DIFFERENTIATION OF EQUINE MESENCHYMAL STROMAL CELLS INTO CELLS OF NEURAL LINEAGE AND THEIR APPLICATION INTO A NOVEL MODEL FOR ACUTE PERIPHERAL NERVE INJURY IN THE HORSE

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Claudia Cruz
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Abstract

Studies have shown that mesenchymal stromal cells (MSCs) are able to differentiate into extra-mesodermal lineages, including neurons. Positive outcomes were obtained after transplantation of neurally-induced MSCs in rats, rabbits and guinea pigs after nerve injury, but the effect of these cells is unknown in horses. Our objective was to test the ability of equine mesenchymal stromal cells to differentiate into cells of neuronal lineage, and to assess differences, if any, in morphology and protein expression. Additionally, we wanted to investigate if horse age and cell passage number contributed to the ability to achieve neural differentiation.

The first part of this research focuses on assessing the potential of equine bone marrow-derived mesenchymal stromal cells to undergo differentiation into cells of neural lineage cells after prior demonstration of their stemness. It describes the optimization of *in vitro* conditions to induce neural differentiation of equine MSCs and the use of neural markers in equine MSCs, which has not been previously reported.

Subsequent research focuses on further commitment of these neural cells into Schwann-like cells for possible transplantation into an acute peripheral nerve injury model in horses. After optimizing the laboratory conditions to induce Schwann cell differentiation of equine MSCs, their detachment from the tissue culture flasks resulted in poor viability. Therefore, undifferentiated MSCs were transplanted in the surrounding fascia after transecting the central portion of the anastomotic branch (*ramus communicans*) of the lateral and medial palmar nerves of the fore limbs in healthy horses. Approximately 45 days after the lesion was created, the whole nerve was removed for histological analyses.

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INTRODUCTION

Horses (*Equus caballus*) are herbivore-domesticated mammals that have played a crucial role in the development of the modern society. Several breeds of horses have been used through the years for transportation, work in agriculture, wars, racing, sources of food, equestrian sports, and pets. Furthermore, the horse industry has a direct effect on the economy of the USA of $39 billion per year, and $102 billion when the industry suppliers and employees factors are taken in consideration.[1] The profound impact of the horse industry on the economy results from the creation of jobs, necessity of industrial supplies, agricultural production, generation of taxes, and entertainment investments. Veterinary medicine is, therefore, one important science to safeguard the stability of the horse industry.

There are several breeds of horses (equines), most of which possess certain corporal characteristics that allow them to perform arduous physical work under various environmental conditions. Some of these anatomic and functional characteristics include a particular musculoskeletal system, a complex gastrointestinal system, and a high efficiency cardiovascular and respiratory apparatuses, that will provide them with satisfactory kinematics, energy, and adequate perfusion and oxygenation, respectively. Because horses are athletic animals and approximately 44-53% of their body weight is constituted solely by muscle,[2] a disturbance of any of the structures of the musculoskeletal system (i.e. muscles, tendons, bones, joints, ligaments, and their associated blood and lymphatic vessels, and nerves) will produce poor performance and, consequently, financial or emotional losses. The musculoskeletal system of the horse is vast; in this research, however, a particular interest is given to the structures that deliver sensory and motor input to the musculoskeletal system of the horse: the peripheral nerves.

CHAPTER I:
LITERATURE REVIEW

Abstract

Peripheral nerve injuries occur commonly in horses but the available options for managing them are limited. Cell therapy via autograft is the preferred method for nervous tissue repair in human medicine but there are high risks for complications at the donor site and the number of Schwann cells yielded from this donor nerve is generally low. Cell therapy via transplantation of autogeneic or allogeneic mesenchymal stromal cells (MSCs) provides an alternative approach for yielding high number of cells without the risks of causing damage to the donor site; it has also revealed promising results after transplantation in peripheral nerve injuries artificially created in laboratory animals or when transplanted in clinical cases in dogs and humans, but this is unknown in horses. Based on *in vitro* studies, MSCs have anti-inflammatory, immunomodulatory, and paraendocrine properties that provide the conditions necessary for tissue repair. The method of delivery of these cells has largely been studied and might depend on the site of lesion and practicability of the procedure. Further research is necessary for evaluating methods of labeling, tracking and delivering these cells in horses suffering from peripheral nerve injuries.

Generalities of the nervous system

The nervous system involves a complex network of cells that ultimately has the functions of acquiring information from the exterior of the animal, processing it, and delivering specific signals to specific organs based on these external stimuli. It is, therefore, that the nervous system is composed of different specialized cells, which originate from the ectodermic layer during the embryonic development.

The nervous system is divided into the central nervous system (CNS) and the peripheral nervous system (PNS). Briefly, the CNS comprises the structures within the encephalon (i.e. brain, brain stem, cerebellum, etc.) and spinal cord. On the other hand, ganglia, cranial and spinal nerves comprise the PNS.[3] It is worth mentioning

that both divisions of the nervous system continuously interact with each other. For purposes of this research, however, a particular interest is given to the PNS.

Additionally, the PNS is subdivided into autonomic PNS and somatic PNS. The autonomic PNS encompasses the nerve fibers (axons) that innervate involuntary or autonomic structures, such as the heart, smooth muscle and glands.[3] The somatic PNS, instead, involves the axons that give input to the musculoskeletal system, which are mostly involved with voluntary activity.

An alternative division of the nervous system is based in its functional properties: afferent (sensory) and efferent (motor).[4]

Peripheral nerves. These include the 12 pairs of cranial nerves located in the encephalon, and the spinal nerves, which arise from the spinal cord. Each nerve consists of parallel bundles of nerve fibers (axons), which can be afferent or efferent. A surrounding sheath of connective tissue provides support to these nerves and their associated blood and lymphatic vessels. Additionally, these axons can be myelinated or non-myelinated.[4] Myelinated axons are surrounded by a myelin sheath formed by supporting cells (also known as satellite cells): oligodendrocytes, in the CNS; and Schwann cells in the PNS. Myelin is composed of the cell membrane from Schwann cells, which is approximately 80% lipid in nature; hence, it has insulation properties. The myelin sheath surrounding the axon is a layer regularly segmented by the nodes of Ranvier that help in the transmission of a nerve impulse in a saltatory and extremely fast manner (**Figure 1.1**). Peripheral nerves, consequently, can also be classified by their speed of conduction (A, B or C fibers) and their fiber size (μm of diameter).[5] The fastest nerve fibers are usually myelinated and thicker (i.e. α and β subtypes of the A fibers, which transmit motor and sensory impulses to skeletal muscle and skin receptors). On the other hand, the slowest nerve fibers are non-myelinated and of small diameter (i.e. C fibers that transmit deep, non-localized pain).

In the CNS, each oligodendrocyte forms myelin sheaths for up to 60 axons. In the PNS, on the other hand, only one Schwann cell exists for each segment of one axon. Moreover, the Schwann cells wrap around the axons in a spiral, which is clockwise in some segments and counterclockwise in others. These wrappings become tight as the axon matures, and the thickness of the myelin sheath will ultimately depend on the number of spirals of a Schwann cell membrane.

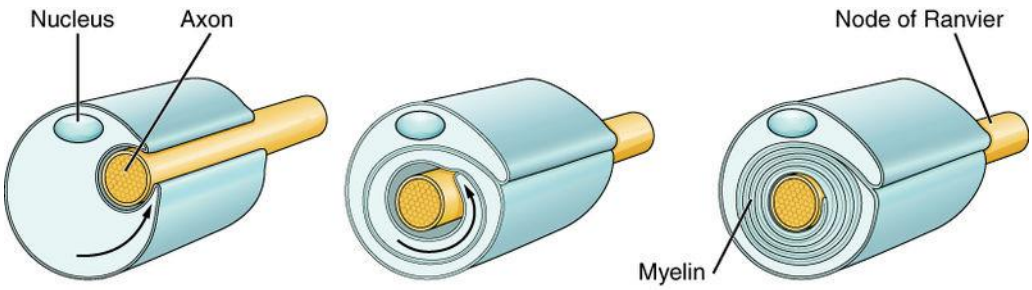

Figure 1.1 Peripheral nerve myelination

Peripheral nerve conduction. Once a stimulus is perceived, an action potential (nerve impulse) starts at the initial segment of an axon and propagates along the plasma membrane of the cell. Before an action potential the interior of a nerve cell is negatively charged at approximately -80 millivolts (mV); this is called the resting membrane potential. The cell membrane allows the opening of ion channels that result in a rapid influx of ions, which will change the charge inside the cell. This change is called depolarization.[3] Ion channels are particular to the cell type. For example, sodium channels are abundant in peripheral nerves; they open fast and allow a massive entrance of sodium inside the cell that will result in an extremely prompt action potential. Calcium channels are abundant on smooth muscle cells; they open in a slower manner, resulting in a more prolonged action potential. Finally, after the impulse has propagated throughout the cell, other ion channels (e.g. potassium) open and allow the exit of positive ions, so the original resting membrane potential can be restored (hyperpolarization).

The speed of conduction is correlated with the axonal diameter of the nerve fiber. Additionally, myelin sheath thickness correlates with nerve diameter. These characteristics and the saltatory conduction of the nerve impulse at the nodes of Ranvier in myelinated nerves make myelinated nerves the fastest nerves for transmission of an impulse, at a velocity of 70-120 meters per second in Aα fibers.

Concepts in regenerative medicine: stem cells

The purpose of regenerative medicine is to create functional tissues for repairing or replacing tissues or organs that have been damaged or lost because of accidents, diseases, and age,[6] usually via transplantation of cells, tissues or organs that were previously cultured or created in the laboratory.

Stem cells are undifferentiated cells that have the ability to proliferate (self-renew) for an indeterminate time during the life of the organism, and to differentiate into mature, specialized cells given the right conditions (plasticity).[6,7] The term "stem cell", however, has been used indiscriminately to denote certain types of cells with specific characteristics and it is, therefore, important to clarify some terms used in the field of cellular therapy for regenerative medicine.

Stem cells can be of embryonic or adult origin. Based on their ability to differentiate, those stem cells capable of differentiating into all cell types, including extra-embryonic membranes and tissues, are called *totipotent* stem cells and they are solely found in the zygote and early embryo.[6,8] *Pluripotent* stem cells are able to differentiate into all cell types from the 3 germinal layers that constitute an implanted embryo (ectoderm, mesoderm and endoderm), but not into extra-embryonic tissues.[6,7] *Multipotent* stem cells are capable of differentiating into various cell types of a specific germinal layer. Finally, *unipotent* stem cells can only differentiate into a single mature cell type.[6]

Types of cell transplants. Depending on the source, cell, tissue, or organ, transplants are called xenogeneic, allogeneic or autologous. Briefly, xenogeneic implies the transplantation of cells, tissue or organs from one species to another; for example, human cell transplants into rats for research purposes. Allogeneic transplantation involves different individuals from the same species; for example, human blood donors. Autologous transplantation involves taking tissue from an individual in order to implant it at another site on that same individual (also known as autograft); for example, removal of a portion of the ischium bone in order to graft it into a bone defect (in a bone other than ischium).

In human and veterinary practice, the source of transplants is either allogeneic or autologous. The concern in performing allogeneic transplantation of cells, tissue or organs is the possibility of rejection, which is due to the immune response of the host. A disadvantage of autologous transplantation is the possibility of causing morbidity at the donor site, which was previously inexistent.

Mesenchymal stromal cells (MSCs)

This term refers to non-hematopoietic (non-blood) adult multipotent stem cells that originate from diverse adult tissues. It is unknown if these cells are the same throughout the different tissues.[8] The term mesenchyme denotes a transient, loose tissue conformed by cells within a mesh of fluid and extracellular protein matrix, during embryonic and fetal development. It is mainly derived from the mesoderm and it directly gives rise to connective tissue throughout the body and to most organs in the body (morphogenesis) after interacting with the epithelium.[9] The mesenchyme, consequently, is crucial in the embryonic life, but can also be found in small quantities in bone marrow, fat, muscles, and dental pulp. The term stromal, on the other hand, refers to cells that constitute the connective tissue of every organ.[6] Certain mesenchymal stromal cells (MSCs) are capable of displaying multipotency and are subsequently called mesenchymal stem cells.[10,11]

Criteria for MSCs

To properly refer to MSCs, the International Society for Cellular Therapy (ISCT) has specified that "multipotent mesenchymal stromal cells" is a term preferred over mesenchymal stem cells, because not all have stem cell characteristics. Three minimal criteria for defining human MSCs have been established: 1. adherence to plastic when maintained in standard culture conditions, 2. expression of specific surface antigens, and 3. potential for multipotent mesenchymal differentiation (plasticity).[10]

It is important to mention that currently there is controversy on the use of the terminology regarding mesenchymal stem cells and mesenchymal stromal cells (MSCs).[12] There is lack of a specific marker expressed solely by MSCs. Several characteristics of these cells, however, have been taken in conjunction to propose the minimal criteria to define MSCs.[10] In addition, there are differences between MSC populations obtained from various sources, adding confusion to the concept of MSC.

Surface antigen expression of MSCs. The recognition of surface antigens permits the identification of a particular cell population. When performing flow cytometry, approximately 95% of the MSC population should mainly express CD73, CD90 and CD105 surface antigens. The MSCs should also lack expression of the hematopoietic antigens CD45, CD34, CD14, CD11b, CD19 and human leukocyte antigen (HLA) class II.[10] It is important to mention that for a proper identification of a MSC population. It is, consequently, important that the three criteria established by the ISCT are taken simultaneously in consideration for defining MSC.

Sources of MSCs

Mesenchymal stromal cells have been isolated from various tissues in humans, pigs, rodents, rabbits, dogs, cats, small ruminants,[13] and horses.[14] The most commonly identified sources for MSC isolation are adult adipose tissue[15] bone marrow, compact

bone, dental pulp,[16] peripheral blood,[17] umbilical cord blood, amniotic fluid, and other fetal tissues.[18,19]

Bone marrow-derived versus adipose tissue-derived MSCs. Because of the relative ease of accessibility, MSCs are most commonly obtained from bone marrow and adipose tissue in human and veterinary medicine. Bone marrow aspiration is considered an invasive procedure in human medicine, but in equine medicine this is not the case because of the relative straightforwardness of the procedure when aspirating bone marrow from the sternum or the hip in horses. Bone marrow aspiration is also faster and leads to less complications than aspirating adipose tissue for obtaining MSCs from a horse.

Both bone marrow-derived and adipose tissue-derived MSCs are effective in their ability to differentiate into cells of mesodermal and extra-mesodermal lineages and both are able to express the aforementioned typical markers for MSCs. Furthermore, cells derived from both sources appear to be similarly proliferative, although it has been reported by some groups that adipose tissue-derived MSCs may yield higher numbers of MSCs.[20] And, most importantly, both sources of MSCs seem to have immunosuppressive and anti-inflammatory properties.

Biological features of MSCs

By definition MSCs are multipotent and self-renew quickly. These features make MSCs an alternative source for tissue regeneration. Additionally, regardless of their source, it has been reported that these cells are well tolerated by the host after transplantation (allogeneic transplantation).

