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How to Understand Regenerative Medicine—What Is It?

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There is still much to learn about the optimal treatment paradigm in regenerative therapies including indications, technique, route, dose, timing, and frequency. In order to fully assess therapies, the treating clinician must have a solid understanding of regenerative medicine techniques. Author's address: Large Animal Surgery, Department of Large Animal Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX 77843; e-mail: awatts@cvm.tamu.edu. © 2014 AAEP.

1. Introduction

Regenerative medicine is the process of harnessing natural healing processes to improve upon tissue repair for a more functional healed tissue. The holy grail of regenerative medicine would be to recapitulate development, resulting in healed tissues that cannot be distinguished from uninjured tissue. Although to date this has not been achieved in musculoskeletal tissues, the potential to substantially improve outcomes with regenerative techniques is considerable. Subsequently, there has been much activity in research and widespread clinical use of regenerative therapies for equine orthopedic applications. Some of the tools for regenerative medicine in orthopedics include stem cells, platelet rich plasma, autologous conditioned serum, growth factors, and gene therapy. Regenerative therapies can be applied by intralesional, perilesional, intrarticular, or intravenous injections.

2. Stem Cells—Defined

Stem cells, unlike their somatic cell counterpart, are self-renewing, highly proliferative, and capable of multilineage differentiation. The ultimate stem cell is made at conception. After fertilization, the zygote consists of totipotent stem cells that are able to form all 3 germ layers as well as placental tissue. Once the zygote becomes a pre-implantation blastocyst, the inner cell mass consists of pluripotent stem cells that will give rise to all 3 germ layers: the ectoderm, mesoderm, and endoderm and can no longer form placental tissues. At this stage the stem cells are embryonic. After day eight, the cells will become either somatic cells (terminally differentiated) or stem cells committed to a specific lineage (multipotent). Subsequently, the stem cells are considered adult-derived, despite their presence in fetal tissues. Local niches of lineage committed multipotent stem cells remain in adult tissue throughout life for normal tissue remodeling and repair. With increasing age, the number, expansion potential, differentiation potential, and so-called potency of stem cells declines; therefore, there is increasing interest in allogeneic embryonic and fetal derived stem cells as well as banking of autologous stem cells from post-natal samples.

Modifications to these classifications of stem cells (embryonic and adult-derived) are also being inves-
tigated and will be briefly discussed. One modification is a fetal-derived stem cell that has been manipulated in vitro to act more like an embryonic stem cell. One such product has been developed and tested in the horse but clinical availability is pending FDA approval. An important benefit of this type of stem cell is its immediate availability as an ‘off-the-shelf’ product and its increased potency due to a pluripotent-like (embryonic-like) state.

Another modification is the induced pluripotent stem cell, where in vitro manipulations are applied to adult somatic cells, such as skin fibroblasts, to de-differentiate them and induce a stem cell-like state. The induced pluripotent stem cell is currently being investigated by several equine research groups.

Because of their broad overlap with other cell populations, mesenchymal stem cells (MSCs) cannot yet be accurately sorted by cell surface markers. Therefore, many labs select and isolate MSCs by expanding the tissue culture plastic adherent population of colony forming cells. This translates to a culture period of 2 to 3 weeks, in vitro, to isolate and expand MSCs from clinical samples for autologous therapy. In the horse, MSCs have been isolated from bone marrow, adipose, tendon, muscle, umbilical cord blood and tissue, gingiva and periodontal ligament, amniotic fluid, and blood (Figs. 1-3). The different tissue sources vary in the ease of harvest, expansion potential, and differentiation capacity. Several academic and commercial laboratories provide for the isolation, expansion, and cryopreservation of stem cells from several different tissue sources; namely, bone marrow, fat and umbilical cord, or blood. Directions for collection and shipping procedures are available from each lab. To date, bone marrow derived MSCs from both the horse and human have been the most thoroughly studied and have the most evidence for the ability to undergo chondrogenesis, tenogenesis, and osteogenesis and might contribute to cartilage, tendon, and bone repair as well as modulate inflammation and soft tissue repair within the joint.

3. Stem Cells—Autologous or Allogeneic

Autologous (self) therapy has been used most in horses to date. Autologous cells are considered safe with minimal risk for disease transmission. A major disadvantage of autologous cells is that unless cells have been banked prior to injury, their use dictates a delay of 2 to 3 weeks for isolation and expansion. Although many labs are offering banking of autologous MSCs, the long-term viability of cryopreserved MSCs has not been fully elucidated. One way to avoid the culture delay for autologous MSCs is to use patient side kits to concentrate stem cells. Several commercial kits are available that enrich for the nucleated cellular portion resulting in a higher concentration of MSCs in a small volume from bone marrow and fat. Another method to avoid delay would be to use allogeneic (nonself) cells. Because MSCs are immune privileged, allogeneic cells can be used in nonrelated individuals and without immune testing. Although this has been demonstrated in most species, it has not yet been thoroughly reported in the horse. Use of an allogeneic stem cell line would allow use of an ‘off-the-shelf’ stem cell product and would have several advantages: reduced variability, allow same day treatment, allow use of younger stem cells, and use in aged horses. Finally, it may reduce costs. However, allogeneic stem cells are considered a drug by the Food and Drug Administration and, as such, are required to undergo the same safety and efficacy
trials and manufacturing processes that are required of pharmaceuticals. Such trials are expensive and time consuming; therefore, commercial allogeneic stem cells are not yet available. In contrast, the use of autologous stem cells in veterinary patients is not currently regulated by the FDA.

4. Platelet Rich Plasma (PRP)
Following wounding, circulating platelets accumulate and become activated when exposed to a basement membrane. Activation causes platelet degranulation and release of many bioactive substances that promote healing, stimulate angiogenesis, recruit endogenous stem cells, and regulate inflammation. Specific growth factors released from activated platelets at high concentrations include platelet derived growth factor (PDGF), transforming growth factor beta (TGF-β), fibroblast growth factor (FGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), and vascular endothelial growth factor (VEGF). Platelet rich plasma (PRP) is a fraction of blood with an increased platelet concentration above baseline and is used largely for its anabolic properties. Other components of PRPs are plasma proteins dissolved in water (adhesive proteins, clotting factors, fibrinolytic factors, proteases and antiproteases, basic proteins, and membrane glycoproteins), varying concentrations of leukocytes, and sporadic erythrocytes and stem cells.

The principal advantage of PRP is that production can be performed patient-side for immediate use, relatively inexpensively. The PRP is produced through centrifugation or filtration of venous blood and can generally be accomplished within 15 min. The blood collection and preparation procedure varies from manufacturer to manufacturer and will influence the composition and volume of the product (platelet and leukocyte fold change, for example). It is likely that the varying reports of efficacy in clinical outcomes are influenced by the PRP composition and the ideal concentration of platelets and leukocytes within PRP remains undefined. Certainly, as platelet concentration increases, so does growth factor concentration. Therefore, higher platelet concentrations may be desirable. In support of this view, in vitro tendon explant data shows...
that tendon and ligament gene expression was improved with increasing platelet concentration. In the same study, increasing leukocyte concentration increased gene expression of collagen type III, the protein composition of scar tissue, which is undesirable.3

Extra doses of PRP can be stored frozen (−20 °C) for later use. It is important to note that leukocytes within the PRP will be lysed and platelets will be activated by this storage process. While some have recommended platelet activation of fresh PRP with varying additives (calcium chloride, thrombin) or by freezing, it is probably not necessary as the local environment should be sufficient to activate platelets for growth factor release. If PRP is to be used as a clot, the addition of thrombin and calcium is required. Clinical anecdotes suggest that PRP is useful for acute tendon and ligament injury when injected intralesionally under ultrasound guidance to acute to subacute lesions. Platelet rich plasma has also been utilized for arthropathies and delayed bone healing. Anecdotally, joint flares have been reported following PRP injection and may be related to the platelet to leukocyte ratio and leukocyte concentration.

