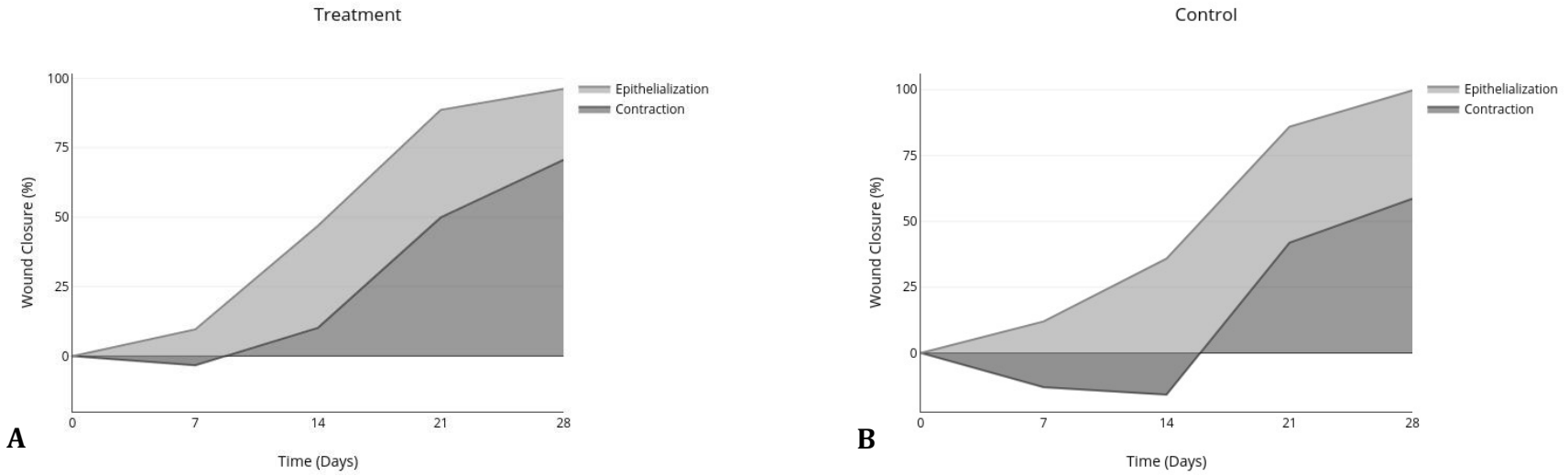

Figure 6.7: Total area of contraction (%) and epithelialization (%) contributing to wound closure of treatment and control limb wounds following IV CB-MSC administration. There was an overall increase in total contraction and epithelialization over all days in the treatment group (A). The lag phase was less pronounced in the treatment group and wound closure was healed with approximately 75% contraction and 25% epithelialization (A), compared to 60% and 40% of contraction and epithelialization, respectively, in the control group (B).

Table 6.6 Histologic Repair and Inflammatory Scores of Limb Wounds following IV allo-CB-MSC Administration in Horses

		Histologic Repair Score					Histologic Inflammation Score				
Group	**Horse**	**Day 1**	**Day 2**	**Day 7**	**Day 14**	**Day 28**	**Day 1**	**Day 2**	**Day 7**	**Day 14**	**Day 28**
Treatment	2	3	4	8	12	N/A	5	5	4	3	N/A
	3	3	4	9	12	15	5	5	4	3	1
	6	3	4	8	12	15	5	5	4	3	1
	7	3	4	8	11	15	5	5	4	3	1
	8	3	4	8	11	15	5	5	4	3	1
	12	3	4	8	12	15	5	5	4	3	1
Control	1	3	4	8	13	15	5	5	4	1	1
	4	3	4	8	11	15	5	5	4	4	1
	5	3	4	8	11	15	5	5	4	3	1
	9	3	4	8	12	15	5	5	4	3	1
	10	3	4	8	12	15	5	5	4	3	1
	11	3	4	8	11	15	5	5	4	3	1

6.3.4 Multiplex Cytokine mRNA Assays

IV allo-CB-MSC administration had an overall treatment effect over all days on proinflammatory cytokines (GEE, 95% CI, -3.81 to -0.172; P = 0.032) with a significant individual treatment effect on day 2 (GEE, 95% CI, -10.4 to -2.26; P = 0.0003) (Figure 6.8). An overall treatment effect over all days was seen where anti-inflammatory mediators were lower (GEE 95% CI, -0.330 to -0.0251; P = 0.022), but there was no significant treatment effect at individual days (Figure 6.9). An overall treatment effect over all days was seen where inflammation resolving mediators were lower (GEE margins 95% CI, -0.558 to -0.0229; P = 0.033), but there was no significant treatment effect at individual days (Figure 6.10). An overall treatment effect over all days was seen where profibrotic mediators were lower (GEE margins 95% CI, -0.431 to 0.0863; P = 0.191), but there was no significant interaction at individual days (Figure 6.11). There was no treatment effect overall or at individual days for antifibrotic mediators (Figure 6.12). In summary, there was an overall treatment effect over all days that lowered proinflammatory, anti-inflammatory, inflammation resolving, and profibrotic mediators, although there were not significant differences at individual days except for proinflammatory mediators on day 2. There were no significant differences at individual days or overall treatment effect between the treatment and control groups for the antifibrotic mediators (Table 6.7).