Immunomodulatory properties of MSCs. After transplantation, MSCs are well tolerated by the host. *In vitro* research has proved that MSCs are immunosuppressive by their ability to inhibit activated cytotoxic T cells and natural killer cell proliferation, and

downregulate B cell proliferation and differentiation.[21,22] Furthermore, this feature appears to be augmented if an active inflammatory response occurs in the body. Likewise in animal models, MSCs were able to ameliorate or inhibit acute rejection and or prolong allograft survival.[22,23] An explanation for this would be the ability of MSCs to downregulate CD86 and MHC class II to consequently impair the ability of macrophages to activate the antigen-specific CD4+ T cells.[24]

More recently, it has been discovered that human bone marrow-derived MSCs express toll-like receptors (TLR) 3 and 4.[25] These TLR are involved in danger signals that certain cells produce after they have been invaded by microorganisms, endotoxins or certain nucleic acids, activating the inflammatory cascade and preventing the suppression of T cell proliferation. Therefore, MSCs have the potential for both inhibitory and stimulatory effects on the immune system.

Anti-inflammatory properties of MSCs. During acute inflammation, the body produces both pro-inflammatory and anti-inflammatory cytokines (mainly, interleukins β1 [IL-β1] and 6 [IL-6], and tumor necrosis factor α [TNFα]) and other factors in an attempt to defend itself from the insult, but also to enhance healing. Over-production of pro-inflammatory factors produces undesirable effects in the body, because it results in an exaggerated inflammatory response. There are reports of MSCs being able to counteract the effects of inflammation. Mesenchymal stromal cells are capable of upregulating TGF-β1, an anti-inflammatory cytokine, shifting the macrophage phenotype from M1 (inflammatory) to M2 (anti-inflammatory), secreting superoxide dismutase, an enzyme that helps reduce oxidative stress, stimulating the synthesis of other chemokines or growth factors, and recruiting other cells to the site of injury.[26-29] On the other hand, MSCs are able to over produce prostaglandin E2 at levels that would suppress the synthesis of the pro-inflammatory cytokines IL-6, TNFα and NF-κB.[24,25]

Additionally, MSCs are capable of migrating and homing into injured tissues and even though the mechanisms have not been discovered yet, it is thought that tyrosine kinase and TLR-3 receptors might mediate their activity.[25,30]

Paracrine influence of MSCs in tissue regeneration. It is alleged that MSCs enhance tissue regeneration not only by their ability to stimulate endogenous progenitor or stem cells or to differentiate into cells of other lineages, but also in an indirect manner by secreting growth factors which are involved in neo-vascularization and tissue protection (i.e. reducing oxidative stress), and by inhibiting the host's immune response after transplantation and promotion of healing.

Growth factors are extra-cellular macromolecules that influence the function of target cells by binding to a cell surface receptor. Similarly, direct cell membrane-to-cell membrane contact mediated by adhesion molecules might also influence the function of other cells.[31] For example, MSCs are known to secrete insulin-like growth factor 1 (ILGF-1), vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (fGF-2), platelet-derived growth factor (pdGF), nerve growth factor (NGF), hepatocyte growth factor (hGF), and several interleukins (IL).[15,26,32-34] The secretion of these factors is triggered by different stimuli, such as hypoxia, ischemia, and free radicals.

The direct and indirect effects of MSCs are vast and still incompletely understood. Research performed in laboratory animals for nervous system diseases such as Alzheimer's disease, spinal cord injuries, or peripheral nerve injuries has demonstrated that the transplantation of MSCs provide neuroprotective effects by reducing neuronal sensitivity to glutamate, reducing oxidative stress and apoptosis, inhibiting the production of extracellular matrix, and by stimulating the synthesis of growth factors needed for regeneration.[26,35,36]

Cell plasticity of MSCs.

As recognized by the ISCT, MSCs should be capable of acquiring phenotypic markers for osteoblasts, chondrocytes and adipocytes, under the proper culture conditions.[37] To demonstrate this, staining with Alizarin Red for osteoblasts, Alcian Blue for chondrocytes, and Oil-Red-O for adipocytes should be performed.[10,38] Mesenchymal stromal cells from bone marrow and adipose tissue, however, have also been demonstrated to differentiate into cells from other mesodermal lineages (i.e. myocytes, cardiac myoblasts, tenocytes, fibroblasts)[39,40] and into cells of extra-mesodermal lineages (i.e. hepatocytes and neuronal cells).[41-43]

<u>*Neural differentiation of MSCs.*</u> *In vitro* research has shown that after induction with specific culture medium MSCs suffer morphological changes, such as contraction of the cell body and appearance of one or multiple processes, which resemble the phenotype of a cell from neural.[41,44-48] Several researchers refer to these cells as "neuron-like cells" but this term has not been defined by the ISCT. Yet, these cells continue to modify their morphology after the culture medium conditions are further changed. For instance, after exposing MSCs to a cytokine cocktail composed mainly of growth factors following previous pre-incubation with beta-mercaptoethanol and all-*trans*-retinoic acid, these cells acquire a different phenotype than the typical fibroblast-like morphology of an undifferentiated MSC; fibroblasts are flat and lack structural assymetry.[44,49-52] The morphological features that these MSCs acquire after exposure to this media resemble those of a Schwann cell, that is an oval-shaped cell body and bipolar or tripolar extensions, giving an overall spindle shape. Furthermore, these cells are able to express specific protein markers of Schwann cell.[34,44,50,53] And, most remarkably, models in laboratory animals have demonstrated that the functional properties of these cells after transplantation during spinal cord or peripheral nerve injury promote myelination and axonal regeneration of the spinal cord or injured nerve segments,[45,48,52,54,55] demonstrating that MSCs have broader plasticity than previously thought. Researchers refer to these cells as "Schwann-like cells".

The complete mechanisms that mediate the fate of these MSCs towards neural commitment are currently unknown. It has been discovered, however, that there is a transcriptional switch of MSCs towards neuroglial lineage, mediated by beta-mercaptoethanol and all-*trans*-retinoic acid.[51] Furthermore, there are also pro-neural genes that code for proteins that will eventually determine the fate of progenitor cells and their differentiation.[56,57]

Caution needs to be taken, nevertheless, not to confuse the term "neuron-like cells" with neurons. This term denotes MSCs (mesodermal origin) that were chemically induced for differentiation and acquired certain morphological characteristics, which is unrelated to the development of any of the nervous system cells, including neurons. During embryological development, the neural crest cells migrate in different directions and give rise to various ectodermal and mesodermal cell populations, including neurons, supportive cells of the nervous system (glial cells, Schwann cells, meningeal cells), endocrine cells (adrenal medulla, calcitonin-producing cells, carotid body cells), and mesodermal derivatives (connective and skeletal tissue of the head, smooth muscle cells on large vessels, and dermis of the neck and face). The fate of the neural crest cells depends on where they migrate and stay, but it is thought that some neural crest cells were already committed to their fate, while others are multipotent.[58] The term "neuron-like cell", consequently, should be avoided when referring to MSCs. A more appropriate terminology should be proposed, but for purposes of this research the term "neuron-like cells" will be replaced with "neural crest-like cells".

Schwann cell trans-differentiation of MSCs. Transdifferentiation implies further commitment or specialization of the cells' fate in the cascade of neural differentiation, in this case, towards a Schwann cell phenotype. Dezawa *et al* (2001) described a protocol for the differentiation of MSCs into Schwann-like cells. This protocol involved pre-incubation of the MSCs with beta-mercaptoethanol for 24 h, and all-*trans*-retinoic acid for 3 days; this was then replaced by a cytokine cocktail containing basic fibroblastic growth factor (bFGF), pdGF, forskolin and heregulin (a type of

neuregulin-1), for 7 days.[52] Other researchers have done some modifications of this protocol.

It has been hypothesized that MAPK signaling is involved in the differentiation of Schwann precursor cells to mature Schwann cells (myelin producing Schwann cells). The combination of some growth factors, such as bFGF and PDGF, stimulate the activation of this MAPK cascade,[59] while the role of forskolin is to upregulate the levels of intracellular cAMP for proliferation.[60] This upregulation of cAMP has additional neuroprotective effects; it retards diffuse axonal damage and has anti-inflammatory and immunomodulatory functions.[61] Other factors, such as Krox-20, activate the myelin genes and thus promote maturation of these Schwann cell precursors;[46,57] Krox-20 also suppresses the Notch signaling that is involved in proliferation of glial cells, including the Schwann cell precursors.[58,62] Similarly, heregulin influences proliferation, survival and maturation of glial cells, by inducing neural crest cells into Schwann cells via the MAPK cascade.[57]

Peripheral nerve injuries in the horse

Nerves can be injured because of trauma, toxicity, metabolic, or infectious disease. Less frequently, degenerative and hereditary disorders pose a threat. Trauma to the peripheral nerves can result from thermal or chemical injuries, compression, crushing, stretching or transection.[26,63] Following injury the blood-nerve barrier permeability increases and a cascade of events leads to the activation of local macrophages and monocytes that will remove myelin and axon debris, and to Schwann cell proliferation that will produce neurotrophic factors and extracellular matrix molecules needed for axonal regrowth and re-myelination. This inflammatory response results in Wallerian degeneration in the distal stump of the injured nerve.[26,30,35,46-48,56,58,63,64]

In veterinary practice, there are numerous peripheral nerve disorders (neuropathies) commonly observed in horses depending on their physical activity, clinical status or environment. Examples of these neuropathies include stringhalt, recurrent left laryngeal neuropathy (laryngeal hemiplegia), suprascapular nerve injury (Sweeney), facial, radial and femoral nerve paralysis, and polyneuritis equi (**Figure 1.2**). All of these are a cause of poor performance and economic loss to the equestrian industry. Furthermore, horses with peripheral nerve disorders are difficult to manage, and clinicians rely primarily on treating affected horses with anti-inflammatory drugs and physical therapy. Their treatment often results in financial hardship for the owner, and the outcome is usually correlated with the severity of the lesion and the interval before initiation of treatment. The final outcome for the horse is sometimes euthanasia.

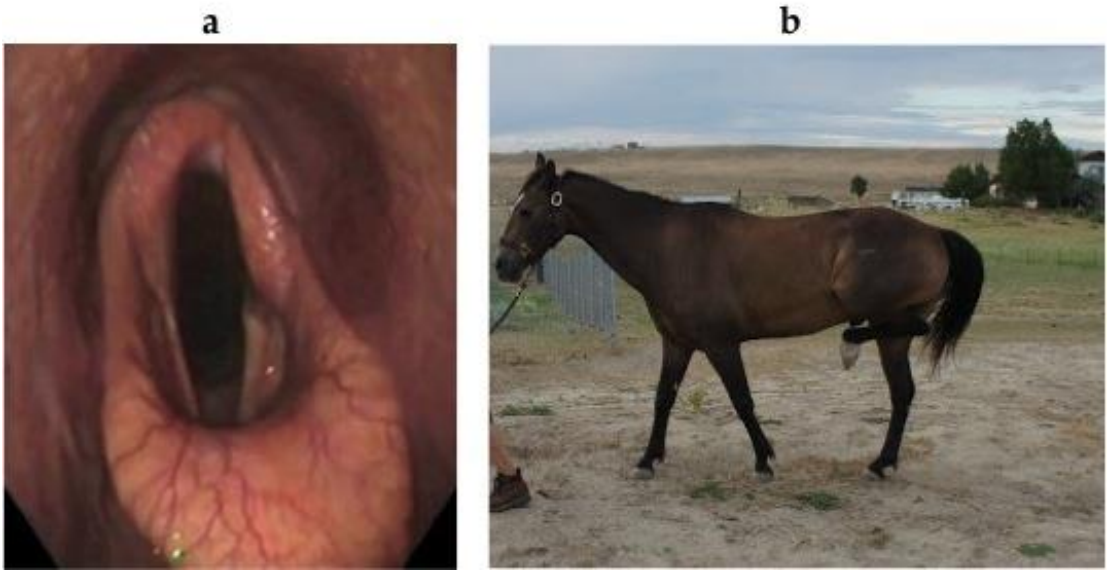

Figure 1.2 Examples of peripheral nerve disorders in horses: laryngeal hemiplegia (a) and stringhalt (b)

Types of nerve injuries

A classification for peripheral nerve injuries based on the severity of damage and nervous components involved was described by Seddon (1943).[65] The least severe damage to the nerve is called neurapraxia, which is characterized by local ischemia

(lack of blood perfusion) and discriminatory demyelination of the myelin sheath; this is reversible and the continuity of the axon itself is intact, thus conduction within the nerve proximal and distal to the lesion remains intact. An example of this is the damage that results from prolonged nerve compression.

The second type of injury is axonotmesis, in which there is disruption of the myelin sheath and the axon. Here, the connective tissue layers surrounding bundles of axons and their associated blood and lymphatic vessels, perineurium and epineurium, remain intact. This injury results in Wallerian degeneration and part of the axon disintegrates distal to the site of injury. Examples of this are nerve damage following displaced fractures and nerve crush injuries.

Finally, the most severe type of nerve injury based on this classification is neurotmesis, in which there is complete nerve transection and, therefore, disruption of the axons, myelin and the endoneurium (the immediate connective tissue layer that engulfs axons). This also results in Wallerian degeneration but there is complete transection of the nervous structures. The prognosis for this type of injury is also the poorest, and even with surgical repair and grafting, the results are not always rewarding. This type of injury can be consequence of severe ischemia or nerve transection.[63]

The role of Schwann cells in peripheral nerve injuries

In the PNS, the Schwann cells are glial cells that produce myelin, which is indispensable for fast conduction of nerve impulses. These cells additionally support axonal regeneration by the provision of several neurotrophic factors, hence, contributing to the creation of a microenvironment that is able to nourish, attract and stimulate other cells to aid in nerve protection and regeneration after an injury.[35,66]

As is the case for other types of cells, there is not a sole marker for identifying Schwann cells; a combination of known markers along with functional studies is preferred

before inferring conclusions on the ability of MSCs to differentiate into Schwann-like cells. Markers frequently used for the identification of Schwann cells are S100, p75, Krox20, CD104, and glial fibrillary acidic protein (GFAP).[46,49,50,56]

S100 is the most commonly reported marker for Schwann cell. It is a calcium binding protein constituted by 2 subunits: α and β (each approximately 10 kilo Daltons). It is considered to be a cell growth regulator but has other functions such as a protein and free fatty acid transport, or causing an increase in membrane permeability to cations, which are likewise attributed to this protein. Cells known to express S100 are glial cells, Schwann cells, melanocytes, chondrocytes, adipocytes, Langerhan's cells, interdigitating cells and some tumor cells (i.e. melanomas, Schwannomas). Consequently, S100 is not a specific marker for Schwann cells; nevertheless, there have been reports of undifferentiated MSCs expressing S100,[46] implying the potential of MSCs towards neural differentiation. In humans, cells expressing S100 have been subclassified into cells that express S100b, S100a and S100ao. Schwann cells and cells from the hypophysis express S100b.[31] Other properties attributed to S100 are nerve protection, nutrition and induction for axonal regeneration.[46]

GFAP is another marker commonly used to identify glial cells, including Schwann cells. We have found, however, that undifferentiated equine MSCs also express this protein, as previously reported by other researchers using undifferentiated MSCs from other species.[67,68] GFAP has neuroprotective properties by modulating the Schwann cell response to nerve injury.[46]

Similarly important, as it was mentioned in previous sections with undifferentiated MSCs, transplantation of either Schwann cells or MSCs differentiated into Schwann-like cells has been demonstrated to directly or indirectly enhance tissue healing by the immunomodulatory, anti-inflammatory and paracrine functions that they exert on target cells after an insult has occurred.