5. Autologous Conditioned Serum

Autologous conditioned serum (ACS) therapy was developed to counteract the inflammatory mediator, interleukin-1, with a naturally occurring antagonist protein, interleukin-1 receptor antagonist protein (IL-1Ra; IRAP). Inhibition of interleukin-1 provides an analgesic as well as an anti-inflammatory effect and, thus, ACS is used for its anticytotoxic properties. Commercially available kits for the production of ACS are available in which blood is incubated overnight in the presence of medical-grade glass beads. This incubation leads to the de novo synthesis and release of stored endogenous substances, including IL-1Ra, by leukocytes and platelets within the blood. Twenty-four hours later, the sample is centrifuged and the supernatant (serum) is collected, sterile filtered (0.2 μm filter), and separated into several aliquots. A portion (usually about 2 ml) of the ACS is injected to the affected region and the extra doses can be stored in a freezer (−20°C). Although IL-1Ra is the target protein made in this process, there is probably a large and diverse set of factors present in ACS that makes it effective.

There has been widespread use of ACS in horses, primarily via intra-articular injection in the treatment of joint disease, osteoarthritis, or synovitis. Joint flare, or serious adverse reaction to joint injection, appears to be infrequent but has occurred. Practitioners have also used ACS for intra- and peri-lesional tendon or ligament injection. Anecdotal evidence suggests that the majority of horses that receive and respond to ACS are those that have become refractory to intra-articular corticosteroid, except on a very frequent re-injection schedule.

Timetables employed for ACS therapy vary among practitioners. Some administer each injection weekly for 3 to 4 treatments and others administer each injection monthly.

6. Growth Factors and Gene Therapy

The addition of growth factors, either directly as proteins or indirectly through gene therapy techniques to stimulate their production, has been used in several orthopedic applications. Compared to the injection of protein, which has a very short half-life, gene therapy would allow for continued expression of the transgene, increasing the duration of growth factor exposure. Members of the TGF family of growth factors and IGF have been used via proteins or gene therapy in the joint to stimulate synthesis of hyaline cartilage, improve subchondral bone architecture, and inhibit inflammatory responses, bone morphogenic protein (BMP) and gene therapy has been used in fractures and cystlike lesions to stimulate bone production, IGF protein and gene therapy has been used in tendon lesions to stimulate repair, growth-hormone-releasing hormone (GHRH) gene therapy has been used in the treatment of laminitis, and IL-1Ra gene therapy has been used in the joint to minimize inflammation.

Many methods for gene transfer are available. For satisfactory transduction efficiency and effective protein expression, the best-described gene therapy procedures involve viral vectors such as retrovirus, adeno-associated virus, adenovirus, and many others. In the horse, adeno-associated virus and adenovirus have been reported. Nonviral methods are also available but have been less studied. Of the virally-mediated gene therapy techniques, there is great variability in the genomic integration and, subsequently, the duration of transgene expression. It is unknown whether a long duration of transgene expression or permanent transgene expression would be required in orthopedic applications. Gene therapy techniques are not yet available for clinical application but may become a routine part of practice in the future.

7. Conclusion

There is still much to learn about the optimal treatment paradigm in regenerative therapies including indications, technique, route, dose, timing, and frequency. There are several factors that contribute to the lack of evidence. First, most regenerative techniques are unencumbered by federal regulations and, as such, are being employed for a variety of conditions, by differing manufacturing processes, and with differing treatment regimens. Second, autologous regenerative products have differing compositions both between patients and even within the same patient from different collections. Such widespread use of a variable product makes it increasingly difficult to make sound conclusions. Based on the anti-inflammatory effects and the ability of regenerative techniques to orchestrate tissue
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repair and regeneration via endogenous cell recruitment and trophic factors, early treatment and possibly repeated treatments may be advantageous.

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Conflict of Interest

The Author declares no conflicts of interest.

References

1. Introduction

Feet affected by chronic laminitis suffer from detachment of the dermal-epidermal interface and various patterns of pedal bone displacement. Once separation of this interface occurs, horses heal with varying degrees of stability. Healing in the chronic laminitic foot is primarily through lamellar epidermal cell hyperplasia or lamellar wedge formation. The lamellar wedge is composed of hyperplastic/dysplastic epidermal tissue with variation of tissue keratinization. The degree of stability is indirectly related to the thickness of the lamellar wedge. That is, the more hyperplastic undifferentiated thickened tissue is less stable. This tissue is often referred to as “rubbery” and does not secure the pedal bone to the hoof wall and would be considered “unstable.” The more stable laminitic foot heals back with a tighter horn to pedal bone junction with less lamellar wedge formation. Factors which are believed to affect the degree of healing are: the extent of initial lamellar insult, subsequent structural damage, and the magnitude of distractive forces placed on the lamina.

Reducing distractive forces by providing proper digital support (wedging heels, providing axial support, foot casts), and by reducing the load placed on the affected limbs (slings, weight displacement with water, recumbency) early in the course of the disease will likely contribute to the degree of healing.

In spite of the most aggressive treatment and management, many cases heal with inferior tissue quality and chronic instability. Unfortunately, it can take several months to realize the integrity of tissue that heals the damaged lamellar interface. The laminitic horse may spend months enduring painful rehabilitation, only to be euthanized because the quality of tissue regenerated is found to be inferior. These horses’ feet suffer frequent setbacks from sepsis and digital instability. This all too common scenario is extremely frustrating and costly to the owner. Mesenchymal stem cell (MSC) therapy has recently become an attractive treatment modality because of its potential to influence the quality of tissue that repairs a lesion. Mesenchymal stem cell therapy has been shown to improve...
the quality of healing in various orthopedic lesions. To date, no studies have been completed to determine the effects MSC therapy may have on healing or improvement in success rates when used adjunctively to treat laminitic cases. Stem cell activity in a foot damaged by laminitis may play a role in the type of healing. Decreased stem cell expression has been observed in lamellar tissue samples from feet with chronic laminitis. Stem cell therapy has also been shown to have an anti-inflammatory effect and, therefore, may have a temporary palliative effect on chronic laminitic cases after treatment. This study looks at laminitic cases treated with mesenchymal stem cells as an adjunctive therapy between 2010 and 2013. Success rates are compared to previously published data in cases treated (without stem cell therapy) from our practice.

2. Materials and Methods

Case Selection

Cases with laminitis categorized as uncompensated/unstable were selected to be treated with stem cell therapy. Uncompensated laminitis cases are defined as having severe damage to one or more of the growth centers (coronary corium or sole corium) of the foot. These cases have no evidence of sole and/or wall growth over time. In more acute cases, venograms show perfusion deficits, indicated by a lack of contrast material in the vasculature in weight bearing and unweighted images. Uncompensated cases often have shear lesion of the coronary band, seroma and abscess formation, and sometimes more serious deeper infections of the pedal bone.