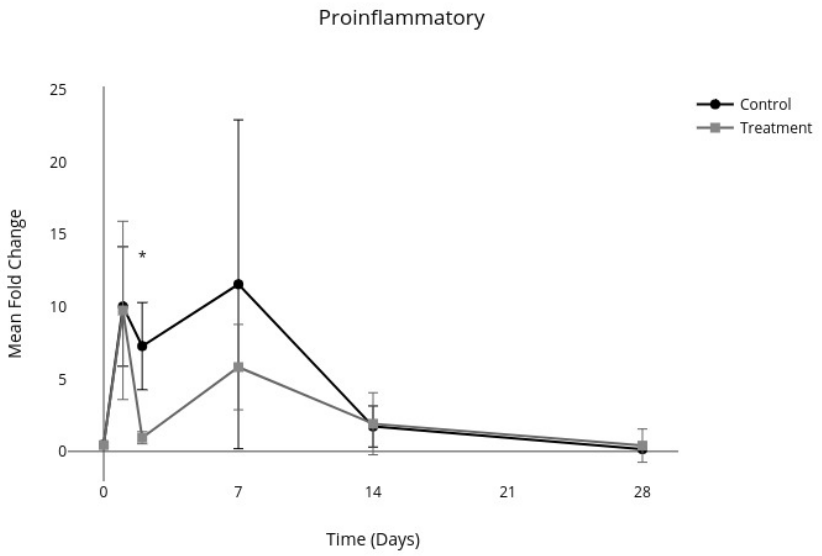

Figure 6.8: Mean fold change (MFC) of mRNA expression of proinflammatory cytokines over time following IV allo-CB-MSC therapy. There was an overall treatment effect over all days characterized by decreased expression of proinflammatory cytokines with an individual significant treatment effect on day 2 (P = 0.032).
(*) = significant difference

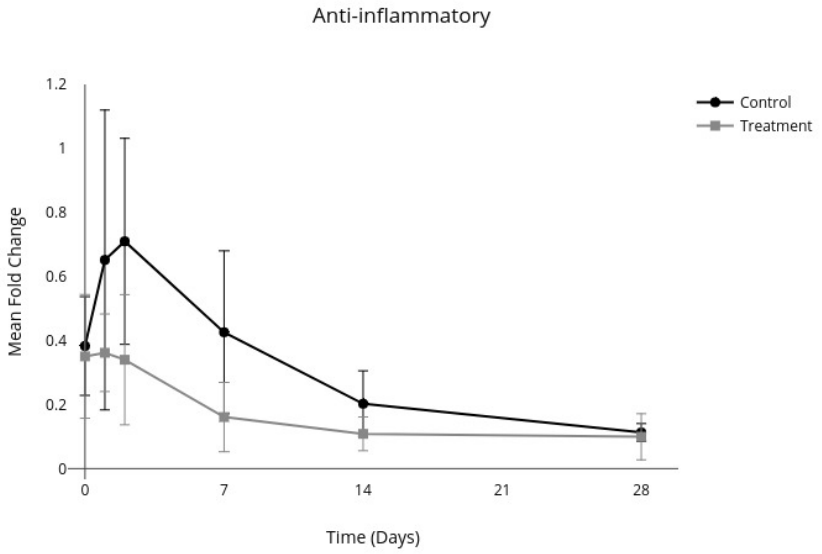

Figure 6.9: Mean fold change (MFC) of mRNA expression of anti-inflammatory cytokines over time following IV allo-CB-MSC therapy. There was an overall treatment effect over all days characterized by decreased expression of anti-inflammatory cytokines with no significant treatment effect on individual days.

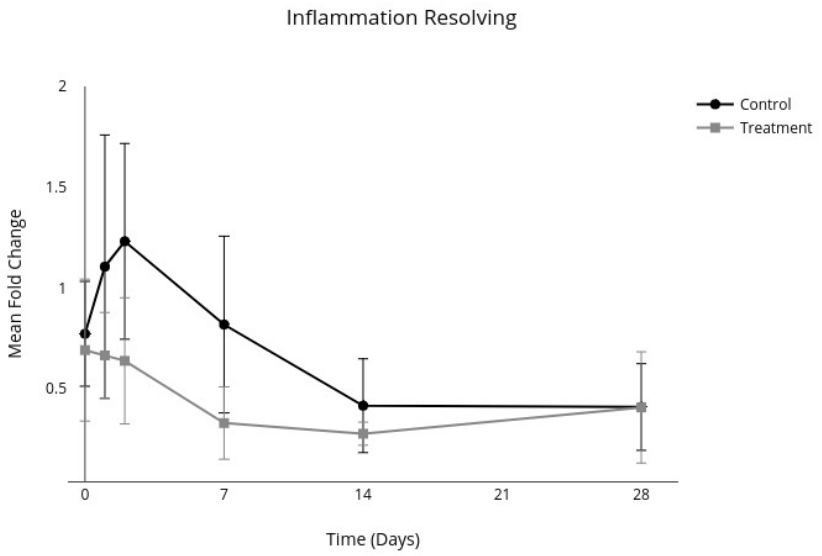

Figure 6.10: Mean fold change (MFC) of mRNA expression of inflammation resolving cytokines over time following IV allo-CB-MSC therapy. There was an overall treatment effect over all days characterized by decreased expression of inflammation resolving cytokines with no significant treatment effect on individual days.