Cell therapy for nervous tissue repair: current research

Studies performed in rats, guinea pigs, and rabbits have demonstrated that peripheral nerves can be regenerated.[26,48,54,69,70] Even though the outcome is often disappointing. The most appropriate treatment for people with nerve injury is the insertion of an autograft into the gap in the transected nerve.[70-72] Limitations, such as available donor nerves, donor-site morbidity, and limited supply of cultured Schwann cells, however, prevent autografting from having practical clinical applications.[26,72] This is one reason why cell therapy with MSCs is intriguing; other factors include the ease of obtaining and expanding these cells *in vitro* to yield a high number for transplantation.

Therapy with MSCs

During the past decade, research for treating peripheral nerve injuries has been directed towards the use of cell therapy alone or combined with platelet rich plasma (PRP), grafts, or tissue-engineered biomaterials. Experimental models in laboratory animals and clinical trials in dogs have revealed that transplantation of either undifferentiated MSCs or Schwann-like differentiated MSCs into animals with peripheral nerve or spinal cord diseases results in positive outcomes.[26,35,36,48,73,74] Better results have been obtained with cells differentiated towards a Schwann-like cell phenotype, based on histological and functional analyses.[44,50,75] This emphasizes the importance of cell therapy as a promising alternative to nerve grafting.

The processes by which undifferentiated MSCs or differentiated MSCs increase nerve regeneration have not entirely been determined. It is known that MSCs possess certain features that allow them to enhance tissue regeneration. When transplanted after sciatic, femoral or facial nerve transection models in rats, MSCs appear to promote the pattern for axonal regeneration and myelin production, as compared to controls (usually, the contralateral limb or facial nerve). Quantitative morphometric evaluation of the nerve involves axonal count and axonal diameter; the evaluation of myelin

thickness provides extra information.[76] These parameters seem improved on laboratory animals that had been transplanted with MSCs after a nerve injury, when compared to untreated controls. Functional analyses testing strength, length of stride, and coordination are also superior results to the control limbs and to the animals that were not treated with cell therapy.[50,75]

When the treatment consists of transplantation of purified Schwann cells from a fresh peripheral nerve, or transplantation of Schwann cell-differentiated MSCs, the aforementioned histological and functional results are superior to those obtained with undifferentiated MSCs in laboratory animals.[44,48,50,75]

Delivery method. Cell therapy via transplantation of MSCs is one of the various auxiliaries within the field of regenerative medicine. Other alternatives for aiding in tissue repair include the use of other types of cells, platelet rich plasma (PRP), grafts, scaffolds, and nanoparticles. Tissue engineering, for example, uses a combination of transplanted cells, engineered scaffolds and biochemical factors to replace an injured tissue or organ. Consequently, there is increasing interest on the design of biomaterials capable of supporting cells and of promoting the delivery of factors (growth factors, neurospheres, etc.) that most appropriately improve nerve regeneration.

In laboratory animals the use MSCs or Schwann cell-differentiated MSCs for treating peripheral nerve injuries (with or without a scaffold or autograft) is commonly performed *in situ* immediately after nerve injury. There are several reports, conversely, on clinical trials and experimental designs on peripheral nerve and spinal cord injuries in which the transplantation of cells occurred intrathecally, by administering the cells into the cerebrospinal fluid (CSF); intra-arterially (IA), or intravenously (IV), via systemic or local administration.[74,77,78] When MSCs are transplanted IV, they are caught in the capillary beds of several tissues, mainly the lungs.[79] It is important to note that these cells were undifferentiated MSCs. To the knowledge of the author, there are no reports regarding the administration of

Schwann cell-differentiated MSCs via IV, CSF or IA routes for treating either spinal cord or peripheral nerve injuries.

There are no current reports regarding the most suitable method of delivery of MSCs for peripheral nerve disease in horses. Likewise, there are not reports regarding the use of Schwann cell-differentiated MSCs for nervous tissue repair in horses.

Tracking MSCs after transplantation. The mechanisms by which MSCs are able to migrate, stay ("homing") and repopulate a tissue are not completely understood. *In vitro* and *in vivo* studies have revealed that stromal derived factor-1 (SDF-1) and its interaction with receptors CXCR1, 4 and CCR2 and 5, play roles in stem cell motility and development. Furthermore, the expression of various types of adhesion molecules, such as integrins, ICAMs and VCAMs, are also involved in stem cell mobilization homing and proliferation.[80-82] The phenomena of migration and homing were evident in the repair of heart, bone, cartilage, CNS and PNS.[47,81]

For the sake of research it is important to be able to trace these cells after transplantation. Relying solely on functional analyses or by improvement of symptoms in humans does not assess the ability of cell therapy to contribute to tissue repair, especially if medications and physical therapy were part of the treatment. Methods to trace cells include labeling them with retroviral vectors to express fluorescent proteins, and the use of commercial dyes. These methods, however, require sacrificing of the animal to dissect the tissues. Alternatively, cells have been tracked *in vivo* by imaging them via magnetic resonance (MRI), computed tomography (CT), positron emission tomography (PET), and multiple photon microscopy.[83,84]

Finally, the feasibility of these methods must be taken in consideration because of issues regarding affordability and availability of equipment and reagents, false positive signals, interference with the biological properties of MSCs, and most importantly, the ability to accomplish any of these scans in a horse.

Neurotrophic factors and nerve regeneration

A particular interest has been given to the paracrine features of MSCs to secrete various trophic factors that aid in tissue repair. This is a topic of current focus because of the ability of MSCs (in this case, differentiated towards a Schwann cell phenotype) to be able to secrete neurotrophic factors that promote axonal regrowth and re-myelination. These factors are thought to provide an appropriate microenvironment for neuronal nourishment, survival and proliferation.[34,66,72]

Efforts have been made to understand the mechanisms of secretion, release and the properties that neurotrophic factors exert over neural cells, regardless of their sub-type. The ultimate purpose is to remove damaged cells and to restore myelin and axonal regrowth after nerve injury by recruiting Schwann cells and other cells via cell signaling. The exact mechanisms for secretion and release are not completely understood yet. *In vitro* studies strengthen this hypothesis, after revealing that MSCs from human adipose tissue or bone marrow are capable of expressing neurotrophic factors and that they promote neurite process outgrowth when these cells are co-cultured with injured nervous tissue.[30,33,34,53]

Some of the growth factors that have recently been investigated because of their effects on nervous tissue repair are: NGF, brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), ciliar neurotrophic factor (CNTF), ILGF-1, and VEGF. Briefly, NGF acts on neurons to stimulate their proliferation and differentiation; its action is potentiated when combined with scaffolds made of biomaterials.[85] Similarly, BDNF enhances MSC differentiation towards cells of neuronal lineage *in vitro*, after nerve injury.[85,86] In nerve fibers that were experimentally transected, it was found that GDNF promotes myelination and axonal re-growth on the early stages of the injury.[87] Finally, CNTF, ILGF-1 and VEGF are auxiliaries of nerve regeneration and survival, the last one mainly due to its location between nerve fibers and blood vessels during nerve regeneration.[31,88]

Overall, the ability of MSCs to inhibit the immune response created after allogeneic transplantation, as well as their ability to contribute to tissue repair by direct or indirect mechanisms makes MSCs an attractive therapeutic alternative in the field of regenerative medicine.

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CHAPTER II:
DIFFERENTIATION OF EQUINE MESENCHYMAL STROMAL CELLS INTO NEURAL CELLS: POTENTIAL FOR CLINICAL APPLICATIONS

Claudia Cruz Villagrán[1], Lisa Amelse[2], Nancy Neilsen[3], John Dunlap[4] and Madhu Dhar[2]

Departments of Comparative and Experimental Medicine[1], Large Animal Clinical Sciences[2], and Biomedical and Diagnostic Sciences[3], University of Tennessee College of Veterinary Medicine, Knoxville, TN.

Advanced Microscopy and Imaging Center[4], University of Tennessee, Knoxville, TN.

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Abstract

Studies have shown that mesenchymal stromal cells (MSCs) are able to differentiate into extra-mesodermal lineages, including neurons. Positive outcomes were obtained after transplantation of neurally-induced MSCs in rats, rabbits and guinea pigs after nerve injury, but this is unknown in horses. Our objective was to test the ability of equine MSCs to differentiate into cells of neural lineage *in vitro*, and to assess differences, if any, in morphology and lineage-specific protein expression. Additionally, we wanted to investigate if horse age and cell passage number affected the ability to achieve neural differentiation. Bone marrow-derived MSCs were obtained from 3 young and 4 adult horses. Following demonstration of stemness, MSCs were neurally-induced and microscopically assessed at 3, 6, 12, 24 and 48 h. Results showed that commercially available nitrogen-coated tissue culture plates supported proliferation and differentiation. Morphological changes were observed as early as 3 hours and all the cells surviving the treatment displayed a neural crest-like cell phenotype. Expression of neural progenitor proteins, nestin, vimentin, β_3 tubulin and glial fibrillary acidic protein (GFAP), was assessed via western blot or immunofluorescence. In our study, MSCs generated from young and middle-aged horses did not show differences in their ability to undergo differentiation. The effect

of cell passage number, however, is inconsistent and further experiments are needed. Ongoing work is aimed to trans-differentiate these cells into Schwann cells for transplantation into a peripheral nerve injury model in horses. The ultimate goal is to provide practical resources for treatment of neuropathies in horses.

Keywords: Horse, mesenchymal stem cell, neuron, peripheral nerve, regeneration

Introduction

Spinal cord and peripheral nerve injuries in horses occur after trauma, toxic/metabolic and infectious diseases. Less frequently, degenerative and hereditary diseases also pose a threat. These events trigger an inflammatory cascade of events that results in poor performance, disability or death. Additionally, the impact of peripheral nerve injuries in horses is reflected by big financial and emotional investments. Peripheral nerves can be injured by thermal or chemical injuries, compression, crushing, stretching or transection.[1,2] After injury, the blood-nerve barrier permeability increases and an inflammatory response is initiated resulting in proliferation of Schwann cells and activation of local macrophages that respond to tissue damage.[2,3] Several of these cells secrete neurotrophic factors and other substances that eventually enhance axonal regrowth and re-myelination, depending on the magnitude of the lesion and the chronicity of it.[1,2,4-10] A loss of axonal continuity along with external nerve structures (neurotmesis, in the Seddon classification) has the poorest prognosis for recovery.[1]

Mesenchymal stromal cells (MSCs) from bone marrow and adipose tissue have been demonstrated to trans-differentiate into cells of other lineages other than mesodermal lineages. In vitro research has shown the ability of rodent and human MSCs to acquire a neural crest-like cell phenotype after induction with specific culture medium.[7-9,11-13] Neural protein markers, mainly, vimentin, nestin, β_3 tubulin and glial fibrillary acidic protein (GFAP) were expressed in these neurally-induced cells, although results from

different studies are contradictory and hence, inconclusive.[10,13-18] Interestingly, a novel study involving the evaluation of K^+ and Na^+ currents in neural crest-like cells revealed that after chemical induction of rat MSCs, these cells appeared to have lost the aforementioned electrophysiological property when compared to non-induced MSCs; concluding that despite morphological and molecular changes similar to neural cells, these chemically induced cells lacked the functional properties of neurons.[14] Nevertheless, several studies have shown positive correlation between in vitro and in vivo results.[2,7,9,12,14,19-21] For instance, bone marrow and adipose-derived undifferentiated MSCs have revealed positive outcomes when these cells were allogeneically transplanted in neurologically injured rats, rabbits and dogs.[2,9-11,18-20,22,23] On the other hand, studies in rats have also shown beneficial responses and regain of nerve function after transplanting either chemically-induced MSCs into a Schwann cell-like phenotype, or pure Schwann cells obtained from fresh peripheral nerve tissue.[9,11,23,24] It is important to mention, however, that in these studies cell therapy occurred immediately after nerve or spinal cord injury.

To our knowledge, there are no reports in the literature that describe the ability of equine bone marrow-derived MSCs (eBM-MSCs) to differentiate into cells of neural lineage, or demonstrate the clinical benefits after transplantation into horses suffering from neuropathies. We have previously reported that to improve the clinical outcomes related to stem cell therapies, it is important to assess the biology and function of MSCs prior to their application in clinical cases.[25] In view of our long term goal of using equine MSCs in neuropathies, the present study was designed as a first step to evaluate the proliferation and survival of eBM-MSCs in vitro, and their ability to differentiate into neural crest-like cells after chemical induction. We compared these properties and assessed changes, if any, in horses (donors) of two age groups. Additionally, we compared changes in neural differentiation relative to the passage number of eBM-MSC cultures. We used the expression profiles of the neural progenitor markers nestin, vimentin, β_3 tubulin and GFAP to investigate these changes.

Materials and methods

1. Animals and bone marrow aspiration. Bone marrow aspirates were obtained from the sternum of 2 young mixed breed (range: 1-4 years old) and 4 adult American Quarter Horse (range: 9-13 years old) mares, and 1 young American Quarter Horse gelding as described previously.[26] All procedures were carried out as per an approved protocol by the Institutional Animal Care and Use Committee of the University of Tennessee, Knoxville, TN. Briefly, horses were sedated with 0.01-0.02 mg/kg of detomidine hydrochloride, intravenously. A 10 cm band was clipped and surgically prepared on the ventral aspect of the sternal area. Three mL of 2% lidocaine hydrochloride were injected subcutaneously and intramuscularly at the level of the 5th or 6th sternebrae at the ventral midline, followed by a stab incision with a 15 blade on the skin, subcutaneous tissue and musculature. A Jamshidi needle was inserted perpendicular to the skin until it reached the periosteum. The needle was then forced into the sternum and bone marrow from either the 5th or the 6th sternebrae was obtained by gentle aspiration with a syringe loaded with 1000 IU/10 mL of heparin sulfate.

2. Isolation and expansion of MSCs. These procedures were as described earlier.[25-27] Briefly, bone marrow aspirate was diluted with 1X PBS and layered on top of the lymphocyte separation media, Ficoll™. After centrifugation at 200g for 20 min at room temperature, the buffy coat containing the mononuclear cells (MNCs) was obtained. The MNCs were resuspended in growth media containing Dulbecco's modified Eagle medium/Ham's F-12 [(DMEM-F12), Cellgro™, Manassas, Virginia], 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Roughly 20x10^6 cells were seeded in 175 cm^2 vented tissue culture flasks (Thermo Scientific™, Rochester, NY), and maintained at 37 °C and 5% CO_2. First growth medium change was carried out between 5-7 days when clusters of colonies were visible under the phase contrast microscope. When 70-80% confluency was reached, the cells were harvested with 0.25% trypsin-EDTA for 2 min at 37 °C (passage 1: P1). After centrifugation, the cell pellet was reconstituted with either the growth medium to start a new experiment, or

with freezing media containing 50% DMEM-F12, 50% FBS and 5% DMSO for cryopreservation under liquid nitrogen for future experiments. These steps were repeated as needed in order to obtain cells from passage 2 to 4 (P2 to P4; low passage), and cells from P9-P12 (high passage) and used in future experiments.