Cases treated for chronic unstable laminitis were divided into 3 groups: acute rotation only (<30 days), sinkers, and chronic cases with advanced bone disease. Success rates for each category treated at the authors’ clinic have previously been published. Records from cases with laminitis and adjunctive treatment with mesenchymal stem cells since August 2010 were evaluated. The cases treated with MSCs were classified according to the specific type of laminitis existing in each. Cases were placed into three categories: (1) severe rotation with mild to no bone disease, (2) chronic laminitis with significant bone disease, (3) sinking. Success rates were compared to the previously published data.

Stem cell therapy was performed either by retrograde venous digital perfusion or intra-arterial injection (Fig. 1) at the level of the palmar/plantar digital vein/artery. Each patient was sedated with detomidine. Perineural anesthesia was performed at the basissesamoid level with 2% mepivicaine hydrochloride. For intravenous perfusion, surgical tubing tourniquet was placed around the metacarpal/phalangeal joint. The medial or lateral palmar/plantar digital vein site was scrubbed and prepped. A 26-g ¾ inch butterfly catheter was placed into the digital vein and the limb slowly perfused with a solution containing 20 to 30 million cells suspended in saline (Fig. 2). The foot was unweighted during at least half of the injection process. The tourniquet and perfusate was left in place for 25 to 30 minutes before removing the catheter and tourniquet. For intra-arterial injection, the medial or lateral palmar digital artery site was scrubbed and prepped. The palmar digital artery was palpated and isolated between the thumb and index finger. A 25-g ¾ inch needle was inserted into the palmar digital artery in a proximal to distal direction, once in the artery a pulsating stream of arterial blood is seen from the needle. The syringe is gently attached to the needle hub, and the stem cell dose is slowly injected.

Treated limbs were then bandaged following the procedure. Each case received an allogenic umbilical cord blood derived mesenchymal cell dose as the first treatment. Bone marrow from the sternum was harvested, cultured, and expanded for subsequent treatments. All cases were treated at monthly intervals, and the average case received 3 to 4 treatments per affected foot.

3. Results

There is a relationship between time to first treatment and success. The median time to first treatment was 71.5 days. For horses who were first treated sooner than 71.5 days, 87% (13/15) were successful. For horses whose first treatment was more than 71.5 days, 53% (8/15) were successful ($P = 0.0464$).

Although not quite statistically significant, age is also related to success. The median age of the horses was 11. For horses less than 11, 82% (14/17)
were successful. While for older horses, 50% (6/12) were successful ($P = 0.0636$).

Is it age or time to first treatment that impacts success? It’s both. For horses that are older than 11 and treatment after more than 71.5 days, only 33% (3/9) were successful, while for all other horses 86% (18/21) were successful ($P = 0.0041$)

HLZ improvement was defined as the average HLZ measurement at time 1 minus the average HLZ at the last treatment.

For horses that had time to first treatment more than 90 days, 17% (2/12) had improved HLZ, while for horses with first treatment up to 90 days, 69% (11/16) improved ($P = 0.0062$).

Among the variables investigated, only severe bone disease was related to improved HLZ. For horses with severe bone disease, 0% (0/6) had improved HLZ, but 59% (13/22) of others had improved HLZ. The authors think that this result would be expected.

It might be interesting to point out that HLZ improvement does not appear to be related to age. The time from onset of laminitis to first injection of stem cells was as follows: (<30 days, 10 cases; 30–60 days, 4 cases; 60–90 days, 2 cases; >90 days, 14 cases). Each case received between 1 and 4 doses of 20 to 30 million cells per foot. Of the 30 cases, 21 (70%) were successful. Deep digital flexor tenotomy (DDFT) was performed on at least one of the affected limbs in 15 (50%) of the cases. Of those cases receiving stem cells <30 days from onset of laminitis, 100% were successful. Cases receiving stem cells >90 days from onset of laminitis showed a 50% success rate (Figs. 3 and 4).

4. Discussion

Umbilical cord blood derived MSC treatment has been shown to be safe with no adverse reactions when injected intradermally in horses. This allows for a readily available dose to be injected.
Intra-arterial and venous regional limb perfusion has been shown to be an effective method of delivering MSC to the digit. Intra-arterial perfusion has been shown to result in a more reliable distribution to the foot. The venous route was used in most of these cases. It is currently not known when the ideal timing of the first dose should be instituted. Reason would justify that for the benefits during the repair phase of the disease, stem therapy would be most useful before lamellar wedge or epidermal cell hyperplasia occurs. This has been shown to take place within the first 30 days of pedal bone displacement. In chronic advanced cases with bone disease and lamellar wedge formation, stem cell therapy had no effect on prognosis. These cases still maintained a thick lamellar wedge, which did not change in appearance on radiographs. In one case, with bone disease and a thick lamellar wedge, the dorsal hoof wall and lamellar wedge was resected prior to stem cell therapy. This was done to strip the unstable tissue in hopes of reseeding the interface with more patent tissue. This case continued to suffer from abscesses, instability, and continued discomfort and was euthanized. There is an intricate relationship between the health of the parietal surface of the pedal bone and the epidermal lamellae, lamellar, and sublamellar dermis. This microenvironment has an effect on the regenerative

Fig. 3. Thoroughbred mare with bilateral sinking and rotation. Pre (A) and post (B) treatment with stem cells, tenotomy, and shoeing.

Fig. 4. Another horse before (A) and 8 months after (B) treatment with stem cells, tenotomy, and shoeing.
potential of the lamellar interface. Simply stated, for good quality tissue to be produced and maintained requires a healthy osseous foundation. Stem cell therapy had a significant effect on prognosis in cases treated early, within 30 days. The majority of these cases healed with a decreased distance between the bone and hoof wall (see Figs. 3 and 4). Studies are currently underway to evaluate lamellar biopsy samples from cases treated with MSCs to further evaluate the quality of tissue which repairs this interface.

Acknowledgments

Conflict of Interest

The Authors declare no conflicts of interest.