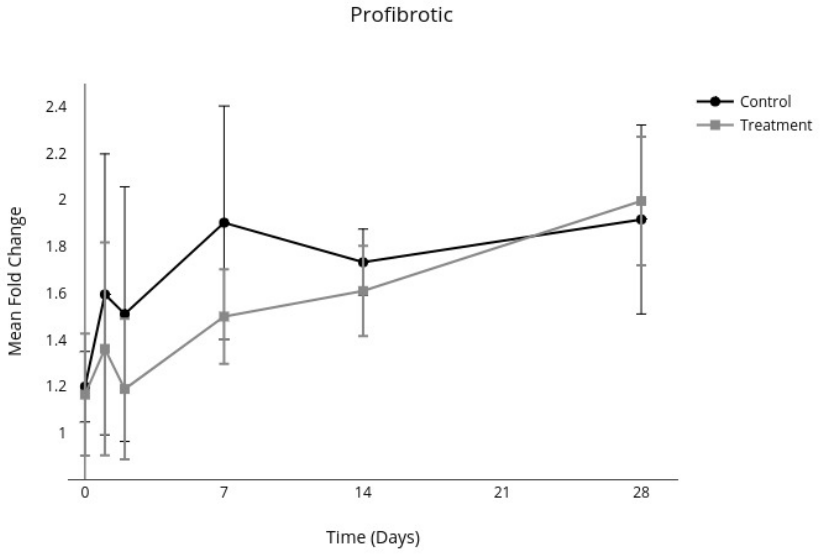

Figure 6.11: Mean fold change (MFC) of mRNA expression of profibrotic cytokines over time following IV allo-CB-MSC therapy. There was an overall treatment effect over all days characterized by decreased expression of profibrotic cytokines with no significant treatment effect on individual days.

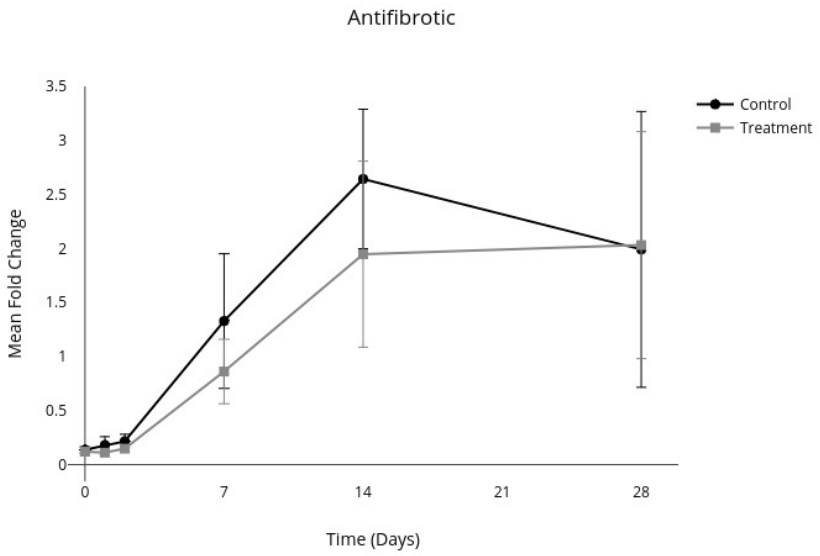

Figure 6.12: Mean fold change (MFC) of mRNA expression of antifibrotic cytokines over time following IV allo-CB-MSC therapy. There was neither overall treatment effect over all days nor significant treatment effect on individual days.

Table 6.7 Summary of mRNA Cytokine Expression of Limb Wounds Following IV allo-CB-MSC Administration

	Proinflammatory			Anti-inflammatory			Inflammation Resolving			Profibrotic			Antifibrotic		
	Contrast	**95% CI**	**P Value**	**Contrast**	**95% CI**	**P Value**	**Contrast**	**95% CI**	**P Value**	**Contrast**	**95% CI**	**P Value**	**Contrast**	**95% CI**	**P Value**
Overall Treatment effect	-1.99	-3.81 to -0.172	0.032*	-0.177	-0.330 to -0.0251	0.022*	-0.290	-0.558 to -0.0229	0.033*	-0.173	-0.431 to 0.0863	0.191*	-0.213	-0.715 to 0.289	0.406
Individual Day Treatment Effect	**Contrast**	**95% CI**	**P Value (Adjusted)**	**Contrast**	**95% CI**	**P Value (Adjusted)**	**Contrast**	**95% CI**	**P Value (Adjusted)**	**Contrast**	**95% CI**	**P Value (Adjusted)**	**Contrast**	**95% CI**	**P Value (Adjusted)**
Day 0	-0.0322	-0.331 to 0.266	1.00	-0.0324	-0.364 to 0.300	1.00	-0.0807	-0.668 to 0.506	1.000	-0.0339	-0.439 to 0.371	1.00	-0.0161	-0.0810 to 0.0487	0.987
Day 1	-0.271	-10.2 to 9.70	1.00	-0.290	-0.939 to 0.359	0.808	-0.440	-1.36 to 0.481	0.755	-0.234	-1.25 to 0.782	0.991	-0.0700	-0.181 to 0.0407	0.455
Day 2	-6.34	-10.4 to -2.26	0.0003**	-0.370	-0.881 to 0.141	0.296	-0.592	-1.37 to 0.182	0.238	-0.322	-1.16 to 0.517	0.895	-0.0683	-0.167 to 0.0300	0.343
Day 7	-5.73	-21.5 to 10.1	0.917	-0.265	-0.636 to 0.106	0.312	-0.488	-1.12 to 0.147	0.232	-0.403	-1.13 to 0.323	0.607	-0.467	-1.39 to 0.460	0.708
Day 14	0.191	-3.27 to 3.65	1.00	-0.0940	-0.249 to 0.0610	0.505	-0.139	-0.460 to 0.183	0.830	-0.123	-0.445 to 0.199	0.897	-0.696	-2.14 to 0.748	0.747
Day 28	0.245	-1.30 to 1.79	0.999	-0.0134	-0.117 to 0.0903	1.00	-0.00212	-0.471 to 0.467	1.00	0.0794	-0.579 to 0.738	1.00	0.0399	-2.18 to 2.26	1.00

(*) – indicates threshold for overall treatment effect over all days (P≤0.2).
(**) – indicates significant treatment effect at individual days (P≤0.05).