3. Demonstration of stemness on low passage equine MSCs from 7 donors:

a) **Colony forming unit assay (CFU).** Passage 1 of equine MSCs were seeded at a density of $1x10^6$ in 100mm tissue culture dishes (Thermo Scientific™, Rochester, NY) and maintained in growth medium for 7 to 10 days until clusters of colonies were observed. The medium was replaced after every 2-3days. Colonies were fixed with 4% paraformaldehyde and stained with 0.5% of crystal violet (Sigma-Aldrich®, Saint Louis, MO). After staining, the dishes were allowed to air dry and images were acquired with a Fujifilm™ LAS-4000 imaging system (GE Healthcare Life Sciences).

b) **MTS proliferation assay.** Low passage (P2-P4) of equine MSCs were seeded at a density of $2x10^4$ per well in a 24-well tissue culture plate. The medium was replaced every 2-3 days. Cell proliferation was measured at 2, 4 and 7 days post seeding. A CellTiter 96 Aqueous non-radioactive (MTS) assay (Promega™, Madison, WI) was used following the manufacturer instructions. Briefly, MTS reagent in a 5:1 ratio relative to the media was added to each well and incubated for 3 h at 37 ° C and 5% CO_2. The absorbance at 490 nm was measured and data was obtained using Gen5 data analysis software (Biotek®, Winooski, Vermont). Growth medium only with no cells was used as a blank to correct the readings for each of the samples.

c) Induction of adipogenic, osteogenic and chondrogenic differentiation. Low passage (P2-P4) of equine MSCs were seeded at a cell density of $2x10^5$cells in 60 mm tissue culture dishes (BD Falcon™, New Jersey) and maintained at 37 ° C and 5% CO_2 in growth medium. When the cells were roughly 70% confluent, the medium was removed and was replaced with lineage-specific differentiation media as described earlier.[27] Briefly, adipogenic differentiation was induced by the addition of DMEM-F12 medium containing 15% rabbit serum, 1 μmol/L dexamethasone, 10 μg/mL

recombinant human insulin, 20 μmol/L indomethacin, and 0.5 mmol/L 3-isobutyl-1-metylxanthine. Osteogenic differentiation was induced by the addition of DMEM-F12 medium containing 100 nmol/L /mL of dexamethasone, 0.25 mmol/L ascorbic acid and 10 mmol/L β-glycerophosphate. Chondrogenic differentiation was induced by the addition of DMEM-F12, 100 nmol/L of dexamethasone, 0.25 mmol/L ascorbic acid and 5 ng/mL transforming growth factor β_1. Medium was replenished every 2-3 days and differentiation was monitored via microscopic evaluation. Undifferentiated MSCs maintained in regular growth medium without any differentiation reagents, for the same number of days served as controls. Differentiation was confirmed using lineage-specific cell staining using previously published methods.[27] Specifically, adipogenic cells were stained with Oil Red-O, osteogenic cells were stained with Alizarin red, and chondrogenic cells were stained with Alcian blue. Images were acquired with an electronic camera (Nikon™ DS-Fi2, Japan) connected to a Zeiss® microscope and evaluated with the NIS-Elements™ imaging software (Nikon™).

4. Neural differentiation on low and high passage equine MSCs from selected donors.

These experiments were performed on equine MSCs obtained from one young and one middle-aged donor. The donors were chosen based on their rates of proliferation as demonstrated by the MTS assay described above.

a) Cell culture. Low (P2) and high (P9) of equine MSCswere seeded at a cell density of 8 to $10x10^6$ into either polystyrene, or Primaria™ nitrogen-coated 100 mm tissue culture dishes (Becton Dickinson Labware, Bedford, MA). Cells were maintained in regular growth medium at 37 ° C and 5% CO_2, for at least 48 h to allow attachment. Neural differentiation was induced using a combination of previously described methods [13,18]. Briefly, the growth medium was removed and cells were pre-incubated with medium containing DMEM-F12, 20% FBS and 1 mM β-mercaptoethanol (Sigma-Aldrich®) at 37 °C and 5% CO_2, for 18-24 h. Subsequently, the cells were induced by the addition of the neural medium containing DMEM-F12, 2% DMSO and 200 μM butylated hydroxyanisole (BHA; Sigma-Aldrich®) and cells were incubated at 37 °C and 5% CO_2, for 3, 6, 12, 24 or 48 h. Undifferentiated MSCs maintained in regular

growth medium for the same number of hours were used as corresponding controls. Undifferentiated and differentiated cells were assayed at the end of each experiment as described below.

b) **Nuclear/cytoplasmic staining.** Nuclear/cytoplasmic fluorescent staining was used to show the neural-cell like morphology of MSCs after neural differentiation. Low and high passages of equine MSCs were seeded at a density of 1.25×10^6 on Primaria™ nitrogen-coated 60 mm tissue culture dishes and maintained in regular growth medium at 37 °C and 5% CO_2, for at least 48 h to allow attachment. When cells were 80-90% confluent they were chemically induced for neural differentiation as described above. Undifferentiated control MSCs described above were maintained with regular growth medium. For cytoplasmic staining, neurally-induced and undifferentiated MSCs at 12 h were stained with 5 µg of WGA (wheat germ agglutinin, Alexa Fluor® 488 conjugate; Life Technologies™) for 10 min, at room temperature. To stain the nucleus, cells were further washed and stained with 5 µg of TO-PRO®-3 iodide stain (Life Technologies™, Grand Island, NY) for 10 min, at room temperature. After washing, the cells were mounted with Slowfade® Gold antifade reagent (Molecular Probes®, Grand Island, NY) and images were obtained with a laser scanning spectral confocal microscope (Leica TCS SP2; Leica Microsystems©, Wetzlar, Germany), at 20x and 63x magnification.

c) **Protein extraction and western blot.** Total cell lysates were prepared from undifferentiated and neuronally-induced equine MSCs from low and high passages 12 h post-differentiation using standard protocols. Cells on each dish were gently washed with HBSS buffer and collected via cell scraping. To obtain total proteins in each sample, cells were lysed in 200 µL of RIPA buffer (Boston Bioproducts™, Ashland, MA), sonicated and supernatants were obtained by centrifugation. Total protein in each sample was quantitated and concentrations were obtained using modified BCA assay at 660nm (Pierce®, Thermo Scientific™). Equal concentrations of total proteins from neurally-induced and undifferentiated MSCs were electrophoretically separated in a 10% acrylamide gel and transferred onto

nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin (BSA) and incubated with mouse anti-β_3 tubulin (1:1000; Santa Cruz™) and mouse anti-GFAP (5 µg/10 mL; 1:1000; BD Pharmingen™). HRP goat anti-mouse IgG (1:5000; BD Pharmingen™) was used as the secondary antibody. Antigen detection was performed after exposure to ECL-2 reagent (Pierce®, Thermo Scientific™). Beta actin was used as a loading control.

d) **Immunofluorescence (IF).** Low and high passages of equine MSCs were seeded at a density of 1.25×10^6 on Primaria™ nitrogen-coated 60 mm tissue culture dishes and maintained in growth medium at 37 °C and 5% CO_2, for at least 48 h to allow attachment. When cells were 80-90% confluent they were chemically induced for neural differentiation as described above. Undifferentiated MSCs used as controls were maintained with regular growth medium. Neurally-induced and undifferentiated MSCs were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 (Sigma®) for 10 min, at room temperature, and blocked with 5% normal serum for 30 min, at room temperature. Cells were washed and incubated overnight with 5 µg/sample of primary antibodies against nestin (BD Pharmingen™) and vimentin (BD Pharmingen™), at 4 °C. After washing with HBSS buffer, cells were incubated with the secondary antibody (Alexa Fluor® 647 donkey anti-mouse IgG at 5 µg per sample; BD Pharmingen™) for 20 min, at room temperature. The cells were mounted with Slowfade® Gold antifade with DAPI reagent (Molecular Probes™) and images were obtained with a laser scanning spectral confocal microscope (Leica TCS SP2; Leica Microsystems©, Wetzlar, Germany)

Statistical analysis. A two-tailed Student's t-test was used to compare the average of neural – like cells, which adhered to the polystyrene-coated with those to the Primaria™ nitrogen-coated tissue culture plates. Similarly, the expressions of nestin and vimentin between cells of P2 and P9 from middle-aged horse, and between cells of P2 from young and middle-aged horse were compared from the IF data. The expressions of the two proteins were also compared between undifferentiated and

neuronally-induced MSCs. Differences were considered statistically significant when $P<0.05$.

Results

a) Stemness of equine MSCs

The stemness of cells from all equine MSC cultures was assessed solely in low passage cells. Once the properties of stem cell were demonstrated, only 1 donor from each group (young and middle-aged) was selected and the population of MSCs was passaged for neural experiments.

Morphology. Equine MSC cultures expanded from the bone marrow harvest adhered to the polystyrene surface and displayed spindle-shaped fibroblastic morphology. No morphological change was observed in eBM-MSC cultures generated from young and middle-aged donors.

Proliferation. Equine MSCs from young (**Figure 2.1:** horses 1-4) and middle-aged donors (**Figure 2.1:** horses 5-7) were capable of self-renewal as demonstrated by their proliferation and viability measured at days 2, 4, and 7. As demonstrated in the figure, there was a 3-6 fold increase, in absorbance for all the donors over a period of 7 days confirming their proliferation and viability. As expected and previously reported cellular proliferation, was variable between each horse. Horses that showed the highest proliferation and thus generating enough numbers of MSCs in a given period of time were selected for all neural experiments (Donors 2 and 6).

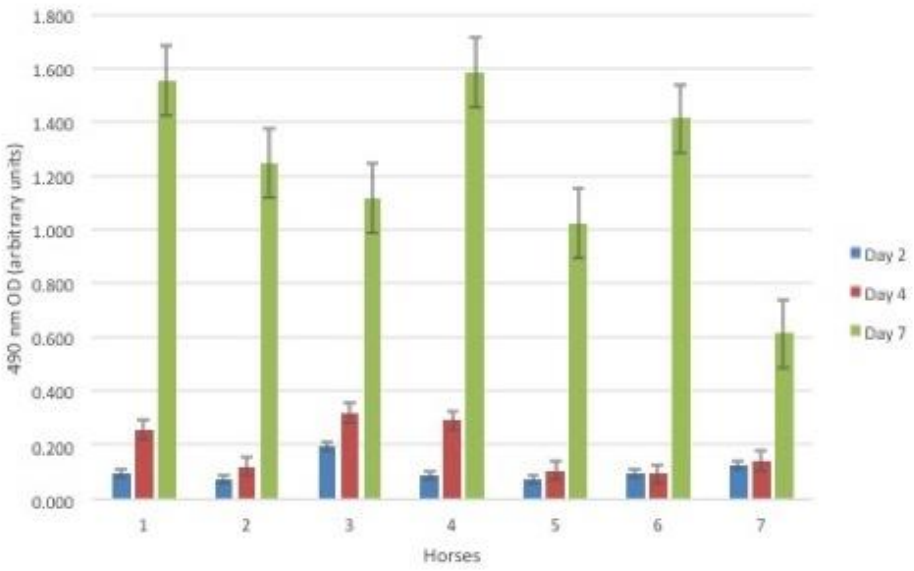

Figure 2.1. MTS proliferation assay on eBM-MSCs

CFU. Equine MSCs of P1 of young and middle-aged horses were capable of growing in clusters over the polystyrene - coated tissue culture dishes at day10, suggesting that the eBM-MSC cultures established represent the MSCs or the progenitor cells. A representative CFU assay is shown **(Figure 2.2).**

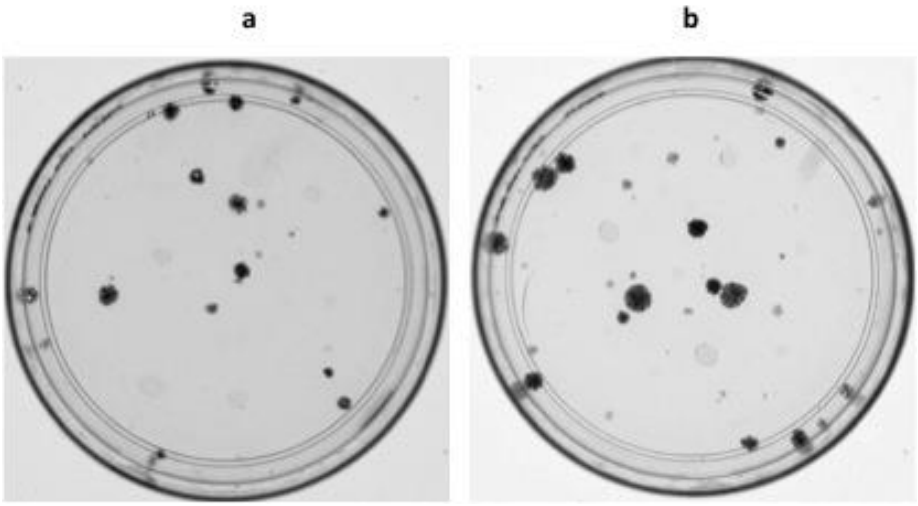

Figure 2.2. Colony forming unit (CFU) assay of eBM-MSCs

Mesodermal tri-lineage differentiation. Low passage equine MSCs from both young and middle-aged donors were capable of differentiation into adipogenic, chondrogenic and osteogenic cells under lineage-specific chemical induction **(Figure 2.3)**. At day 5, adipogenic cells displayed a typical pattern of lipid droplet formation, which could be stained with oil-red-o. Osteogenic and chondrogenic differentiation potential was confirmed by Alizarin red and Alcian blue staining, respectively after 10-15 days of chemical induction. No morphological differences were observed between horses. Variability existed, however, on the time of reaching a differentiated phenotype, as judged by specific staining. Undifferentiated controls remained with a spindle fibroblastic shape at all times and were not positive for lineage-specific staining. Of importance and in accordance to our previously published paper[25], osteogenic and chondrogenic differentiation of MSCs from one middle-age donor (horse 6), consistently occurred earlier than the rest.

All data presented above confirm that equine MSCs generated from each donor are progenitor cells, and they satisfy the criteria to be classified as the adult mesenchymal stromal/progenitor cells.