References

How to Select Cases and Use Platelet Rich Plasma for Tendon and Joint Injuries

Lisa A. Fortier, DVM, PhD, DACVS

1. Introduction
Regenerative medicine is a commonly used term that broadly applies to the use of biologics to enhance tissue repair. In the context of equine medicine, regenerative therapies are more commonly used to enhance the quality of repair in musculoskeletal tissues following injury. The most commonly used biologics include platelet rich plasma (PRP), stem cells, and interleukin-1 receptor antagonist protein (IRAP). The popularity of biologics is increasing, with owners looking for more “natural” therapies and their reluctance to treat their horses with drugs such as corticosteroids. Traditionally, PRP was used for tendons and ligaments, IRAP for joint injections, and stem cells for either soft tissue or joint injections. However, there is evidence for use of PRP for tendon or joint injuries. It should be remembered that all biologics are heterogeneous in their final composition because they are based in biology, not chemistry. Biologics are not drugs and there are many subtypes of each biologic. For example, PRP preparations vary considerably in the concentration of platelets and leukocytes. There is tremendous controversy over which type of biologic (PRP, stem cell, or IRAP) is best for a specific tissue or injury and then what specific type of PRP, etc, is best for a given application. Current evidence suggests that leukocyte-low PRP preparations with platelet concentrations 2 to 4-fold over baseline are optimal for increased tissue repair. There are no head-to-head comparison studies to answer those questions, thus some extrapolation between studies is required to formulate a recommendation for clients. Recent reviews on regenerative medicine in horses are cited in the following text for further reading.¹–⁶

2. Methods and Results
PRP for Joint Pain
There is evidence that PRP can increase hyaluronic acid (HA) synthesis by synoviocytes and decrease joint pain and inflammation, which should help enhance early rehabilitation.¹ Platelet rich plasma is not just for tendons and can be considered as a first line of joint therapy. Case selection is those horses with mild to moderate joint disease. Many practitioners use PRP as a postoperative adjunctive therapy following arthroscopic chip removal or fracture repair. The first injection is given 3 weeks postoperatively with a second injection 2 weeks later.
be by centrifugation of blood or by gravity filtration using a bag system. Approximately 2 to 4 mL/fetlock or carpus or 5 to 8 mL/stifle is injected twice at 10 to 14 day intervals. Based on evidence in the human literature, a third injection is not necessary. In long-term outcome data regarding the use of PRP for treatment of human knee arthritis, PRP is as, or more, effective as HA in restoring function and decreasing pain, but the effect lasts longer. The effects of HA typically last 6 months, while the effects of PRP are reported to last for a year or longer.

No activation step such as thrombin is necessary. When platelets in PRP are exposed to joint tissue, they are activated and degranulate, thereby releasing their growth factor contents. Residual PRP can be frozen and used at a later date without loss of growth factor activity (unpublished data). There is no need for rest after a PRP injection. One of the major benefits of intra-articular PRP injections is diminished pain signaling, which should allow for aggressive rehabilitation in cases of mild to moderate arthritis. If there is no response within a week after a second injection, it is unlikely that a third injection will help. If there is a response, then routine joint maintenance (with 2 injections, as previously described), or treatment as needed can be performed, according to your practice philosophy. There are no published “flare rates” following PRP injections, but they have been anecdotally reported.

PRP for Tendon or Ligament Injury
Platelet rich plasma injections into tendons and ligaments increases normal tendon matrix synthesis, such as collagen type I, and decreases cytokines associated with matrix degradation. If PRP is administered into a lesion within a few days of injury, it can stop the progression and growth of a lesion. The effectiveness of PRP for tendonitis appears to be related to the chronicity and severity of the lesion. Therapeutic intervention with PRP should be considered as soon as a lesion is diagnosed, not after weeks or months of failed rehabilitation. Once scar tissue has formed and remodeled, the regenerative capacity of tissues is limited. This time frame for successful intervention depends on the site and severity of the injury, but is likely limited to those injuries less than 3 to 6 months old.

Ultrasound guidance should be performed for injections, so two sets of hands, one to run the ultrasound and one to do the injection, is ideal. A convex probe is best for ultrasound guided injections. A sterile probe cover should be used. The volume of PRP for injection is simply enough to fill the lesion, which can be visualized under ultrasonography. A small 23 gauge needle can be used to minimize damage to normal tendon during injection.

After PRP treatment, the horse resumes active rest with a minimum of 30 min in hand walking per day. A recheck examination in 2 to 3 weeks and a second injection can be considered if the lesion has not significantly improved with a 50% improvement in palpation, lameness, and ultrasound appearance of the lesion. Platelet rich plasma injections into bowed tendons appear to result in a more rapid resolution of lameness and early return to linear fiber orientation in the injured region. It is unclear if the ultimate time to return to performance is diminished by the injection of PRP.

3. Discussion
For tendon and joint injections, PRP appears to both enhance tissue repair and to decrease pain. The diminished pain allows for a faster return to rehabilitation, which is particularly important in middle-aged horses that are prone to secondary lameness issues with extended rest. The use of PRP is an attractive method of therapy because of the growth factors that are delivered, but it must be remembered that none of the biologics are drugs and, therefore, each preparation will vary, as will the clinical outcome. It is unclear which biologic is best for any given situation, but given the devastating loss of time and performance associated with musculoskeletal injuries in horses, a dual PRP/stem cell approach is commonly employed. Regenerative therapies should be applied early after injury, within a week if possible, so that they can change the healing environment before scar tissue begins to form.

Acknowledgments

Conflict of Interest
The Author is a consultant to Arthrex, Inc.

References
How to Choose Cases and Utilize Mesenchymal Stem Cells in Joint Injury

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1. Introduction
The use of “stem cells” for treating orthopedic disease is rapidly growing. We are currently experiencing an exponential growth in the number of peer reviewed and Medline indexed publications (Fig. 1). This means that the equine practitioner is faced with greater information to help in the best selection of cases in which to utilize stem cells. This does not mean that unproven sources (i.e., commercial sources) or indications of mesenchymal stem cells (MSCs) does not exist but this manuscript will provide the author’s opinion on current available information and case selection as it pertains to stem cell use for joint disease.

2. Treating With MSC: Points to Consider
The first point of consideration is the tissue source of stem cells. Multiple studies have been published comparing various tissue sources of mesenchymal stem cells (MSCs) and current thought suggests bone marrow-derived cells provide the best source for joint-related treatments.¹ Specific locations and volumes of aspirates have also been assessed and a low volume (5 mL) aspirate from the ilium has been shown to have significantly better results as it relates to cartilage matrix production.² This is not to say that bone marrow draws from the sternum do not produce usable MSCs, it is more to say that the ilium draws produce cells which may be more desirable. Specific studies assessing the dose of MSCs to be used in joint tissues have not been published; however, the range of doses being used in studies

Fig. 1. A frequency table by publication year from a PubMed search on February 11, 2014 using the key words “stem cell” and “joint.”
with successful outcomes is 10 to 50 million cells in roughly a 10 to 50 mL joint. A recent clinical study in human patients with medial meniscectomy suggested 50 million MSCs performed as good, if not better, long-term compared to 150 million; this agrees with the author’s clinical experience as well. The timing of the treatment, as well as the use of multiple treatments, has also not yet been definitively worked out. The best evidence exists in a study looking at MSC treatment/timing in tendon injury. This work demonstrated significantly better long-term outcomes by delaying MSC treatment past the inflammatory phase of the injury. This is also consistent with the author’s experience in clinical cases. Finally, most of the published reports in treating joint related injuries have, to date, utilized a single treatment; thus, compelling clinical results to pursue multiple treatments is lacking.

3. Case Selection

In joint disease there are four main tissue types that become injured: (1) the articular cartilage, (2) fibrous joint capsule/synovial membrane, (3) soft tissue structures such as the meniscus or collateral ligaments, and (4) subchondral bone. In many cases of joint disease, some or all of these tissues are involved to some extent and the severity and extent are what should dictate the treatment protocol. Currently, in cases with generalized osteoarthritis and no or minimal soft tissue involvement, the MSC treatment is not the primary mode of therapy utilized by the author. Rather, other types of medical treatments such as corticosteroids, hyaluronic acid, polysulfated glycosaminoglycans, or biologics such as interleukin-1 receptor antagonist are the first line of treatment. However, in cases that are refractory to such treatments and no surgical options exist, there is evidence of significant beneficial effects of administration of intra-articular (IA) stem cells free in the joint space. This benefit is mainly through a reduction in inflammatory mediators such as prostaglandin E2 but this can have a significant long-term effect on overall joint health and the author has had palliative success.