6.4 Discussion

This study utilized an IV dose range of 1.51 – 2.46 x 10^8 viable CB-MSCs administered to horses, which is the higher than the previously highest dose that our team had administered in the pilot project (see Chapter 4). This dose of cells was determined by the maximum amount of CB-MSCs that the supplier was able to provide as resources and facilities allowed. As far as the authors are aware, this is the highest dose administered to a horse experimentally via any route. Contrary to our hypothesis, 50% of the horses in the treatment group experienced a clinically adverse reaction. Interestingly, one control horse (17%) also had a clinically adverse response to the placebo suspension (Table 6.3). Previous studies by other researchers examining IV MSC administration in horses did not detect any clinically adverse reactions, although the amounts administered were relatively small[12,71,89,94–96]. In our previous studies, the treatment horses received twice the previously highest reported IV dose[71], and did not have any clinically measurable responses (see Chapter 4). However, potential adverse reactions were still considered possible in the treatment group secondary to major histocompatibility complex (MHC) reaction[12,70,71] and pulmonary vascular injury[21,91]. A clinical response was not anticipated in the control group as the placebo injection contained only chilled diluted HTS-FRS cellular suspension medium. Possible reasons for the responses to the administered perfusate in both treatment and control groups are 1) thermic response to a chilled intravenous solution, 2) immunological response to the cells and/or a substance in the suspension medium, and 3) physiologic response to concentrated electrolytes within the perfusate. Although possible, administration of relatively small volumes of a chilled solution is unlikely to cause systemic reactions. Saline chilled to 4°C is commonly administered to hyperthermic human individuals at approximately 1 mL/kg/min for 30 minutes without complications[145], far more than the 0.0089 mL/kg/min of the HTS-FRS that our horses were administered. The make up of the HTS-FRS contains several electrolytes at elevated concentrations that may explain the clinical effects that were seen. HTS-FRS is a cell preservation medium designed to maintain cell viability of chilled or frozen mammalian cells. The composition of HTS-FRS is not public, but is similar to the concentration of intracellular solutes[71]. An earlier public version of an intracellular-like HTS-maintenance solution intended as a blood substitute had a concentration of 42.5 mEq/L potassium[146]. The safe dose of potassium is 0.5

mEq/kg/hr[147], or 3.75 mEq/min for the average 450 kg horse. Assuming HTS-FRS has a similar concentration of potassium as the original maintenance solution, administration of 60 mLs of 50% diluted HTS-FRS over fifteen minutes would be equivalent to 0.085 mEq/min for the averaged sized horse, well within the safe rate. Similarly, Williams et al[71] determined that a 10 mL bolus of undiluted HTS-FRS, alone or containing 5×10^7 mismatched CB-MSCs, did not induce clinically detectable adverse reactions when administered to ponies. In our study, live cells were not filtered from the delivered solution to avoid further cell stress and cell death. Lysed cells could potentially release a significant amount of intracellular potassium that may cause transient symptoms of hyperkalemia in the treatment group, but obviously would not have altered the cell-free HTS-FRS placebo suspension administered to the control horses. Some individuals may be particularly sensitive to alterations in circulating potassium concentrations, and/or the patented HTS-FRS may contain a concentration of potassium higher than the original maintenance solution. Furthermore, it is possible that the HTS-FRS contains a preservative that could cause an immunological response in some individuals. Regardless, the symptoms experienced were transient and mild, although one should consider administering HTS-FRS at an even slower rate to potentially avoid reactions.

Intravenous administration of CB-MSCs did not accelerate time to wound closure or improve the histologic repair score, contrary to our hypotheses (Table 6.6). However, CB-MSC therapy did have a treatment effect over all days where the treated horses had more contraction and less epithelialization, characterized by significantly less epithelialization on day 14 (Table 6.5; Figures 6.4, 6.5, 6.6). Furthermore, although not significant, there was a trend in the treatment group for a less pronounced lag-phase during contraction in the first two weeks, and the treated horses wounds healed with approximately 75% contraction and 25% epithelialization compared to the control group that had 60% and 40% contraction and epithelialization, respectively (Figures 6.5, 6.7). In all equine wounds, during the lag phase the wound edges will retract and the wound will initially get larger and then decrease in size as myofibroblasts begin to infiltrate the wound to contract the dermal edges centripetally to the center of the wound, allowing for a smaller area requiring repair through epithelialization[10,22]; this is a common observation in equine wound healing

studies[3,6,42,45]. A cutaneous wound healed primarily with epithelialization is more fragile and weaker than normal skin, and on horse limbs only achieves 60% of its original strength of normal skin[37]. Furthermore, it has a poorer cosmetic and functional outcome as it consists mainly of acellular collagen and is devoid of components such as hair follicles and sebum and apocrine glands, whereas a wound that has closed mostly with contraction has an intact dermis[10]. Although ultimately IV CB-MSC therapy did not improve the time to wound closure or histologic quality of healing, a positive healing response was still appreciated because the smaller visible dermal scars from more contraction and less epithelialization would have a potentially better cosmetic and functional repair than untreated cohorts.