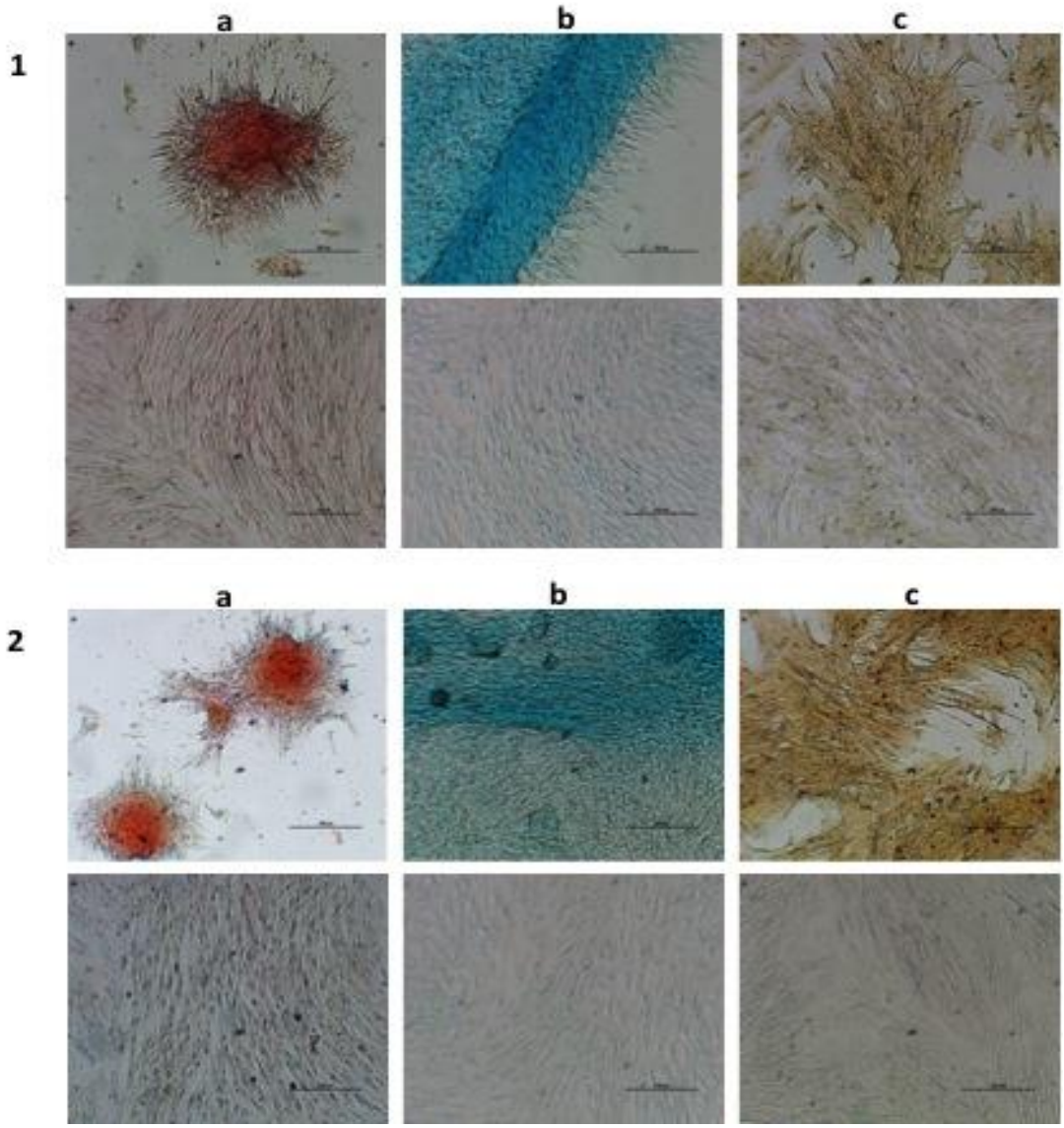

Figure 2.3. Mesodermal tri-lineage differentiation assays of eBM-MSCs

b) Neural differentiation

After ensuring selection of MSCs from low passage cells from the previous experiments, eBM-MSCs were further expanded, passaged, and tested for neural differentiation.

Neural crest-like cell morphology. For easy visualization, fluorescence microscopy was used to show an intact nucleus and a "healthy" cytoplasm. TO-PRO®-3 stain, the most sensitive probe for nucleic acid detection, along with WGA, specific to the cell

membrane, were used to demonstrate the nucleus and the cytoplasmic structure of the neural-like cells. Low and high passage eBM-MSCs from the selected young and middle-aged horses were capable of adopting neural crest-like cell morphology as early as 3 h after chemical induction. These morphological characteristics consisted of elongation of the cell, cell body contraction, and formation of one or multiple cell processes **(Figure 2.4)**. After 12 h of neural differentiation, all the cells displayed neural crest-like cell morphology. Moreover, these cells appeared to grow in a cluster in a mesh-like pattern. Undifferentiated controls had the spindle fibroblastic appearance of a MSC. No differences were microscopically observed on the phenotypical characteristics of neural crest-like cells of low passage between young and middle-aged horses. When analyzing high passage cells, subtle morphological differences were detected. The majority of these cells also displayed the aforementioned neural crest-like cells characteristics; however, they seemed to lose their cytoplasmic integrity and some of them appeared rounded and small. Nuclear and cytoplasmic staining helped in visual assessment of the morphological characteristics of these differentiated cells **(Figure 2.5)**. These morphological differences were more specific to the passage numbers, i.e. can be attributed to the process of cell culture itself, and not to the age of the donor.

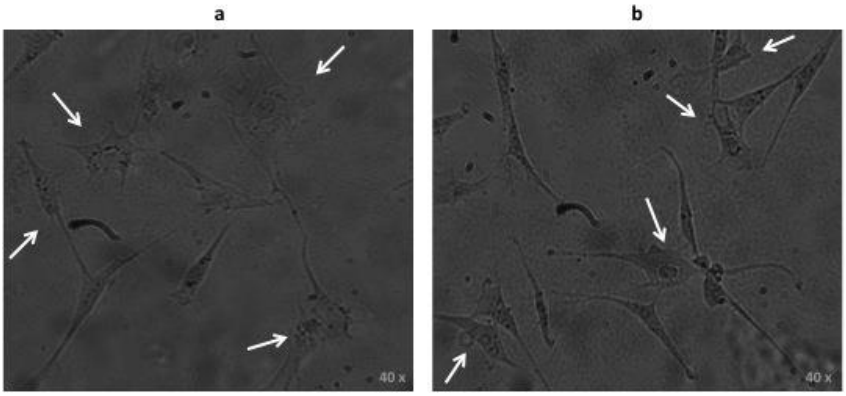

Figure 2.4. Neural crest-like cell differentiation assay of eBM-MSCs

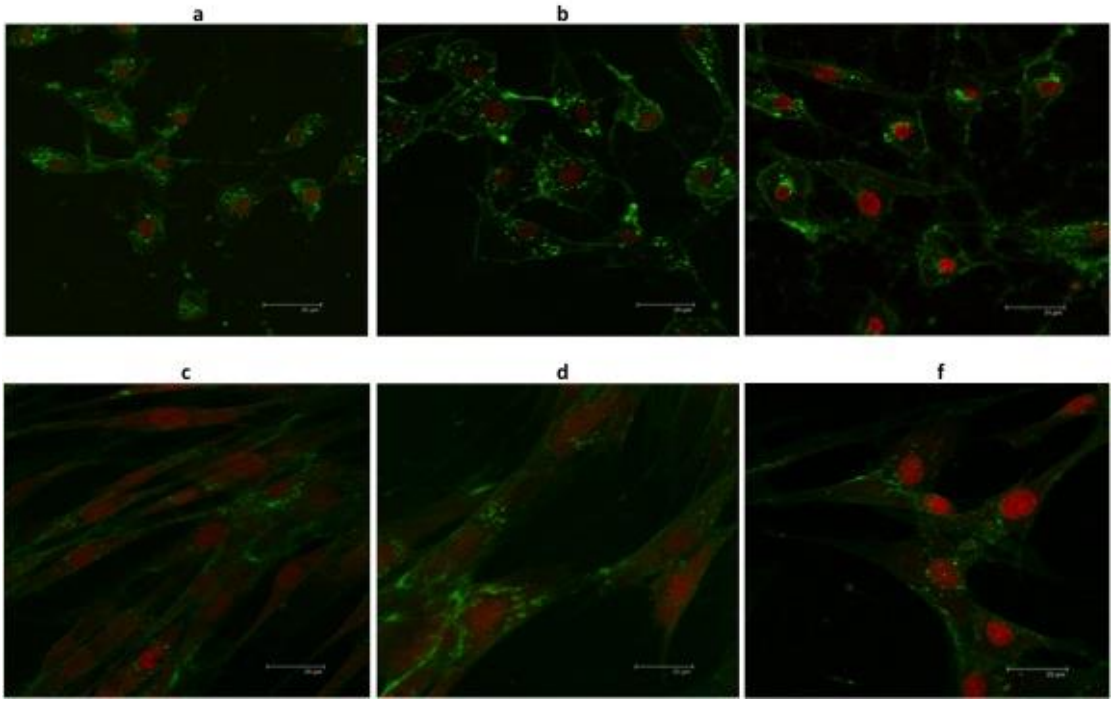

Figure 2.5. Nuclear (TO-PRO-3-iodide)/cytoplasmic (WGA) staining of neural crest-like eBM-MSCs (a, b, c) and undifferentiated eBM-MSCs (d, f). Scale bar= 25 µm

Finally, as judged by the numbers of cells adhered to the 2 different types of tissue culture plates; the nitrogen-coated plates (Primaria™) significantly enhanced cell survival and proliferation roughly, 2-fold higher than the polystyrene – coated plates (P=0.0319) after chemical induction (**Figure 2.6)**.

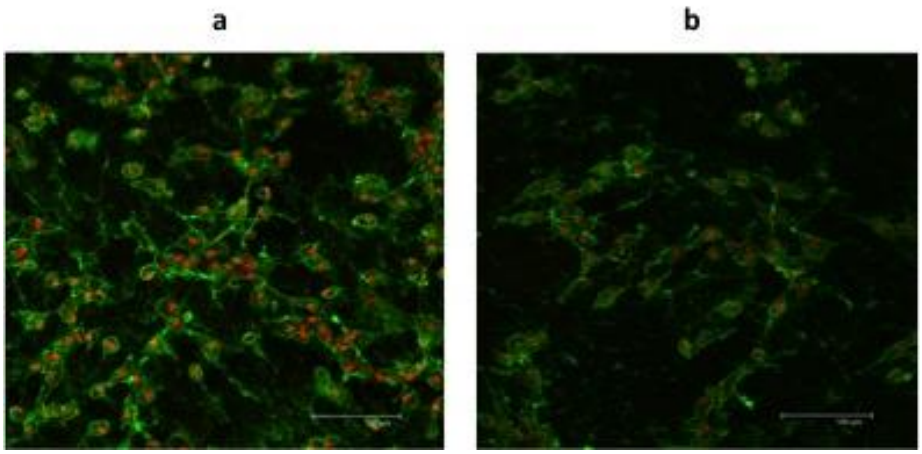

Figure 2.6. Nuclear (TO-PRO-3-iodide)/cytoplasmic (WGA) staining of neural crest-like eBM-MSCs on Primaria™ (a) and polystyrene-coated (b) tissue culture plates. Scale bar= 100 µm

Expression of neural progenitor proteins. The expression of vimentin was confirmed by IF (**Figures 2.7 and 2.8**). Vimentin was expressed by cells of low passage for both age groups with no significant differences (P=0.7653); cell passage number did not affect vimentin expression on the middle-age horse (P=0.7774), and no difference on vimentin expression was noted between undifferentiated MSCs and neurally-induced MSCs for young (P=0.9660) or middle-aged (P=0.1577) horses.

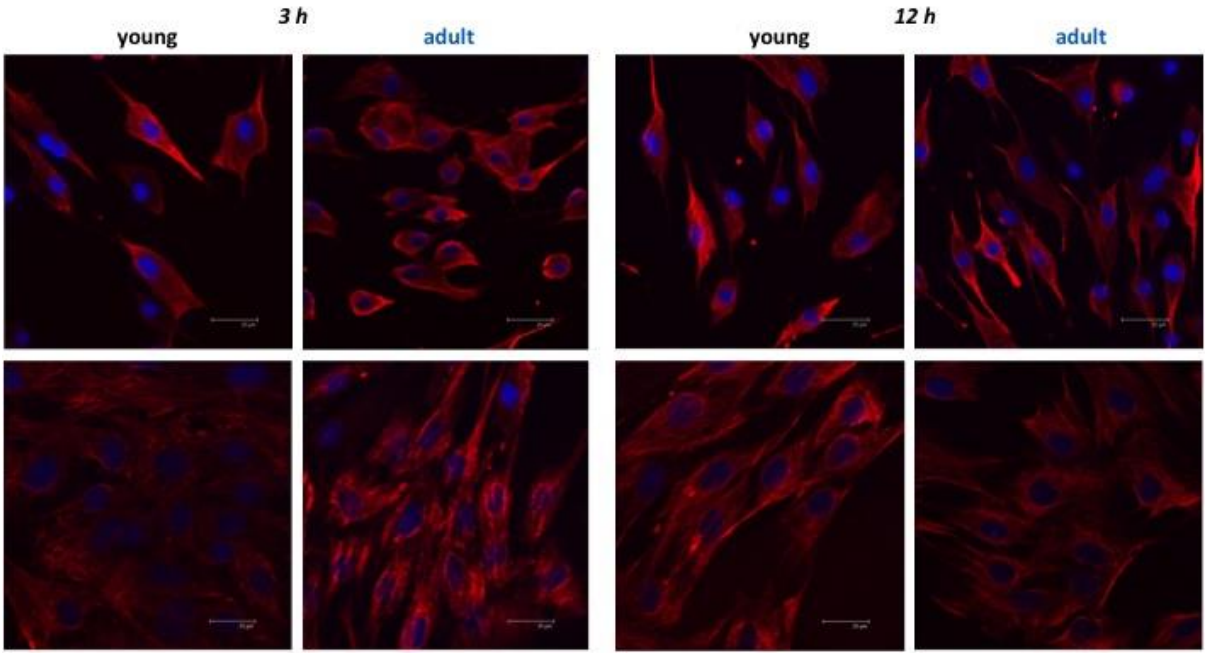

Figure 2.7. Expression of vimentin in undifferentiated (top row) and differentiated low passage eBM-MSCs (bottom row). Scale bar= 25 µm

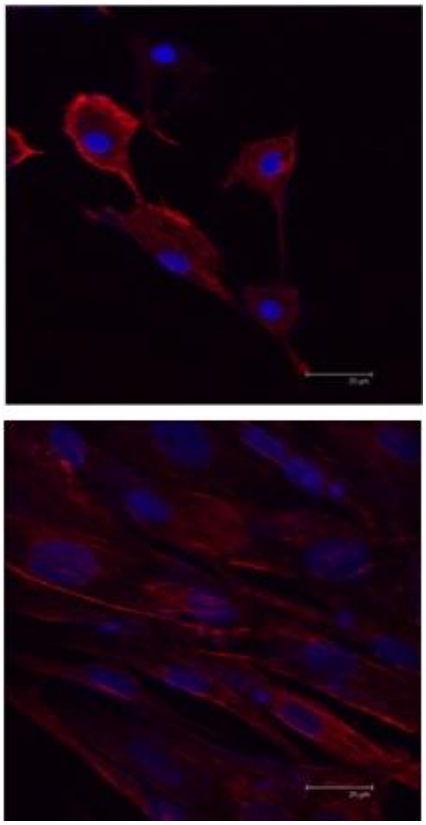

Figure 2.8. Expression of vimentin in undifferentiated (top) and differentiated (bottom) high passage eBM-MSCs. Scale bar= 25 µm

Immunoblot analysis was carried out to assess the expression of GFAP and β_3 tubulin (**Figure 2.9**). Differentiated cells from low passaged cells obtained from middle aged and young MSC cultures showed the expression of both the GFAP and β_3 tubulin. β_3 tubulin, was not expressed in the differentiated cells from high passaged cells obtained from the young MSC culture. The importance, if any, is not known at this time. Interestingly, low and high passaged undifferentiated cells generated from both donors expressed both proteins, suggesting plasticity of MSCs outside of mesodermal lineages.