In cases where the primary issue is cartilage loss, i.e., a focal cartilage lesion, numerous studies have shown the lack of effectiveness of MSCs contained in a scaffold or fibrin gel for direct repair of an articular cartilage defect. Conversely, administration of IA MSCs free within the joint following a cartilage resurfacing technique, such as subchondral bone microfracture, has been shown to provide added long-term benefit over cartilage resurfacing alone. This combination of surgical debridement followed by MSC therapy 30+ days postoperatively is utilized by the author in cases with cartilage lesions.

In cases where soft tissues within the joint cavity are injured, published studies have shown a significant improvement in soft tissue healing as well as a decrease in osteoarthritis progression following the utilization of MSCs free within the joint. Specifically, building on experiential studies a recent double blind randomized placebo-controlled study in human patients demonstrated long-term significant benefit following administration of 50 million bone derived culture expanded stem cells post medial meniscectomy. These significant improvements were not only in the regeneration of meniscus but also in pain for the patient two years post-treatment. A recent equine study has been published with long-term follow up of horses undergoing stifle surgery that were diagnosed with meniscal disease. Surgical debridement was performed followed by treatment with IA bone derived MSCs post-surgery. The study demonstrated an improved ability of these horses to return to performance when compared to similar published cases undergoing surgery alone.

Cases with significant subchondral bone disease have been treated using a regional perfusion of MSCs but published scientific evidence of efficacy is currently not available. Thus, the author reserves MSC therapy as a last resort in such cases. In these cases the vein supplying the area is aseptically prepared with a tourniquet above and/or below, depending on the region. The author uses 10 to 20 million MSC diluted in a 20 mL volume of a buffered polyionic solution. The horses are typically sedated and the tourniquet left in place for 20 to 30 min.

4. Summary

Strong evidence exists for the use of bone-derived culture expanded stem cells in cases with cartilage or other soft tissue lesions within the joint in various species. Similar evidence has been reported specific to the horse. The dose typically administered after the inflammatory phase has subsided (which for the author is 4–6 weeks postinjury or surgery) is a one-time treatment (usually in the presence of a 20 mg dose of hyaluronan). The dose is in the range of 20 to 50 million cells for 10 to 50 mL joints with a nonsteroidal antiinflammatory drug administered parenterally at the time of injection. The dose is administered free in the joint, i.e., not within a restrictive scaffold, and other routine surgical options can be employed in addition to the stem cell treatment. The author avoids the use of corticosteroid post IA MSC injection for 6 months or greater but the use of hyaluronic acid-based products are encouraged. Routine rehabilitation protocols are utilized in most cases but typically include 4 months out of full work or competition. In general, hand walking commences within days of the MSC treatment and continues for 4 to 6 weeks with pony-exercise at a trot beginning for an additional 4 to 6 weeks. This is followed by work with a rider increasing intensity and duration of exercise over the next 4 to 6 weeks.
Acknowledgments

Conflict of Interest

The Author is a shareholder of Advance Regenerative Therapies, Fort Collins, CO.

References and Footnote


*Frisbie DD, unpublished data, February 2014.
How to Select Cases and Utilize Stem Cells in Tendon Injury

Roger K.W. Smith, MA, VetMB, PhD, DEO, FHEA, DECVS, MRCVS

1. Introduction
The use of stem cells for the treatment of tendon injuries has gained acceptance over the past decade. While there has been no randomized controlled trials for the use of mesenchymal stem cells (MSCs) in the treatment of tendon overstrain injuries, an increasing amount of experimental1–3 and clinical data4 have mostly demonstrated improved quality of equine tendon healing with cellular treatments.

2. Case Selection
The cells are most commonly delivered by intratendinous injection and therefore require a defect within the tendon that is surrounded by an intact paratenon to retain the cells after implantation. The size of the lesion is not critical, although small lesions are more difficult to inject and generally carry a better prognosis and therefore cellular therapies are less indicated for the smaller lesions. Cell retention is very poor if the paratenon is not intact (unpublished data). Hence, percutaneous lesions, where this is not the case, are not appropriate for treatment unless at the stage when a granulation bed is present that can act as this retaining layer.

Analysis of the clinical data shows improved outcome with earlier implantation although not always significantly different statistically. For racehorses, there was an average of 46 days between injury and implantation for successful cases compared to 54 days for cases that subsequently re-injured4; for sports horses, the re-injury rate was lower for horses treated within 1 month of the injury compared to those treated after a month (unpublished data). Autologous treatments are a two-step procedure requiring first the aspiration of bone marrow (or other tissue) to obtain the MSCs and second, after a variable time depending on the preparation used, to implant the cells. In the intervening time, the horse is either maintained on box-rest (if lame or severely injured) or given up to 10 minutes hand-walking per day. For bone marrow, a period of approximately 3 weeks for culture is needed, which limits any earlier implantation, and so bone marrow should be aspirated as soon as is possible after injury. The use of banked or allogenic cells allows even earlier implantation although it is logical to target the cell implantation to when there is a vascular supply established to support the cells after implantation—normally at least 1 to 2 weeks after injury.

3. Cell Choice
There are a number of options with respect to cell choice. The most commonly used preparations are
MSCs recovered from bone marrow or fat, with more recent additions of umbilical cord cells and fetal tendon cells. The major differences between these cell products are more related to the preparation methods rather than the source. Of the autologous treatments, cells can either be 'minimally manipulated,' when cells are released from tissue by enzymes and a cell mixture returned to the veterinarian for implantation (often the case when fat is chosen as the tissue source), or MSC populations are expanded by culture in a laboratory. Culture and expansion is necessary if it is deemed important to produce an abundant and homogenous stem cell preparation because of the low numbers of MSCs in the source material (e.g., only 1 in 100,000 of the nucleated cells in bone marrow is an MSC). Allogenic cells simplify the process and may become more commonly used, providing legislation allows, as it avoids the first step and is easier and cheaper. Allogenic cells do not appear to cause immunological reactions because of the immunosuppressive effects and appear to be well tolerated (unpublished data). However, such products have not been extensively researched or well characterized to date to ensure quality and effectiveness and, therefore, should be used with caution.

4. Dose

There have been no good studies documenting a dose response. Initially, 10 million cells (suspended in bone marrow supernatant at a concentration of 5 million cells/mL) were used for an ‘average-sized’ lesion occupying up to 30% of the cross-sectional area of the superficial digital flexor tendon. More recent studies have shown that there is limited retention of cells after implantation and an analysis of treated horses showed a reduced re-injury rate in horses treated with 20 million or more cells when compared to the original empirical doses of 10 million cells/lesion. Therefore this ‘routine’ dose has been increased to 20 million cells (at the same concentration of 5–10 million cells/mL) with the option to increase this twofold for larger lesions.