A secondary objective of this study was to determine whether IV CB-MSC therapy would significantly alter the mRNA expression of several inflammatory mediators. There was a treatment effect over all time points with less proinflammatory, anti-inflammatory, inflammation resolving, and profibrotic cytokine mRNA expression, although no significance at individual days was detected other than significantly less expression of proinflammatory mediators on day 2 (Table 6.7; Figures 6.8, 6.9, 6.10, 6.11). To the authors' knowledge, no comparable wound cytokine analysis studies have been performed in horses following IV MSC therapy. In laboratory species, IV MSC therapy modulates the proinflammatory response systemically similar to what we measured in our horses. In a bladder obstruction model using rats, IV MSCs significantly lowered expression of proinflammatory (TNFα) and profibrotic (TGFβ1) genes and had decreased collagen deposition in the detrusor muscle, improving bladder function[148]. Also, IV MSCs decreased expression of proinflammatory (TNFα) genes and improved histologic scores in a rat colitis model[80]. In a burn wound healing model using rats, IV MSCs decreased expression of proinflammatory (TNFα) proteins and had improved healing time and histologic appearance[84]. However, all three of these laboratory animal models also saw an increase in anti-inflammatory mediators, contrary to the decrease that we measured in our horses. Furthermore, in an equine *in vivo* study where CB-MSCs were injected locally in the dermis surrounding limb wounds, the profibrotic mediator TGFβ1 was significantly increased compared to controls one week after administration[20], in contrast to what was measured in

the rat bladder obstruction model[148] as well as our horses. MSCs exert their influence through immunomodulation both locally and systemically[16]. After IV infusion, MSCs become entrapped in the lungs, but still suppress inflammation through secretion of anti-inflammatory cytokines which are absorbed by the pulmonary vasculature and are distributed systemically, having effects at local sites of injury[16,79]. MSCs also modify the immune response through "licensing" - where activated MSCs secrete anti-inflammatory cytokines after exposure to proinflammatory cytokines[117]. This process appears to be somewhat dose dependent to initial exposure of TNFα and/or INFγ. Although speculative, it is possible that our treated horses had a lessened initial systemic proinflammatory response after MSC lung entrapment, which then likewise decreased a reciprocated licensed release of endogenous anti-inflammatory and inflammation resolving cytokines at the wound site. This cytokine profile may have promoted the trends for increased contraction and decreased epithelialization in the treatment group, although investigating this relationship was beyond the scope of this study. Further studies examining the cytokine profile and wound closure relationship are indicated.

Allo-MSCs were chosen for this study rather than auto-MSCs. Although allo-MSCs are MHC-I and MHC-II negative *in vitro*[12], MHC surface expression upregulates after allo-MSCs engraft and interact with endogenous mononuclear cells, leading to early rejection and stimulation of an immune response by the host[71–74]. Likewise, *in vivo* allo-MSCs have been shown to stimulate an immune response in equine studies[12,70,71]. Regardless, allo-MSC therapy still has favourable outcomes equable or nearly equable to auto-MSC therapy[73,75], and allo-MSC therapy has had favourable outcomes in equine experimental synovitis[63,76] and naturally occurring arthritis studies[77,78] . Furthermore, allo-MSCs collection is not invasive to the recipient, and they can be characterized, expanded, then cryo-stored long term, allowing prompt administration during the acute inflammatory stage when MSC therapy may have its most advantageous effects, unlike auto-MSCS that can take many weeks to be expanded[18,62,78–80]. Thus, allo-MSC therapy has value in the clinical setting for unanticipated urgent surgical procedures and unexpected injuries.

There were several challenges met in this study. First, the total amount of cells administered to treatment horses in each sub group varied from 1.51 – 2.46 x 10^8 CB-MSCs,

a variation of 1.05 x 10^8 cells. We conducted previous feasibility studies with smaller amounts of cells and determined that a viability rate of 80% was expected following transportation of the cells (See Chapter 4). However, on arrival the first two shipments had viability rates of 60% and 66%, far below the anticipated 82% viability achieved in the third shipment rate. No explanation for the variation in viability rates was immediately apparent, other than acknowledgement of the fragile nature of expanding and transporting large amounts of live mammalian cells. Admittedly, this may be a confounding practicality for clinical practice. Regardless, the effective cell dose to modulate the inflammatory and healing response is unknown, and even the lowest administered dose in our horses was three times higher than previously highest administered IV dose[71], and cumulatively a positive effect was seen with increased contraction, decreased epithelialization and decreased overall wound size. Second, we intentionally did not check for cross-mismatch of the administered cells to the recipient horses. It is possible the three treatment horses that did not have a clinically adverse response failed to react because their haplotypes matched the MHC of the donor cells. However, this is extremely unlikely considering the donor cells were pooled from five separate donors and horses have upwards of twenty-nine different haplotypes[121], although it is possible that the degree of clinical adverse reaction could be related to whether there was a MHC mismatch of only one donor compared to all five. Third, the mRNA expression levels of the cytokines were measured rather than the translated functional proteins. Messenger RNA expression does not necessarily equate similar fold change expression of a functional protein, as the actual expressed protein can be quite variable from the measured mRNA. However, recent studies have determined that in mammalian cells in a state of homeostasis, an mRNA to protein expression ratio can be predictably measured for different genes across all tissues, with 85% of a proteins translation being determined at the mRNA level[149]. In addition to the decreased technical complexity, improved measurement accuracy, and decreased cost of measuring mRNA using multiplex assay technology compared to other methods such as qPCR[139,140], an 85% accuracy in predicting protein expression based off measured mRNA can be considered an acceptable screening tool before quantifying actual protein expression using more expensive and technically challenging methods such as ELISA. Finally, the small sample size of horses was a limiting factor in truly enumerating the risk of clinical adverse reactions

and detecting statistical significance amongst the tested variables. A larger sample size with adequate power would allow for better statistical analysis and firmer conclusions.