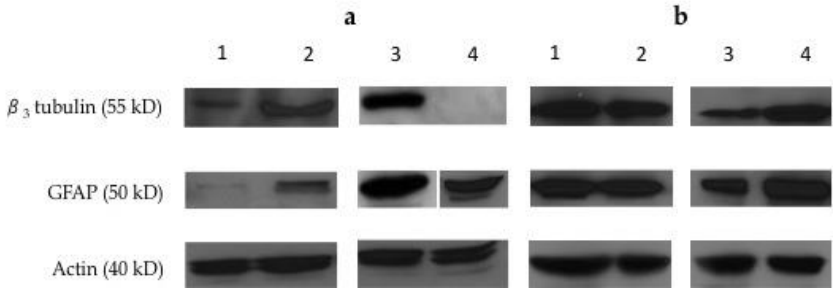

Figure 2.9. Western blot analysis of the expression of neural progenitor proteins β_3 tubulin and GFAP in differentiated (a) and undifferentiated (b) eBM- MSCs generated from low and high passaged cells from middle-aged (lanes 1 and 2, respectively) and young (lanes 3 and 4, respectively) horses

We were not able to detect nestin expression by western blot analysis, even when 3 different primary antibodies with various dilutions (1:1000, 1:2000) or secondary antibody concentrations (1:5000, 1:10 000). Interestingly, nestin expression was evident in undifferentiated and neurally-differentiated MSCs of low and high passages for both age groups by IF (**Figure 2.10)**. Nestin expression on cells of low passage for both age groups was observed with no significant differences (P=0.7325). Additionally, cell passage number did not affect significantly the expression of nestin on the middle-aged horse (P=0.3467), and no difference on nestin expression was noted between undifferentiated MSCs and neurally-induced MSCs for young (P=0.9616) or middle-aged (P=0.0830) horses. A perinuclear location of nestin was

evident on undifferentiated and neurally-differentiated MSCs of low passage, and on undifferentiated MSCs of high passage both age groups (**Figure 2.10**). The location of nestin in neurally-differentiated cells of high passage, however, was inconsistent, with some cells displaying a perinuclear location and others showing a more diffuse, cytoplasmic expression **(Figure 2.11)**. It is possible that cells of high passages undergo changes that alter the structure of some filamentous proteins (i.e. nestin).

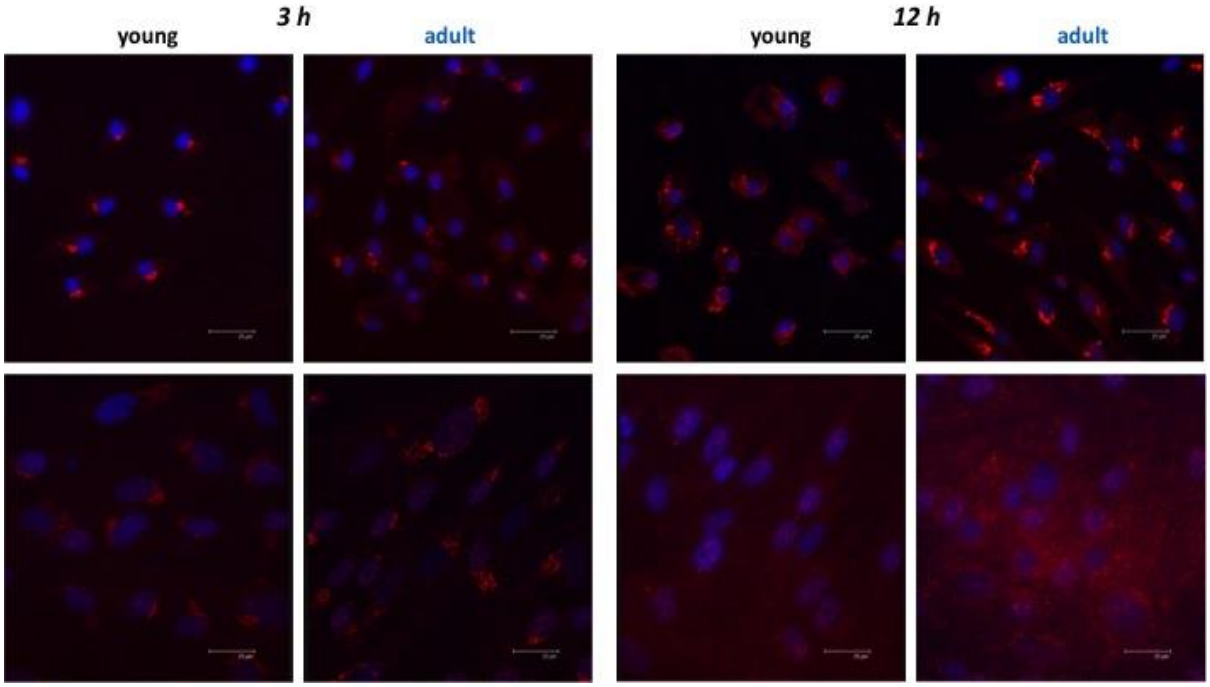

Figure 2.10. Expression of nestin in differentiated (top) and undifferentiated (bottom) low passage eBM-MSCs. Scale bar= 25 µm

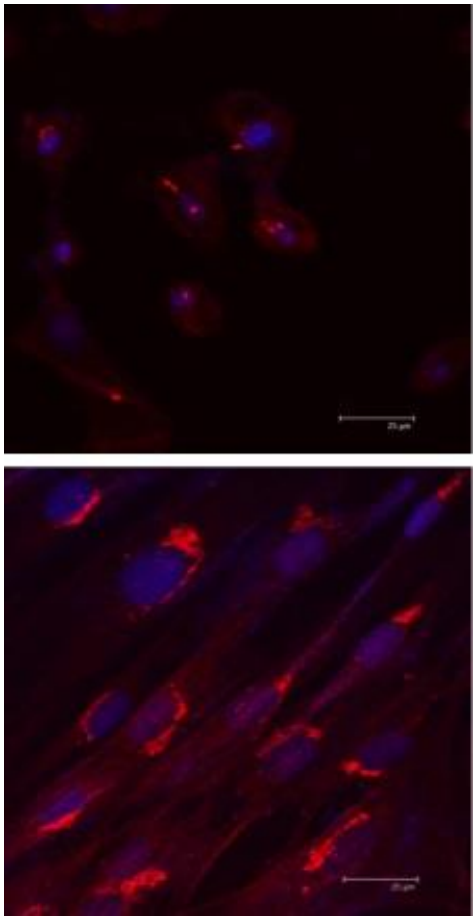

Figure 2.11. Expression of nestin in differentiated (top) and undifferentiated (bottom) high passage eBM-MSCs. Scale bar= 25 µm

Discussion

Peripheral nerve injuries are a cause of poor performance in horses. These injuries are difficult to manage and treatment mostly relies on physical therapy and anti-inflammatories; however, the long-term effects are time and personnel consuming. The development of neuronal cells is divided into various stages. After determination of their fate, these cells migrate to specific locations of the nervous system and accomplish different functions.[18,28] Previous studies have demonstrated that undifferentiated MSCs are able to express some neural protein markers, leading to the question whether MSCs are in advance committed to a neural lineage.[29-32] Moreover,

as neural progenitors develop into more specialized cells, changes in protein markers are also evident.[4,13,17,30,33]

Adipose-derived and bone marrow-derived MSCs from humans and rats are able to differentiate into neural lineages.[4,13,18,28,29,33-37] Several reports have described different methods of chemical induction for neural differentiation.[13,30,33,37,38] Medium containing trans-retinoic acid or butylated hydroxyanisole is the most frequently reported. Additionally, co-culture systems with MSCs and cells from nervous tissue have also proven successful for the induction of neural differentiation of MSCs.[29]

We conducted chemical induction of bone marrow-derived MSCs from young and middle-aged horses to characterize their viability and plasticity into mesodermal and neuronal (ectodermal) lineages. Cells from all horses were capable of self-renewal and underwent differentiation into adipogenic, osteogenic and chondrogenic lineages. This was consistent with a previous report published from our laboratory[25] and, thus, confirmed the stemness of the cultured MSCs. For the neural differentiation, fluorescence microscopy revealed that morphological changes were observed as early as 3 h after chemical induction in all horses. Using nuclear/cytoplasmic staining we were able to demonstrate that differentiated cells acquired a neural crest-like cell morphology in which retraction of the cell soma and formation of multiple cell processes were observed. Most importantly, the proliferation of differentiated MSCs under the neural media was higher on the nitrogen-coated plates (Primaria™) than on the polystyrene tissue culture plates, suggesting stabilization of the cell membrane by providing a positive charge.

By the combination of western blot and IF analyses, we found that all MSCs, undifferentiated and differentiated, expressed neural progenitor markers, namely, vimentin, nestin, GFAP and β_3 tubulin were evident. The expressions of GFAP and β_3 tubulin in undifferentiated MSCs were particularly interesting and data is supported by previous studies in rats and humans, suggesting their ability for neuronal differentiation.[39,40] Nestin has been shown to be expressed in muscle and neural

progenitor cells, as well as in highly proliferative cells (i.e. following injuries, high mitotic rate, etc.) or undergoing development.[41-43] In IF data, nestin subjectively appeared weaker in cells from high passage than cells from low passage, suggesting the loss of cellular mechanisms for proliferation and plasticity as the cell ages. We detected a perinuclear location of nestin in undifferentiated and neurally-differentiated MSCs from P2. In differentiated cells from P9 in nestin location extended from the perinuclear region towards other regions in the cytoplasm. This was similar to previous reports[43,44] and may suggest post-translational modifications of nestin[42-44], the analysis of which was beyond the scope of our study. Similarly, the effect of cell passage number on the expression of these markers also suggested modifications or structural changes as the cells age, but further studies are needed to confirm this.

Our study mainly relies on morphological changes and neural marker protein expression to describe the events occurring during neural differentiation of eBM-MSCs. Our results agree with previous *in vitro* studies performed in bone marrow-derived MSCs from rat, human and dogs in which eBM-MSCs are chemically induced into cells that display morphologic, genetic and protein characteristics of neural progenitors. To our knowledge, this is the first report describing the plasticity, morphological characteristics and protein expression changes of eBM-MSCs into cells of neural lineage after chemical induction. We have not only demonstrated the in vitro differentiation patterns but also validated the cross-reactivity of human neural-specific antibodies with equine protein samples. Further studies are however, warranted for investigating the functionality of these horse cells *in vivo*.

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CHAPTER III:

A PRELIMINARY, *IN VIVO* STUDY EVALUATING THE EFFECTS OF MESENCHYMAL STROMAL CELLS IN SPEEDING REGENERATION OF TRANSECTED PERIPHERAL NERVES OF HORSES

Claudia Cruz Villagrán[1], Jim Schumacher[2], Nancy Neilsen[3], Robert Donell[3], and Madhu Dhar[2]

Departments of Comparative and Experimental Medicine[1], Large Animal Clinical Sciences[2], and Biomedical and Diagnostic Sciences[3], University of Tennessee College of Veterinary Medicine, Knoxville, TN.

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Abstract

Peripheral nerve injuries cause poor performance in horses and their outcome is frequently unfavorable. Autografting a peripheral nerve is the ideal approach for repairing a nerve. This, however, represents difficulties in obtaining enough tissue and in causing morbidity to the donor site. Alternative approaches include the insertion of biocompatible scaffolds and transplantation of mesenchymal stromal cells (MSCs) into the affected individual. Transplantation of MSCs differentiated into Schwann-like cells is still under investigation, with promising results in laboratory animals. There are no current reports of bone marrow-derived MSCs from horse and their ability to differentiate into Schwann-like cells. Likewise, there are no reports describing a model for peripheral nerve injury in horses. Our objectives were to evaluate the ability of equine bone marrow-derived MSCs to differentiate into Schwann-like cells, to propose a model for peripheral nerve injury in horses, and to evaluate the effects of MSCs on nerve regeneration after transplantation *in situ*. Equine bone marrow-derived MSCs were differentiated into Schwann-like cells for 7 days, and assessed via microscopy, immunofluorescence and western blot by using Schwann cell markers. Results showed that MSCs from young and middle-aged horses displayed Schwann cell morphology and were able to express glial fibrillary acidic protein and S-100b at day 7. Furthermore, histological analyses were performed on peripheral nerve sections from 3 horses, 45 days after nerve transection followed

by *in situ* transplantation of undifferentiated MSCs. Our fundamental goal is to provide practical resources for treatment of peripheral nerve disease in horses.

Introduction

In veterinary medicine, horses can suffer peripheral nerve injuries after traumatism, metabolic, toxic, hereditary, degenerative and infectious diseases. The sequels of nerve injury in horses are frequently undesirable, resulting in poor performance, disability or even death. This, consequently, causes a profound financial and emotional impact afterwards.

Peripheral nerves have the ability to regenerate after injury due to the secretion of cytokines and neurotrophic factors from the damaged cells (mainly by Schwann cells), and because phagocytosis is initiated by local macrophages to remove cell debris.[1-3] The regaining of nerve function, however, will depend on the magnitude of the nerve lesion and its chronicity.[4] The prognosis for recovery is poorest when a nerve is transected and its nerve fibers and surrounding fascia (connective tissue) have been completely disrupted. This is known as neurotmesis in Seddon's classification for peripheral nerve injuries.[5,6] Surgical techniques for nerve repair involve suturing the nerve stumps together or inserting a graft that bridges the gap. In tissue engineering, some of the most commonly utilized materials for fabricating grafts or scaffolds consist of vein, artery, nerve, silicone, collagen, laminin, gels made of platelet rich plasma (PRP), and synthetic polymers.[7,8] Regardless of the technique used or the duration of the nerve repair, clinical results are often disappointing.[6,9]

Currently, research regarding cell therapy with mesenchymal stromal cells (MSCs) suggests this might be an alternative approach for nervous tissue repair, including spinal cord and peripheral nerve injuries.[10-12] The neuroprotective effects of MSCs have been widely described, and they involve anti-inflammatory, immunomodulatory, angiogenic and nurturing mechanisms.[13-16] Furthermore, MSCs

from bone marrow and adipose tissue are able to differentiate into Schwann-like cells after chemical induction with specific conditions.[12,17,18] In experimental models in laboratory animals, when Schwann-like cells are transplanted into nervous lesions, the results for axonal and myelin repair as well as the sensory and motor functions, appear promising. This is believed to be consequence of the secretion of specific neurotrophic factors that promote nerve repair.[11,12,16]

To our knowledge, there are no reports in the literature regarding outcomes of peripheral nerve injuries after cell therapy in the horse. Similarly, we can find no reports that describe the ability of equine bone marrow-derived MSCs (eBM-MSCs) to differentiate into myelinated Schwann-like cells. We have recently reported that eBM-MSCs are able to display morphological and protein characteristics of neural progenitors. In this preliminary study, we evaluated the ability of eBM-MSCs to differentiate into Schwann-like cells after chemical induction. Additionally, we propose a new model for peripheral nerve injury in the horse and its outcome after transplantation of undifferentiated MSCs.

Materials and methods

1. Animals. Three healthy American Quarter Horse crossbred mares from our Institution's teaching herd were used for the study. The mares were transplanted allogeneic undifferentiated MSCs from a donor whose cells have been previously characterized. These cells were transplanted immediately after the first surgery (see No. 2). All procedures were carried out as per an approved protocol by the Institutional Animal Care and Use Committee of the University of Tennessee, Knoxville, TN.