Step 1—How to Obtain a Bone Marrow Aspirate

**Equipment Needed**
- Ultrasound machine with 7.5 MHz or above linear transducer
- Sedative
- 2 mL syringe (for local analgesia)
- Local anaesthetic (mepivicaine)
- Heparin (stock solution concentration: 5000 IU/mL)
- 11 scalpel blade
- 11 G 10 cm Jamshidi needle
- Two 10 mL or one 20 mL syringe
- At least two 5 mL syringes
- Sterile swabs
- Sterile containers and transport pack (to ship the bone marrow to the laboratory for culture).

**Technique: Sternum**

As the bone marrow has to be transported to the laboratory after it is obtained, aspirations should only be done on a Monday to Thursday to avoid prolonged delivery over weekends.

The horse is first sedated with a combination of an α2 agonist and opioid (e.g., detomidine HCl and butorphanol tartrate). A 10 cm wide band overlying the sternum is clipped and scrubbed with surgical scrub (e.g., chlorhexidine) and surgical spirit.

While some don’t advocate ultrasound, ultrasound is advised to ensure accurate aspiration and therefore minimize the risks of inadvertent penetration of the heart. The sternum is examined ultrasonographically to identify the three most caudal sternebrae by the appearance of their intersternebral spaces (Fig. 1). The most caudal one is avoided as it is thinner and hence penetration of its deeper surface is easier.

![Image 1](https://via.placeholder.com/150)

**Fig. 1.** A, longitudinal section of the equine sternum (caudal to the left). Usually the two most caudal sternebrae (6 and 7) are fused and the first intersternebral space encountered when scanning from caudal to cranial is between sternebrae 5 and 6 (arrow). The easiest sternebra to aspirate is the 5th. Either the 4th (further cranial between the forelimbs but complicated by the presence of the ventral ‘keel’) or 6th (more caudal but with less dorsoventral thickness so care should be taken to avoid inserting the Jamshidi needle too deep) can also be aspirated. Care should be taken to avoid the caudal border of the sternum, which is thin and close to the apex of the heart. B, the ultrasonographic appearance of the intersternebral space between sternebrae 5 and 6. This space is usually level to the caudal aspect of the elbow.
The position of the most caudal aspect of the sternum and the intersternebral spaces between 5 and 6 and 4 and 5 are marked on the adjacent hair with a marker pen (or white correction fluid for a dark-coated horse; Fig 2). As a double check, the space between 5 and 6 is usually level with the caudal aspect of the forearm.

The area of the sternum is prepared aseptically and local anesthetic is placed at the predicted aspiration entry points to aspirate bone marrow from sternebrae 5 and 4 (or 6), as shown in Fig. 3.

Prior to aspiration, syringes are aseptically pre-loaded with heparin (to give a final concentration of 250 IU/mL bone marrow). Commonly, a 10 mL syringe is preloaded with 0.5 mL of a 5000 IU/mL heparin solution into which 9.5 mL bone marrow is aspirated.

The area is then scrubbed a final time before a small stab incision is made through the skin with a No. 11 scalpel blade. The Jamshidi needle is introduced through the stab incision and advanced until it contacts with the ventral surface of the sternebra on mid-line (Fig. 3A). The index finger is placed 2 cm from the skin surface on the needle shaft and the needle is gradually advanced using rotating movements until the index finger is against the skin surface. This ensures that the needle does not penetrate the deep surface of the sternebrae.

The central trocar is removed from the Jamshidi needle. Typically, bone marrow does not initially flow spontaneously from the needle but requires gentle aspiration with an attached syringe. This can be, but only initially, associated with a small amount of discomfort to the horse, usually manifested by a slight guarding of the abdomen. Thereafter, bone marrow flows easily into the syringe (Fig. 3B) and it spontaneously drains from the needle when the needle is disconnected.

At least two more 4 mL samples are also obtained without heparin and transferred immediately to sodium citrate glass blood tubes. Care should be taken when transferring bone marrow to citrated tubes or universal containers to avoid the possibility of contamination. These samples are used to derive the bone marrow supernatant used to resuspend mesenchymal stem cells for implantation. The tubes should be vigorously agitated to prevent clotting, with thumbs over the lids. For very large lesions (over 30% cross-sectional area) more than two sodium citrate tubes should be submitted (two more per 10 million additional cells required). Alternatively, a jugular blood sample can be substituted for the bone marrow for later resuspension of the cells.

The second sternebra is then aspirated in the same fashion (or a second insertion of the Jamshidi needle into a separate site in the same sternebra to provide a back-up sample for culture in case of clotting or failed cell recovery).

Once the Jamshidi needle is withdrawn, the portals can continue to bleed but pressure is usually all that is necessary to stop this hemorrhage. Closure is unnecessary.

**Technique: Tuber Coxae**

A 10 cm square area overlying the tuber coxae is clipped. In an average-sized horse the aspiration

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**Fig. 2.** Marking the intersternebral space between the two sternebrae to be aspirated with a marker pen.

**Fig. 3.** A, insertion of the Jamshidi needle. The index finger should be placed 2 cm from the skin surface once the Jamshidi needle makes contact with the ventral surface of the bone to prevent overinsertion of the needle. Note that the entry point is close to the caudal aspect of the forearm. B, successful aspiration of bone marrow.
site is a point 2 cm caudal to the ridge running from craniodorsal to caudoventral and approximately one quarter to one half dorsoventrally.

The area is prepared aseptically and 5 mL local anesthetic is placed subcutaneously along the aspiration path and deeper over the periosteum.

Prior to aspiration, the 20 mL syringe is preloaded with 1 mL of 5000 IU/mL heparin. A larger syringe can be used to allow greater negative pressure during aspiration (required for tuber coxae aspirations compared to sternum).

The area is scrubbed a final time before a small stab incision is made through the skin at the aspiration site with a No. 11 scalpel blade.

The Jamshidi needle is introduced (horizontally) perpendicular to the skin through the stab incision and advanced until it contacts the surface of the tuber coxae. The needle is then gradually advanced approximately 5 cm into the bone using rotating movements (Fig. 4). The needle can alternatively be introduced more caudally to enter the wing of the ilium 2 to 3 cm from the tip of the tuber coxae. The latter technique offers the advantage of avoiding the dense outer cortex of the tuber coxae, which can be difficult to penetrate with the Jamshidi needle.

The central trocar is removed from the Jamshidi needle. Bone marrow does not initially flow spontaneously from the needle; aspiration with an attached syringe is required. The preloaded syringe is attached to the Jamshidi needle and full negative pressure is applied in order to aspirate 19 mL of bone marrow (making a total volume of 20 mL).

The bone marrow sample is gently agitated in the syringe to ensure adequate mixing of the anticoagulant with the marrow (bone marrow clots extremely quickly). The sample is then transferred into the two universal plastic containers provided, divided into two 10 mL aliquots. Continue to gently mix the samples once in these containers.

An additional sample, without heparin, is then taken and transferred immediately to two or more sodium citrate glass blood tubes (4 mL bone marrow into each tube).

Once the Jamshidi needle is withdrawn, the portal can continue to bleed but pressure is usually all that needed to stop the hemorrhage. Closure is unnecessary.