Although administering this amount of IV allo-CB-MSCs is feasible, ultimately it did not accelerate time to wound closure or improve histologic quality of healing. However, the treated horses had significantly reduced epithelialization on day 14 and a trend for increased contraction as well as an overall reduced mRNA expression for several cytokines. There are financial and logistical implications that likely would limit the clinical usefulness of IV allo-CB-MSCs for wound healing, particularly in horses that are otherwise healthy and probably would have healed satisfactorily anyways. However, IV allo-MSC therapy may have more benefits in horses that are truly immunocompromised, or in those that have sustained large wounds with an inappropriate inflammatory response, rather than small surgical wounds created in a controlled environment. Techniques that may further increase the effectiveness of IV allo-MSC therapy include priming or preconditioning MSCs prior to administration[20,87,117,150], repeating injections[79,93], and administering cells via regional limb perfusion[89,94,96].

6.5 Conclusions

This is the first equine study to demonstrate changes in wound healing following IV MSC administration. Furthermore, in this study the highest ever dose of MSCs, intravenous or otherwise, was administered to equids. We achieved our primary objectives of administering 1.51 – 2.46 x 10^8 CB-MSCs intravenously and showed that large doses of IV allo-CB-MSCs can be administered with minor transitory adverse clinical reactions, at least in this subpopulation of horses, contrary to our hypothesis. The reasons for these adverse reactions are unknown, and were also seen in one control horse and may be related the cell suspension solution, MHC mismatch, and/or hyperkalemia. In addition, horses that received allo-CB-MSCs had smaller dermal scar size overall characterized by a trend for more contraction and significantly less epithelialization on day 14, which may provide a cosmetically and functionally superior repair, although the time to wound closure and quality of histologic repair were not improved, again contrary to our original hypothesis. We also met our secondary objective of measuring gene expression of several inflammation-associated cytokines and were able to determine that there was a difference

in expression after IV allo-CB-MSC therapy, and although exploring the significance of these variations was beyond the scope of this study, initial findings suggest that there is a general anti-inflammatory effect that may have influenced final dermal scar size. At this time, we cannot recommend IV allo-CB-MSC therapy as the sole treatment for improving cutaneous wound outcomes, although further research may demonstrate support its use as ancillary therapy in complicated wounds or immunocompromised patients when paired with basic good wound management. Further studies are needed to describe the profiles of immune modulators involved in wound healing and how IV MSC therapy alters their expression, and to determine ideal effective doses, route of administration, number of treatments, and optimal timing of administration.

7. GENERAL DISCUSSION AND FUTURE EXPERIMENTS

We are able to report the highest dose of IV MSCs administered to an equine; first 1.02 x 10^8 allo-CB-MSCs in the pilot project (see Chapter 4) followed by 1.51 – 2.46 x 10^8 allo-CB-MSCs in the main study (see Chapter 6). This is two to five times higher than the other highest reported dose by Williams et al.[71]. In the pilot project, neither of the mares had any adverse reactions, and tolerated the injection well. However, in the second study, 3 of the treatment horses (50%) had reactions and, surprisingly, one of the horses in the control group (17%). Although recognized as a possibility, this was not entirely expected as the horses in the pilot project did not have a response and all the previous reports of horses receiving IV MSCS via the jugular or palmar digital vein did not report a clinically detectable response[12,64,71,89,94–96]. The reactions are consistent to what is reported to occur with antigenic stimulation during a plasma or whole blood transfusion in horses, and our horses responded appropriately when the infusion was slowed[151]. However, this does not account for the response seen in the single control horse. The formulation of the HTS-FRS is not publicly available, but has similar concentrations of intracellular electrolytes including potassium to preserve mammalian cells during shipping[71,146]. HTS-FRS is not formulated for IV infusion use, but part of the intention of Williams et al.'s study was to determine if HTS-FRS was a safe medium for IV administration because separating and then re-suspending CB-MSCs in saline can cause increased cell death[71]. Our treatment of sixty mLs of 50% diluted HTS-FRS delivered over fifteen minutes is a low rate and well below the safe mg/kg/min dose of potassium, although some individuals may be sensitive to additional IV potassium. In addition, as the HTS-FRS formulation is currently unknown, it's possible it may contain a preservative or other additive that the single control horse responded to.

Pulmonary injury is a potential complications following IV MSC infusion[21,91]. Even though none of the horses developed any complications for the remainder of both studies, we cannot ascertain that there was no damage to the lungs because no histopathology was performed on lung tissue. A full necropsy and subsequent histologic examination would have been interesting but was deemed ultimately unnecessary as the horses had no other clinical symptoms apart from the initial infusion reactions. However, the subject horses were not followed long term to determine if they developed any possible sequela of lung

injury that may affect their future athletic performance and welfare. Further investigation into the safety of IV HTS-FRS and IV MSCs with larger numbers of horses, histopathology, and long term follow up is needed.

For the first time in the equine literature, we are able to report evidence of homing and engraftment of allo-CB-MSCs to cutaneous wounds in horses following jugular IV administration (see Chapter 4). As far as the authors are aware, there are no comparable studies in the equine literature examining tracking of MSCs and cutaneous wounds. Although not necessary to promote healing[16,17,79,80,85], engraftment of MSCs at the site of injury is considered beneficial for secretion of immune mediators and have a local paracrine effect to promote healing[16,84,86,87]. There are several different avenues to enhance engraftment including delivery method, priming and preconditioning the cells, and preconditioning the host environment.