2. Transection of the *ramus communicans* with the horses standing. Horses were sedated with 0.01-0.02 mg/kg of detomidine hydrochloride and 0.01-0.02 mg/kg of butorphanol tartate, IV. The palmar aspect of both metacarpal regions was prepared

for aseptic surgery and 2 mL of 2% mepivacaine hydrochloride were subcutaneously deposited adjacent to the medial palmar nerve and adjacent to lateral palmar nerve proximal to the palpable *ramus communicans*, palmar to the superficial digital flexor tendon (**Figure 3.1**). A scalpel blade was used to create a cutaneous, longitudinal incision over the *ramus communicans*. Using a 6 mm punch biopsy the center of this anastomotic nerve, which connects the medial and lateral palmar nerves, was removed and placed in formalin. Following nerve transection, $10x10^6$ allogeneic MSCs suspended in a volume of 1 mL of sterile saline were instilled into the fascia surrounding this nerve (**Figure 3.2**). The contralateral transected nerve received an injection with the same volume of sterile saline (control). The cutaneous incision was closed with staples, and the distal portion of the limbs was bandaged. The horses received phenylbutazone (4.4 mg/kg, PO) at the time of surgery and the day after surgery (2.2 mg/kg, PO). The bandage was changed every third day until the staples were removed at day 14. The horses underwent general anesthesia for complete removal of both *ramus communicans* approximately 45 days after the cell transplant. Briefly, the horses were induced with 0.5 mg/kg of xylazine and 2.2 mg/ kg of ketamine, IV. They were intubated, placed in lateral recumbence, connected to the ventilator and maintained with isofluorane. A scalpel blade was used to create a cutaneous, longitudinal incision over the medial and lateral aspects of the *ramus communicans* of both fore limbs. The subcutaneous tissue and surrounding fascia were bluntly dissected and once the nerve was identified, it was exposed and transected with scissors. The nerves were placed in Carson's fixative and sent to pathology for histological analyses. The horses received phenylbutazone (4.4 mg/kg, PO) prior to surgery and the day after surgery. The bandages were changed every third day until the staples were removed 14 days after surgery.

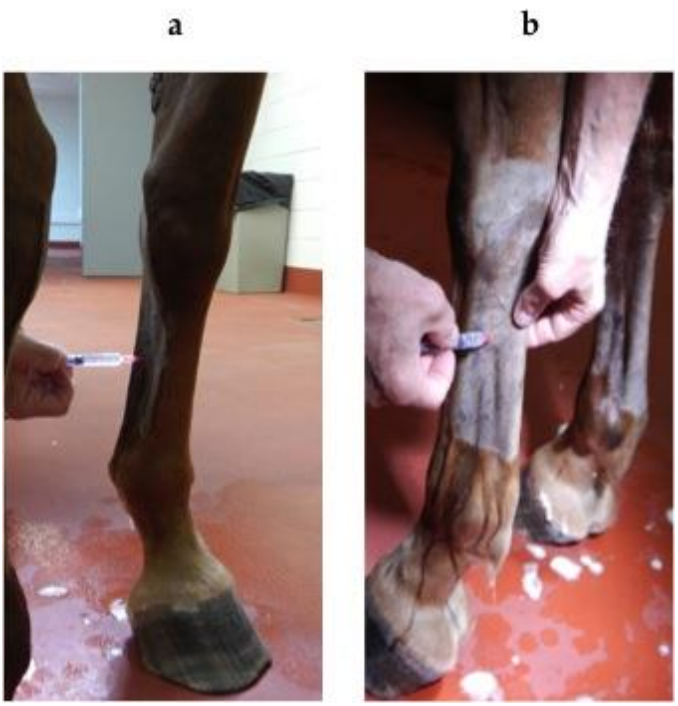

Figure 3.1. Local anesthesia (nerve block) of the medial (a) and lateral (b) palmar nerves of a horse

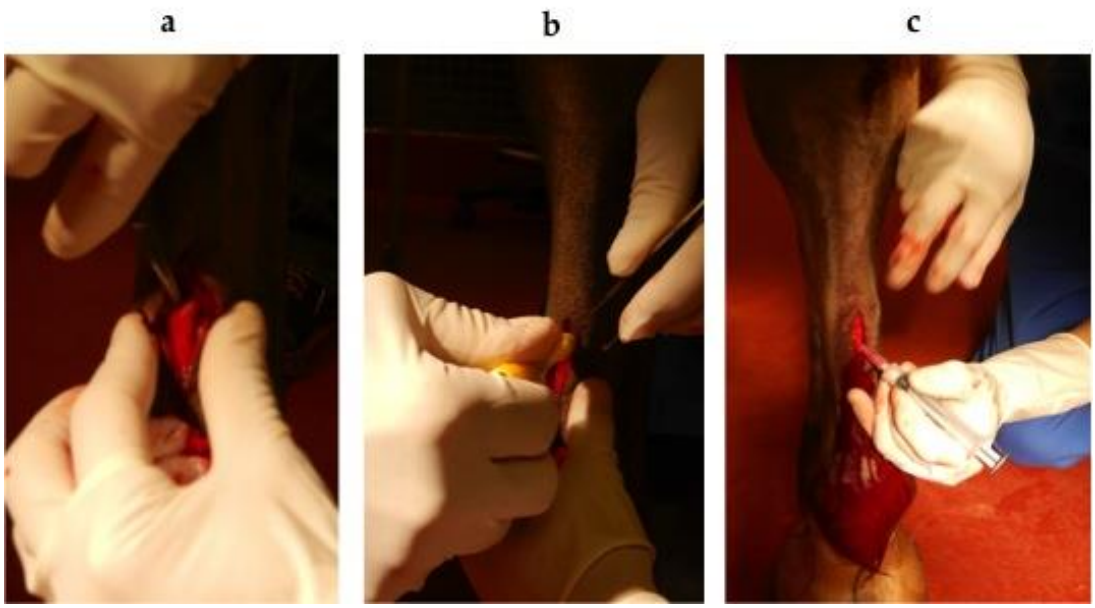

Figure 3.2. Dissection (a) and transection (b) of the *ramus communicans* of the fore limb of a horse, followed by transplantation of undifferentiated MSCs (c)

3. MSCs. Low passage (P1 to P4) bone marrow-derived MSCs previously cryopreserved from an allogeneic horse donor were used for cell expansion or for cell transplantation into the mares. These MSCs had been previously characterized, as described in the section of materials and methods of Chapter II (see "Demonstration of stemness on low passage equine MSCs from 7 donors").

4. Schwann cell differentiation.

a) Cell culture. Low passage (passage 1-5) equine MSCs were seeded at a cell density of 8 to $10x10^6$ into 100 mm Primaria™ nitrogen-coated tissue culture dishes (Becton Dickinson Labware, Bedford, MA). Cells were maintained in regular growth medium containing containing Dulbecco's modified Eagle medium/Ham's F-12 [(DMEM-F12), Cellgro™, Manassas, Virginia], 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, at 37 ° C and 5% CO_2, for 48 h to allow attachment. Neural differentiation was induced using a previously described method.[12] Briefly, the growth medium was removed and cells were pre-incubated with medium containing DMEM-F12, 20% FBS and 1 mM β-mercaptoethanol (Sigma-Aldrich®) at 37 °C and 5% CO_2, for 18-24 h. Subsequently, the cells were induced by the addition of the neural medium containing DMEM-F12, 10% FBS and 35 ng/mL all-*trans*-retinoic acid for 3 days. The medium was then replaced with DMEM-F12, 10%FBS and a cytokine cocktail containing: 5 μM forskolin, 200 ng/mL recombinant human heregulin-β1 (HRG-β1), 5 ng/mL platelet-derived growth factor, and 10 ng/mL recombinant human basic fibroblast growth factor (all reagents from Sigma-Aldrich®), for 7 days, replacing the medium every 72 h.

b) Nuclear/cytoplasmic staining. Equine MSCs were seeded at a density of $8\text{-}10x10^6$ on 100 mm Primaria™ nitrogen-coated tissue culture dishes and maintained in regular growth medium at 37 °C and 5% CO_2, for 48 h to allow attachment. After 48h when cells were 80-90% confluent they were chemically induced for Schwann cell differentiation as described above. Undifferentiated MSCs (control) mentioned above were maintained with regular growth medium. For cytoplasmic staining, Schwann-like cells and undifferentiated MSCs at 12 h were stained with 5 μg of WGA (wheat

germ agglutinin, Alexa Fluor® 488 conjugate; Life Technologies™) for 10 min, at room temperature. To stain the nucleus, cells were further washed and stained with 5 µg of TO-PRO®-3 iodide stain (Life Technologies™, Grand Island, NY) for 10 min, at room temperature. After washing, the cells were mounted with Slowfade® Gold antifade reagent (Molecular Probes®, Grand Island, NY) and images were obtained with a laser scanning spectral confocal microscope (Leica TCS SP2; Leica Microsystems©, Wetzlar, Germany), at 20x and 63x magnification.

c) Protein extraction and western blot. Total cell lysates were prepared from Schwann-like cells and undifferentiated MSCs at 12 h using standard protocols. Cells on each dish were gently washed with HBSS buffer and collected via cell scraping. To obtain total proteins in each sample, cells were lysed in 200 µL of RIPA buffer (Boston Bioproducts™, Ashland, MA), sonicated and supernatants containing total proteins were obtained by centrifugation. Total proteins in each sample were quantitated and concentrations were obtained using BCA assay at 660nm (Pierce®, Thermo Scientific™). Equal concentrations (20 µg per lane) of total proteins from Schwann cell-induced and undifferentiated MSCs were electrophoretically separated in a 12% acrylamide gel and transferred onto nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin (BSA) and incubated with mouse anti-S-100b (1:1000; BD Pharmingen™), mouse anti-β_3 tubulin (1:1000; Santa Cruz™) and mouse anti-GFAP (1:1000; BD Pharmingen™). HRP goat anti-mouse IgG (1:5000; BD Pharmingen™) was used as the secondary antibody. Antigen detection was performed after exposure to ECL-2 reagent (Pierce®, Thermo Scientific™). Beta actin was used as a loading control.

d) Immunofluorescence (IF). Low passage (P1-P5) MSCs from young and middle-aged horse were seeded at a density of 8 to $10x10^6$ cells on 100 mm Primaria™ nitrogen-coated tissue culture dishes and maintained in growth medium at 37 °C and 5% CO_2, for at least 48 h to allow attachment. When cells were 80-90% confluent they were chemically induced for neural differentiation as described above.

Undifferentiated MSCs used as controls were maintained with regular growth medium. Schwann cell-induced and undifferentiated MSCs were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 (Sigma®) for 10 min, at room temperature, and blocked with 5% normal serum for 30 min, at room temperature. Cells were washed and incubated overnight with 5 µg/dish of primary antibodies against S-100b (BD Pharmingen™) and GFAP (BD Pharmingen™), at 4 °C. After washing with HBSS buffer, cells were incubated with the secondary antibody (Alexa Fluor® 647 donkey anti-mouse IgG at 5 µg per dish; BD Pharmingen™) for 20 min, at room temperature. The cells were mounted with Slowfade® Gold antifade with DAPI reagent (Molecular Probes™) and images were obtained with a laser scanning spectral confocal microscope (Leica TCS SP2; Leica Microsystems©, Wetzlar, Germany).

5. Histological analysis of nerve tissue. The central portion the *ramus communicans* transected from both fore limbs of each horse (n=3) during the first surgery was placed on Carson's fixative for histological analyses. Approximately 45 days after cell transplantation, the whole nerve was collected from both fore limbs on these horses placed on Carson's fixative for histological analyses.

Results

1. Schwann cell differentiation

Schwann-like cell morphology. For better visualization, fluorescence microscopy was used to show the integrity of nucleus and cytoplasm on the cells. TO-PRO®-3 stain, the most sensitive probe for nucleic acid detection, along with WGA, specific to the cell membrane, were used to demonstrate the nucleus and the cytoplasmic structure of the Schwann-like cells. Low passage MSCs from young and middle-aged horses were capable of adopting a Schwann-like morphology 7 days after chemical induction. The cells elongated until they displayed spindle-shape morphology and

one or 2 cell processes appeared (**Figures 3.3 and 3.4**). At 4 days of chemical induction, some of these cells began displaying this morphology, but at 7 days, most of the cells were as described. Moreover, these cells seemed to grow in a "whorl-like" pattern. Undifferentiated controls had the typical fibroblastic appearance of a MSC. No differences were microscopically observed in the phenotypic characteristics of Schwann-like cells between young and middle-aged horses. Subjectively, approximately 80% of the cells had acquired a Schwann cell phenotype at 7 days after chemical induction.

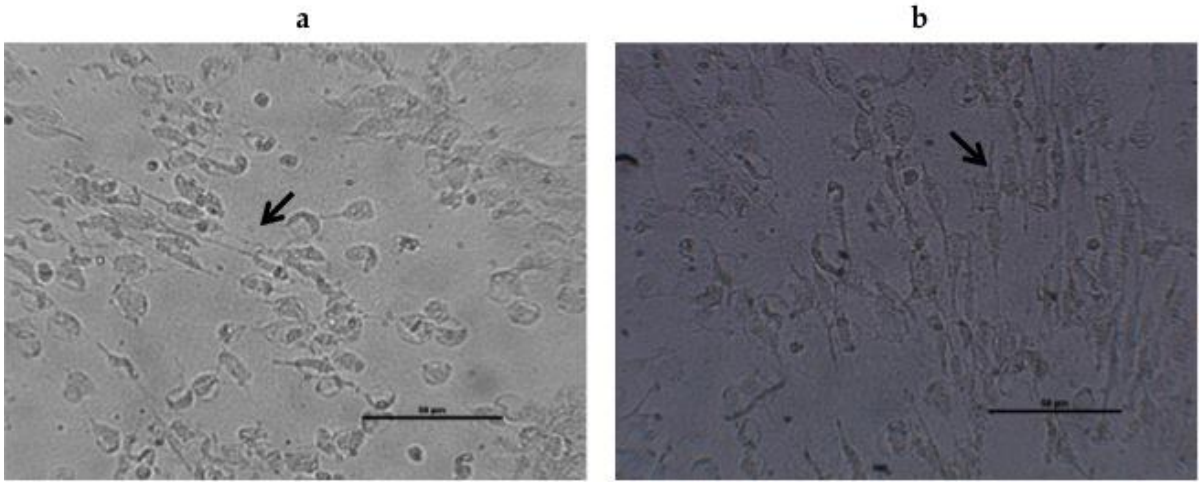

Figure 3.3. Phase-contrast microscopy of Schwann-like cells (black arrows) from young (a) and middle-aged (b) horses

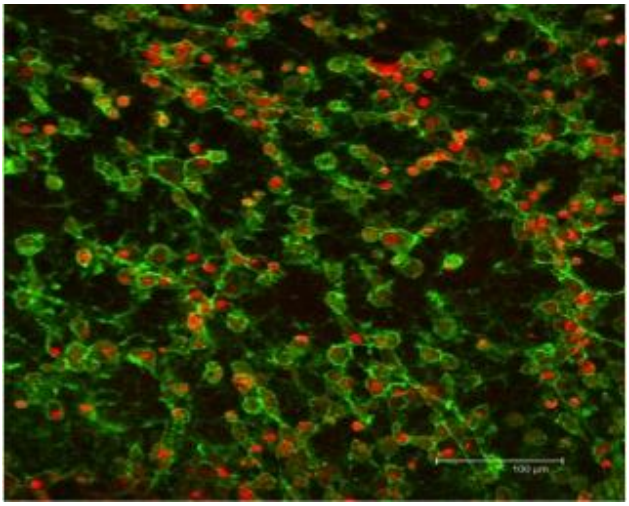

Figure 3.4. Nuclear (TO-PRO-3-iodide)/cytoplasmic (WGA) staining of equine Schwann-like cells. . Scale bar= 100 µm

Expression of Schwann cell markers. The expression of S-100b and GFAP was confirmed by IF in Schwann-like cells from young and middle-aged horses (**Figure 3.5**). Immunoblot analysis via western blot also demonstrated the expression of S-100b, GFAP and β_3 tubulin on these Schwann-like cells. As reported in our previous work (see Chapter II), undifferentiated cells generated from young and middle-aged horses also expressed GFAP, suggesting that the ability of MSCs to differentiate into other cell lineages goes beyond of mesodermal lineages. The expression of S-100b was not evident in undifferentiated MSCs (**Figure 3.6**).