Step 2–How to Implant Stem Cells Into Injured Tendons or Ligaments

Equipment Needed

- Ultrasound machine with 7.5 MHz or above linear transducer
- One sterile drape
- Two 2 mL syringes and 20 G or 19 G, 1.5, or 2 inch needle (for stem cells)
- Four 2 mL syringes and 23 G 1 inch needles (for local analgesia)
- One arthroscopic camera sleeve (or sterile ultrasound probe sleeve)
- One intrasite gel (or sterile ultrasound gel)
- Robert Jones bandage material
- Local anesthetic (mepivicaine)
- Sedative

Technique

The cell delivery time and place should first be arranged. It is important that the cells are implanted as soon as possible after arrival; therefore, implantation will normally need to be carried out in the
morning and only on a Tuesday to Friday (to avoid delayed transit of the cells over a weekend).

The horse is restrained in stocks and sedated with a combination of α2 agonist and opiate (e.g., detomidine HCl and butorphanol tartrate).

The leg to be implanted is clipped to include the subcarpal local anesthetic sites. The area is then cleaned with surgical scrub (e.g., chlorhexidine) and surgical spirit (alcohol).

An ultrasonographic examination is used to identify the core lesion, its extent, and the appropriate site for stem cell implantation.

The site for local analgesia is aseptically prepared. To ensure complete desensitization of the skin overlying the superficial digital flexor tendon (containing the lesion to be treated), both the palmar nerves deep to the metacarpal fascia and the subcutaneous nerve supply superficial to the fascia have to be ‘blocked’ on both sides of the limb at the subcarpal site. If the accessory ligament of the deep digital flexor tendon or suspensory ligament is being treated, the palmar metacarpal nerves should also be ‘blocked.’ A uniaxial suspensory branch injury would necessitate only uniaxial blocks.

The palmar metacarpal region should then be prepared aseptically.

In a sterile fashion, at least two separate syringes are loaded with stem cell aliquots. The cells are resuspended in the supernatant within the transport vial by gently shaking the vial prior to aspirating its contents into the 2 mL syringes. Usually, only 2 mL (10 million cells/mL) is injected into an superficial digital flexor tendon (SDFT) lesion—larger volumes can be damaging to the tendon—although larger volumes with the same concentration of cells can be used to deliver higher doses (up to 40 million cells) for very large lesions.

The ultrasound transducer is placed into a sterile sleeve (a sterile arthroscopic camera sleeve with the end sealed can be used for this purpose). Contact between the transducer and the skin is optimized with the use of scanning gel within the sleeve and intrasite gel or sterile ultrasound gel, or alcohol on the skin.

With the ultrasound transducer oriented in longitudinal fashion, triangulation of a 20 G 1.5 or 2 inch needle is visualized entering the tendon (Fig. 5) in the weight-bearing limb. Care should be taken to ensure that the whole needle is visible ultrasonographically so that the end does not penetrate the deep surface of the tendon.

Fig. 5. Localizing needle in a core lesion. A, triangulation technique with the needle and ultrasound transducer in line on the palmar aspect of the limb, and B, ultrasound appearance of the needle as it is advanced into the tendon (arrow).

Fig. 6. After injection, air bubbles from the injection are present with the core lesion in both transverse, A and longitudinal, B images.
The stem cells are then injected into 1 to 5 sites depending on the nature of the core lesion. The first site of injection is usually the midpoint of the lesion as there is often good spread of the cells throughout the lesion after implantation when the core lesion is still very hypoechoic (e.g., within 2 months of injury). More advanced healing (e.g., when the core lesion has filled with echogenic tissue—usually around 3 months post injury) requires more injection sites due to less spread. Accurate placement is confirmed by the presence of air bubbles within (and only within) the core lesion (Fig. 6), which will also indicate the degree of spread.

The limb should be bandaged immediately with a Robert Jones style bandage to minimize subcutaneous hemorrhage and loss of injected cells from the tendon. The bandage should be left in place for 5 to 7 days. Thereafter, stable bandages can be used to maintain the temperature of the distal limb (to maximize healing).

Peri-operative antibiotics and nonsteroidal anti-inflammatory drugs are unnecessary.

Repeated delivery of cells is a logical approach to obviate the relatively poor retention of cells after implantation although, often, expense precludes this approach. Some have advocated initial early treatment with allogenic cells (legislation allowing) while others propose a second autologous dose given by regional perfusion 2 to 4 weeks after the first dose. Regional limb perfusions seem to be a less efficient delivery route, but do avoid repeated injections into an injured tendon, which can potentially cause further damage.

After implantation, the horse commences a rehabilitation program, which is not specific for stem cell treatment (Table 1). There is no current evidence that stem cell treatment shortens the rehabilitation phase, which is consistent with the current hypothesized mechanism of action of improving tendon healing by modulating the inflammatory response. This programme is approximately 12 months in length for superficial and deep digital flexor tendon injury (Table 1) but shortened for suspensory desmitis (to 6–9 months) and desmitis of the accessory ligament of the deep digital flexor tendon (to 3–6 months).

**Acknowledgments**

**Conflict of Interest**

The Author was formerly a Director and Technical Advisor of VetCell Bioscience, Ltd.

**References**

4. Godwin EE, Young NJ, Dudhia J, et al. Implantation of bone marrow-derived mesenchymal stem cells demonstrates

### Table 1. Recommended Standardised Rehabilitation Programme for Stem Cell Treatment of a Superficial Digital Flexor Tendon Injury

<table>
<thead>
<tr>
<th>Exercise level</th>
<th>Week</th>
<th>Duration and nature of exercise</th>
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<tbody>
<tr>
<td>Box rest</td>
<td>0</td>
<td>Implant cells</td>
</tr>
<tr>
<td>Walk</td>
<td>1</td>
<td>Box rest; maintain clinical bandage</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>10 minutes walking; replace clinical bandage with stable bandage; maintain stable bandaging</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>15 minutes walking; maintain stable bandaging</td>
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<tr>
<td></td>
<td>4</td>
<td>20 minutes walking; maintain stable bandaging; Repeat ultrasound examination</td>
</tr>
<tr>
<td></td>
<td>5–6</td>
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<td></td>
<td>7–8</td>
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<td>35 minutes walking</td>
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<td></td>
<td>11–12</td>
<td>40 minutes walking</td>
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<tr>
<td>Trot</td>
<td>12</td>
<td>Repeat ultrasound examination</td>
</tr>
<tr>
<td></td>
<td>13–16</td>
<td>35 minutes walking and 5 minutes trotting daily</td>
</tr>
<tr>
<td></td>
<td>17–20</td>
<td>30 minutes walking and 10 minutes trotting daily</td>
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<tr>
<td></td>
<td>21–24</td>
<td>25 minutes walking and 15 minutes trotting daily</td>
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<tr>
<td></td>
<td>24</td>
<td>Repeat ultrasound examination</td>
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<tr>
<td></td>
<td>25–28</td>
<td>20 minutes walking and 20 minutes trotting daily</td>
</tr>
<tr>
<td></td>
<td>29–32</td>
<td>15 minutes walking and 25 minutes trotting daily</td>
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<tr>
<td></td>
<td>32</td>
<td>Repeat ultrasound examination</td>
</tr>
<tr>
<td>Canter</td>
<td>33–48</td>
<td>Introduction of canter work; gradual return to full work</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>Repeat ultrasound examination</td>
</tr>
<tr>
<td>Full work</td>
<td>48+</td>
<td>Treat as normal</td>
</tr>
</tbody>
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<th>Exercise level</th>
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How to Select Cases and Use Autologous Conditioned Serum to Treat Proximal Suspensory Desmitis

J. Lane Easter, DVM, DACVS*; and Ashlee E. Watts, DVM, PhD, DACVS

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1. Introduction

The suspensory ligament (SL) can be divided into 3 regions subject to injury: the proximal region, the body, and the medial and lateral branches. In this presentation, only treatment of the proximal region is discussed.