Compared to regional limb perfusion (RLP) and direct intralesional injection, IV delivery appears to have the lowest rates of engraftment[16,152]. Using a natural occurring tendinopathy clinical trial, Becerra et al. tracked technicium99m-labelled MSCs using nuclear scintigraphy following IV jugular administration and found that there was no homing of cells to the tendon lesion, small amounts following RLP, and the most after direct intralesional injection[152]. However, the horses were only administered 1.0×10^7 MSCs and the injuries were not considered acute[152], both factors that would negatively influence homing and engraftment[16,86,87]. Homing and persistence of MSCs seems to be most efficient with direct intralesional injection in tendons in horses[96,119,152,153]. However, direct intralesional injection can be used only if the lesion is discrete and accessible by needle, and there is concern that injecting a substance into already compromised tissue may cause further iatrogenic injury to the tissue[16,88,89]. RLP can successfully deliver MSCs to an active lesion, and although homing and engraftment appears to be less efficient than direct injection[96,152], RLP may be more advantageous in cases where the lesion cannot be easily accessed with a needle, such as in the hoof capsule, or where there is diffuse disease or injury while decreasing the risk of an adverse response from large doses of jugular MSCs. Furthermore, fewer cells may be required to have the same effect as jugular IV administration, although the benefits of systemic IV MSCs may be missed[16]. Although

successful homing and engraftment of MSCs is possible following IV administration, exploring other delivery methods such as RLP may deliver the same beneficial effects while avoiding possible systemic adverse reactions.

Preconditioning the CB-MSCs prior to injection may have increased the engraftment observed in the pilot project (see chapter 4). The purpose of preconditioning is to either increase the cells' efficiency in homing and transendothelial migration, and/or improve the resilience to rejection in the host tissue by exposing the cells to certain conditions and substances[86,87,117]. Exposing the cells to hypoxic conditions prior to injection preconditions them to tolerate a hypoxic environment and causes upregulation of CXCR4, a receptor necessary for transendothelial migration[86,87]. In an equine wound model, however, hypoxic preconditioning did not enhance wound healing, although evidence of engraftment was not examined[20]. Other preconditioning or priming methods include exposure to anti-oxidants, statins, and interferon, and gene therapy to prevent premature apoptosis and to upregulate surface receptors to enhance engraftment[86,87,117]. Methods to precondition the host tissue to be more reception to foreign MSCs include irradiation, post-ischemia conditioning through repeated restriction of blood flow following MSC administration, and systemic statins[87]. Further studies examining the effects and of feasibility of preconditioning of MSCs and the host tissue in the horses with limb wounds is warranted.

In the main project, IV allo-CB-MSCs did not accelerate time to wound closure nor improve the histologic healing score, although over all days there was a decrease in the non-haired dermal scar size characterized by an overall increase in contraction and decrease in epithelialization, with significantly less epithelialization on day 14 (Figures 6.4 - 6.7). In laboratory animals, MSC therapy has been shown to accelerate time to wound closure, enhance contraction, and promote angiogenesis and re-epithelialization[15,17,142]. Similarly, in an equine study, direct dermal injection of CB-MSCs reduced the histologic inflammatory score and accelerated reduction in the wound area, although time at complete wound closure was not recorded[20]. Reduction of the total non-haired dermal scar size is considered beneficial as the repair would be more cosmetic, durable and less prone to reinjury. Although benefits in dermal scar size were appreciated in both our studies, perhaps direct injection of MSCs along the edges of the wounds would be more beneficial than jugular IV

delivery in order to avoid potential systemic adverse reactions, to reduce the number and therefore cost of CB-MSCs therapy, and to improve engraftment rates.

In this study, a single dose of CB-MSCs was administered in the acute phase of wound healing, which has been shown to have more beneficial effects than dosing in later following injury[18,62,73,78–80]. However, several studies in other species have shown that repeat doses have even more beneficial effects than a single acute dose[79,93]. An equine wound model using repeat doses of MSCs during the acute inflammatory, inflammation resolving, and remodelling phases would be interesting to determine whether increasing dosing regimens would further enhance healing and wound closure.

Intravenous allo-CB-MSCs suppressed the mRNA expression of all cytokines in limb wounds except for the antifibrotic cytokine, TGFβ3 (Figures 6.8 – 6.12). Determining the direct relationship between the cytokines and the macroscopic and histologic characteristics of wound closure was beyond the scope of this project, but there are some interesting differences in the cytokines profile of equine limb wounds following IV MSC infusion compared to other studies. In laboratory animal models that received MSC therapy shortly after injury, cytokine profiles typically had a decrease in proinflammatory mediators, but increase in anti-inflammatory and profibrotic mediators[15,16,84,142], in contrast to the decrease in anti-inflammatory and profibrotic cytokines that we measured. Limb wounds in horses share inflammatory characteristics with those of chronic wounds in humans; specifically, a low-grade protracted inflammatory response increases the chance for infection and impairs epithelialization, angiogenesis, and contraction[4,15,24,142]. MSC therapy has been shown to trigger the transition of chronic wounds from a proinflammatory and proliferative cytokine environment to one that resolves of inflammation and promotes remodelling[16,142]. In an acute limb wound in the horse, the initial inflammatory response is less robust than what is seen in thoracic wounds, and it's believed that a restricted initial inflammatory response is inadequate to trigger the cascade of cytokines required for resolution of inflammation and transition into each of the sequential phases of healing, leading to a chronic non-healing wound and the formation of EGT[4,24]. Following IV CB-MSC administration in our study, expression of inflammatory mediators was overall lower throughout the study period with significantly lower initial proinflammatory cytokine

expression on day 2, and positive effects on dermal scar size were still appreciated. This is in contrast to the theory that limb wounds need more initial inflammation to promote healing. Comparing the local cytokine environment of limb wounds to thoracic wounds with and without treatment with IV MSCs will further help us understand the role of MSCs and the cytokine environment in wound healing, and will be described in future manuscripts using samples collected from thoracic wound during this study.