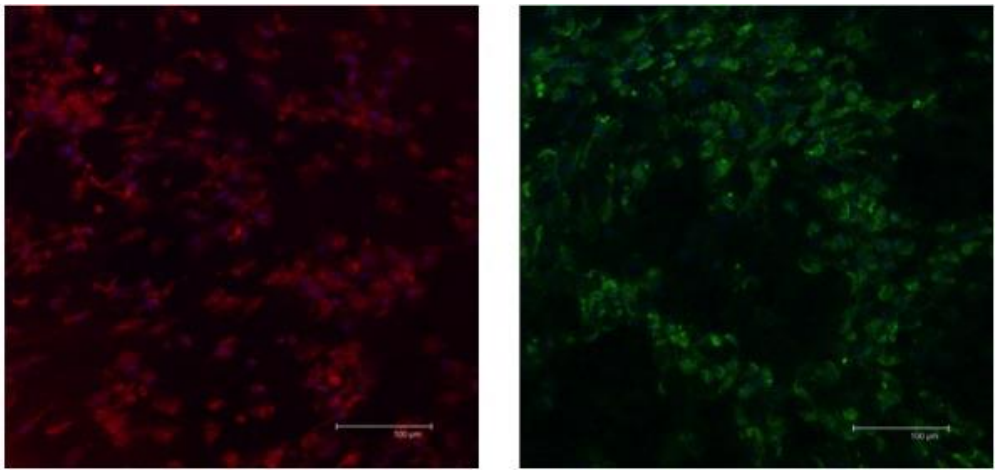

Figure 3.5. Expression of Schwann cell markers S-100b (red) and GFAP (green) in equine Schwann-like cells. Scale bar= 100 µm

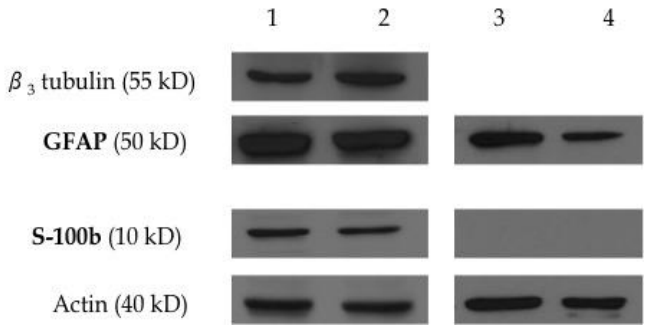

Figure 3.6. Western blot analysis of the expression of β_3 tubulin, GFAP and S-100b on Schwann-like cells from middle-aged (lane 1) and young (lane 2) horses, and undifferentiated MSCs from middle-aged (lane 3) and young (lane 4) horses

2. Peripheral nerve regeneration.

Five micrometer thick sections of Carson's fixed, paraffin embedded tissues from the sites of nerve transection and treatment, as well as adjacent segments of nerves, were examined by light microscopy. The adjacent nerves in all specimens were considered to be within normal limits (**Figure 3.7**). Both the saline and MSC treated sections in all specimens had seroma formation characterized by cleft like spaces rimmed by or partially filled by aggregates of fibrin overlain by/bordered by/ or partially infiltrated

by fibroblastic cells and macrophages (**Figure 3.8**). Both the saline and cell treated sections in all specimens had post traumatic, transactional neuroma formation characterized by haphazard streams, whorls and fascicles of small vessels, fibroblasts and Schwann cells (**Figure 3.9)**. No localized or discrete population of undifferentiated (i.e. MSC) or primitive cells (e.g. neural or Schwann cell progenitors) were recognized.

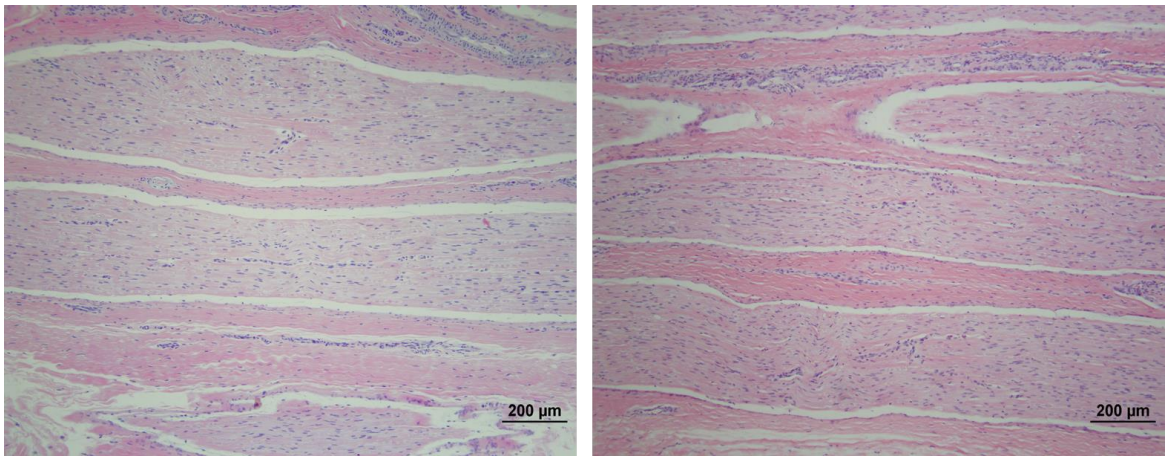

Figure 3.7. Nerves adjacent to the transection sites: saline injected (left) and MSC injected (right). Hematoxylin and eosin stain. 100X magnification

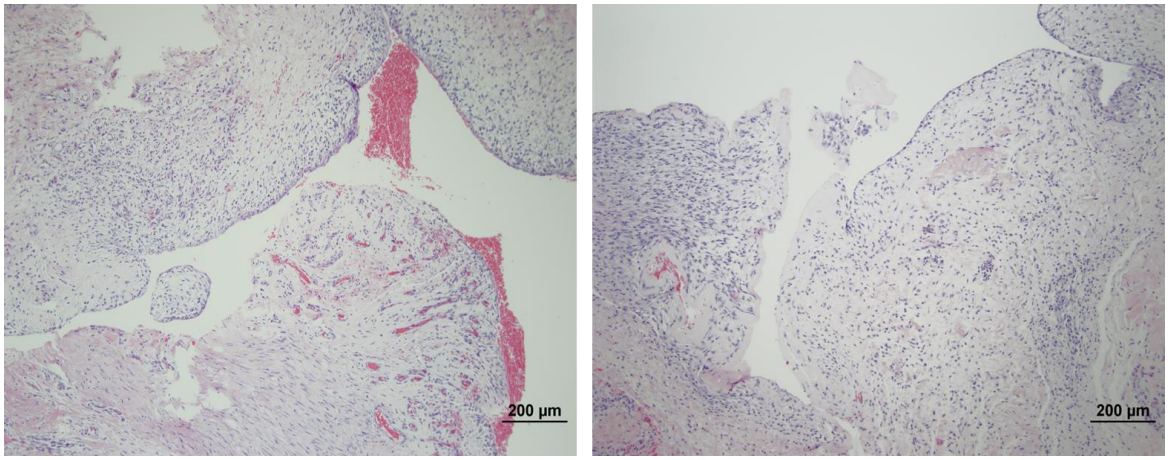

Figure 3.8. Seromas at transection sites: saline injected (left) and MSC injected (right). Hematoxylin and eosin stain. 100X magnification

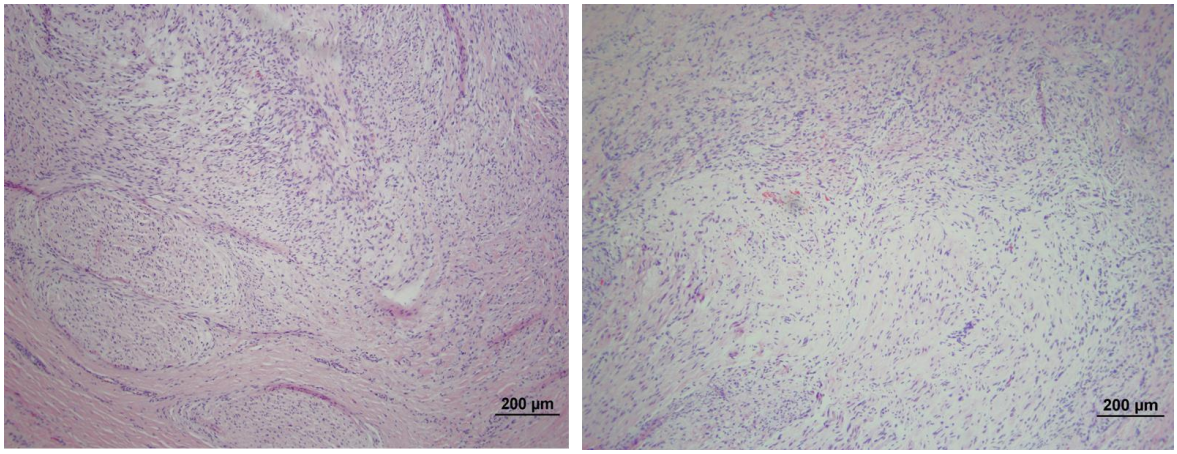

Figure 3.9. Neuromas at transection sites: saline injected (left) and MSC injected (right). Hematoxylin and eosin stain. 100X magnification

Discussion

Peripheral nerves can be injured by chemical, thermal or mechanical trauma.[19] Direct traumatism, metabolic disease, or idiopathic disease such as damage to the branch of the left recurrent laryngeal nerve (laryngeal hemiplegia) appears to be the nerve injuries most commonly reported in the horse. Treatment with anti-inflammatories and physical rehabilitation are often unrewarding. In human medicine when the nerve fibers are transected, the ideal method for repair is closing the gap by suturing the nerve endings and their surrounding fascia, or by inserting an autograft.[7] The use of autografts, however, is non-practical and can cause sequelae.[20]

The field of regenerative medicine is developing possible alternatives for nerve repair by the use of cell transplantation alone or in combination with bioengineered materials with the purpose of providing proper environmental conditions for survival and proliferation of cells that will aid on nerve repair, particularly Schwann cells.[16,21] Based on experimental studies in laboratory animals, the transplantation of MSCs after peripheral nerve injury results in regain of motor and sensory nerve functions.[11,16,22,23] Additionally, previous studies have demonstrated that MSCs are

capable of extra-mesodermal differentiation, including cells from neural lineage. Transplantation of MSCs differentiated into Schwann-like cells has revealed better results on nerve function and morphology after nerve injury.[10,11,24]

We conducted chemical induction of low passage bone marrow-derived MSCs from young and middle-aged horses to describe their plasticity into Schwann-like cells. Phase-contrast and fluorescence microscopies revealed that morphological changes were evident seven days after chemical induction in all horses. Differentiated cells were elongated, with an oval-shaped cytoplasm, and the formation of one or multiple cell processes. These cells appeared to grow in patches as compared to the flat even layer that undifferentiated MSCs produce.

Western blot and IF analyses revealed the expression of the Schwann cell markers S-100b and GFAP in Schwann-like cells. Undifferentiated MSCs also expressed GFAP, as previously reported by our group (see Chapter II, manuscript under review), but not S-100b. This suggests that bone marrow-derived MSCs are able to differentiate into extra-mesodermal lineages[25], including those from horses.

Moreover, we created a core lesion at the center of the *ramus communicans* in both fore limbs of three horses. After this procedure, cell transplantation with allogeneic MSCs was immediately performed in one of the limbs on all horses. Approximately 45 days after these procedures, the horses underwent a second surgical procedure to re-harvest the whole nerve on both fore limbs.

The *in vitro* portion of this study mainly relies on morphological and Schwann cell protein markers to describe the events occurring during differentiation of equine bone marrow-derived MSCs into Schwann-like cells. Our results agree with experiments performed in bone marrow-derived MSCs from rats and humans, in which cells are able to undergo differentiation and express typical Schwann cell markers.[3,17,24,26]

The *in vivo* portion of this study proposes a model for acute peripheral nerve injury in the horse, which has not previously been reported. Additionally, we evaluated the effects on speed and pattern of nerve regeneration after transplantation with allogeneic MSCs. Cell transplantation with MSCs differentiated into Schwann-like cells was not possible due to poor viability of the cells (25%) after detachment from the tissue culture flasks. A method for optimizing collection of equine Schwann-like cells is necessary. Furthermore, no histological differences were observed between nerves treated with MSCs and saline (controls) after transection in all horses. Additionally, there was no evidence of undifferentiated MSCs in the nerves that were treated with MSC.

There are several limitations when working with large animal models. In the present model for acute peripheral nerve injury in the horse, results are inconclusive due to the low number of horses used and due to other factors that could have been involved in nerve regeneration without the formation of seromas, such as surgical technique, anatomical region of the artificially injured nerve (the distal limb of the horse has less vasculature than other areas in the body), method of cell delivery, labeling and tracking of cells, etc. In retrospective, we consider that transecting the center of the *ramus communicans* with the horse under general anesthesia may provide a better exposure of this nerve and the opportunity to close the surrounding nerve sheath and inject the cells directly into the nerve. Likewise, closing the subcutaneous tissue may prevent the formation of seromas.

To the knowledge of the authors, this is the first report describing the morphological features and protein expression changes of equine bone marrow-derived MSCs into Schwann-like cells. We have also validated the cross-reactivity of rat Schwann cell-specific antibodies with protein samples from horse. Further studies are warranted for exploring the viability, homing, and functionality of both MSCs and Schwann-like cells after transplantation in horses with peripheral nerve injuries. Similarly, a practical and affordable method for delivering these cells is essential.

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CONCLUSION

This research focuses on evaluating and optimizing the *in vitro* conditions necessary for equine bone marrow-derived MSCs to differentiate into cells of neural lineage. It also describes the use of neural markers on equine MSCs or on protein samples derived from equine MSCs, which has not been previously reported in the literature. Subsequent work focuses on further optimization of *in vitro* conditions necessary for committing these neural cells into Schwann cells. Finally, an *in vivo* project evaluating a model of peripheral nerve transection in horses is presented for the evaluation of nerve regeneration after undifferentiated MSCs are transplanted into the lesion immediately after nerve injury.

Further research is required to investigate several aspects of peripheral nerve repair in horses, including the optimization of a method for obtaining viable equine Schwann-like cells after cultured *in vitro*, labeling of cells to track their migration after transplantation in horses, studying the effects of inserting a scaffold with MSCs after peripheral nerve injury in horses, and developing a practical and affordable method of delivery of MSCs in the horse. These studies should combine *in vitro* and *in vivo* findings for application in real cases in veterinary practice. The ultimate goal is to provide alternative methods for peripheral nerve regeneration by using cell therapy with MSCs and engineered biomaterials; consequently, improving not only the performance but also the quality of life of horses affected with peripheral nerve and, ultimately, spinal cord disorders.

VITA

Claudia Cruz Villagrán was born in Ciudad de México, México. She obtained her veterinary degree from Universidad Nacional Autónoma de México (UNAM) in 2008. She arrived to the United States on 2008 to pursue profesional development. She became a large animal Diplomate of the American College of Veterinary Internal Medicine in 2013. Her main research interest is regenerative medicine in horses.