Normal healthy horses were used in these studies, and although positive changes were seen, the measured improvement in the wounds was relatively minor and may not be worth either the expense of the procured allo-CB-MSCs or the risk of adverse reactions and pulmonary injury for wounds that likely would have healed normally anyways. However, IV MSC therapy in horses with chronic non-healing wounds may have more benefits. MSC therapy has been shown to help resolve refractory wounds in humans by decreasing proinflammatory cytokine expression while increasing the expression of the anti-inflammatory cytokines IL-4 and IL-10, consequently promoting granulation tissue, contraction, and epithelialization[15,142]. MSC therapy as a sole or concurrent therapy to basic chronic wound management in horses warrants further investigation.

EGT is a fibroproliferative disorder and shares many similarities with keloid formation in humans[38,46]. There is some evidence to suggest that keloids formation may be driven by an autoimmune reaction or hypersensitivity response to components of sebum or glandular structures when the skin is injured[48,50]. A similar process may also potentially be a component in EGT formation in horses, and research examining EGT for characteristics of an autoimmune response and the potential use of immunosuppressive antifibrotic drugs such as intralesional interferon[48] and tranilast[154] would be interesting.

Messenger RNA of the inflammation-associated proteins βarr2, CXCL8/CXCR2, and CXCL10/CXCR3 were identified for the first time in equine cutaneous tissue (see Chapter 5), and initial findings suggest that there are differences in expression for βarr2 and CXCL8/CXCR2 in wounds of the limb compared to the thorax (Figure 5.1), although these suspicions have to be repeated on a larger group of horses that have not been administered MSCs to be confirmed. Chemokines are chemotactic cytokines that are vitally important in

the progression and resolution of inflammation in wound healing[25,26], and several studies have repeatedly shown that deficiencies in the proinflammatory CXCL8/CXCR2 axis early in wounding, or the inflammation resolving CXCL10/CXCR3 axis later on can lead to a delayed wound healing and pathologic scarring[25,26,29,135,155]. Cytokine-based strategies to influence the interaction of chemokines with the wound environment can promote a normal course of healing, such as preventing ligand-receptor binding by administering chemokine and/or receptor specific antibodies and administering "decoy" non-functional chemokines[26]. In addition, preventing or promoting cell surface receptor expression for these chemokines by altering βarr-mediated intracellular signalling is also very promising[130,156]. For example, blocking βarr2-mediated upregulation of CXCR2 can prevent chemotaxis of neutrophils and help resolve chronic inflammation of wounds, while still allowing production of CXCL8 to promote angiogenesis and epithelialization[130]. One common pharmaceutical that has been shown to alter the inflammatory environment of wounds is gentamicin. Gentamicin is an aminoglycoside antibiotic that in addition to having antimicrobial properties, has also been shown to increase expression of CXCL10 when applied topically in ultrahigh doses in a pig wound model[157], and thereby can potentially resolve neutrophilic inflammation while promoting M2 macrophage and lymphocyte infiltration and stopping angiogenesis. This study was performed in the context of skin grafting and thus was not recommended in the acute stages of wound healing when neovascularization is vital. However, this mechanism may be beneficial in chronic equine limb wounds, where halting the protracted inflammation of neutrophil infiltration and stopping granulation tissue proliferation through angiostasis could potentially resolve or prevent EGT formation, and has the added benefit of being widely available and relatively inexpensive compared to other pharmaceutical and regenerative therapies. These mechanisms of altering chemokine-mediated inflammation has potential in promoting healing and resolving inflammation of chronic wounds in horses and warrants further investigation.

Other novel therapies that have promising results in altering the wound environment and improving healing in laboratory animals may also help resolve chronic wounds in horses. These strategies include gene therapy to increase or decrease the expression of cytokines to promote healing, and biomaterials such as microspheres or hydrogels to deliver and

maintain MSCs and other inflammatory mediators at the wound site[158]. As far as the author is aware, these therapies have not been applied to wound healing in horses, and merits further consideration and may provide further valuable ancillary treatment options to chronic equine wounds.

8. GENERAL CONCLUSIONS

Following the administration of >1.51 - 2.46 x 10^8 allo-CB-MSCs, limb wounds had decreased wound area size characterized by increased contraction and decreased epithelialization, but did not accelerate the time to wound closure or improve the quality of repair or inflammation histologically. The cytokine profile was altered within these wounds to have less expression of proinflammatory, anti-inflammatory, inflammation resolving, and profibrotic mediators, which is in contrast to the current belief that more acute inflammation at the time of injury followed by rapid resolution of inflammation, similar to what is seen in body wounds, would result in improved healing of limb wounds. Unexpectedly, half of the horses that received CB-MSCs and one of the placebo injected control horses experienced infusion reactions, although the reactions were relatively minor and resolved quickly when infusion was slowed, and did not experience any further reactions for the duration of the study. However, the minor improvement in wound healing and observed reactions brings into question whether the administration of this high of a dose is worth the expense or the risk of an adverse systemic reactions to promote wound healing in otherwise normally progressing wounds, although it may be of benefit in horses with chronic refractory wounds. In addition, because necropsies and histopathology were not performed, and the horses were not followed long term, we cannot state that there were no immediate or long-term consequences. Further research to avoid these disadvantages of IV allo-CB-MSC therapy in equine cutaneous wound healing include delivery by RLP, preconditioning the cells and the host tissue prior to injection, and repeating doses throughout healing. Other therapies that do not involve MSCs and may benefit wound healing include the use of immunosuppressive antifibrotic medication and administrating drugs that alter the regulation of chemokines and their receptors.

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