Proximal suspensory desmitis (PSD) is a common injury of athletic horses causing pain and lameness. It can occur unilaterally or bilaterally and can affect the hind limbs, the fore limbs, or both.1 Clinical signs displayed by horses with PSD have been well documented1 and pain causing lameness can be localized to the proximal aspect of the suspensory ligament (PSL) by using a variety of local and regional anesthetic techniques.1 The PSL and adjacent bone is usually imaged using ultrasonography and radiography. Nuclear scintigraphic examination of horses affected with PSD can be helpful, but magnetic resonance imaging (MRI) is the most defining method by which the ligament and surrounding structures can be imaged.1

Most horses lame because of acute PSD of the fore limb, respond favorably to stall confinement and controlled walking exercise for 3 months,1,2 but for the 3-year-old Western performance horse, 3 months of confinement may end the horse’s show career, thereby significantly reducing the horse’s worth. The prognosis for return to soundness for horses lame because of PSD of one or both hind limbs is poor when affected horses are treated by confinement and incremental increase in exercise.3,4

Besides stall rest, a myriad of adjunctive therapies have been recommended for horses lame because of PSD, including systemic and topical administration of an NSAID; periligamentous injection of a corticosteroid, hyaluronan, polysulfated glycosaminoglycans, extract of the pitcher plant, or a combination of these drugs; topical application of dimethyl sulfoxide (DMSO), with or without a corticosteroid or other drugs; extracorporeal shockwave therapy; and intraleisional injection of 2% iodine in almond oil, extracellular matrix, mesenchymal stem cells, or platelet rich plasma.1
Surgical treatments of horses for PSD include splitting the ligament, lateral palmar/plantar neurectomy, neurectomy of the deep branch of the lateral palmar or plantar nerve, ulnar or tibial neurectomy, fasciotomy of the fascia overlaying the ligament, and combinations of these surgical treatments.1

In this presentation, we describe periligamentous injection of autologous conditioned serum (ACS) for the treatment of horses lame because of PSD. Autologous conditioned serum is made by collecting the patient’s blood in a proprietary syringe containing glass beads soaked in chromium sulfate. The blood is incubated in the syringe for approximately 24 hours (18–26 h) and then centrifuged, filtered, and divided into dose-sized aliquots, which can be injected immediately or frozen for future use. The processing procedure is designed to specifically harvest anti-inflammatory cytokines,5 but anabolic cytokines and morphogenic proteins may be harvested, as well.6

Commercial veterinary systems are available for the horse for patient-side production of ACS.4–d Autologous, conditioned serum has been used in the horse for several years to modulate synovitis and has become an accepted mode of equine joint therapy.1,7–13 Autologous conditioned serum has also been investigated for modulation of the post-breeding inflammatory response in the mare’s uterus.14 Autologous, conditioned serum is used to treat humans for a variety of synovial and non-synovial orthopedic maladies,15,16 and experimental studies using rats have demonstrated improved tendon healing after ACS therapy.17,18

The purpose of this report is to describe how we select horses lame because of PSD for treatment with ACS using the irap® system. Our criteria for selection of horses for this treatment are based on our experience in treating 271 horses lame because of PSD.

2. Materials and Methods

Case Selection

Horses in this study were those determined to be lame solely because of PSD and that were treated with ACS by one of the authors® between September 2004 and September 2013. All horses included in this study were lame at the time of treatment, showed signs of pain during palpation of the PSL, and had the pain causing lameness localized to the PSL by performing various techniques of diagnostic analgesia using 2% mepivacaine hydrochloride (HCl). Only horses with a minimum follow-up time of 3 months, after the initiation of treatment were included in the study.

Fore Limb Blocks

Pain in the distal portion of the fore limb(s) was excluded as a cause of lameness by observing no improvement in lameness after administering a low four-point nerve block or after administering an abaxial sesamoid nerve block in combination with intrasynovial analgesia of the fetlock joint and, occasionally, intrasynovial analgesia of the digital flexor tendon sheath. Pain was isolated to the PSL by observing substantial improvement in lameness after desensitizing the PSL by anesthetizing the lateral palmar nerve at the level of the accessory carpal bone.

Hind Limb Blocks

Pain in the distal portion of the hind limb(s) was excluded as a cause of lameness by observing no improvement in lameness after administering a low four-point nerve block or after administering an abaxial sesamoid nerve block in combination with intrasynovial analgesia of the fetlock and, occasionally, intrasynovial analgesia of the digital flexor tendon sheath. Pain was isolated to the PSL by observing substantial improvement in lameness after desensitizing the PSL by anesthetizing the tibial nerve or by locally infiltrating the PSL with local anesthetic solution. For some horses, pain originating from the distal joints of the hock was excluded as a cause of lameness by observing little or no improvement in lameness after intrasynovial analgesia of the distal intertarsal and tarsometatarsal joints during lameness exam performed after the effects of local or regional analgesia had dissipated.

Imaging

The metacarpus (Mc III) or metatarsus (Mt III) of all horses were radiographically examined to exclude horses with substantial bone disease at the proximal aspect of Mc/Mt III, and all were examined sonographically in the palmar/plantar metacarpal/metatarsal region. Horses with a stress fracture of the proximal palmar/plantar Mc/Mt III were excluded from the study. Horses with substantial tissue disruption of the PSL or damage to other structures in the area such as the inferior check ligament were excluded. Horses that had enlargement of a PSL or edema of a PSL, as compared to the contralateral limb, were included (Fig. 1). For horses with bilateral PSD, assessment of enlargement and edema of the PSL was based on published guidelines.4

ACS Harvest and Processing

Whole blood harvested from each horse was incubated in the proprietary syringe in which it was collected and processed according to the manufacturer’s guidelines. Some aliquots were used immediately after processing whereas others were stored in a −20°C freezer for future use. Frozen aliquots were thawed in a water bath immediately prior to use, and the ACS was filtered through a 0.2 μm disk filter® prior to injection.
Treatment

Injection Technique

All injection sites were prepared for aseptic injection, and the injector wore sterile gloves. The horse was sedated, and the affected fore limb or hind limb was held in flexion. A 20-gauge, 1.5 in needle, attached to a 6 mL Luer-lock syringe containing 5 mL of ACS, was inserted through the skin approximately 4.5 cm distal to the proximal end of the head of the lateral splint-bone. The needle was directed proximomedially and inserted deep to the PSL until its tip engaged the palmar/plantar cortex of Mc/Mt III axial to the axial border of the lateral splint bone in the space between Mc/Mt III and the PSL. The ACS was injected with little or no resistance if the needle was properly placed. If resistance to injection was encountered, the bevel of the needle was assumed to be within the periosteum of the Mc/Mt III, and the needle was withdrawn until the ACS could be injected with ease.

All horses received a single dose (1 mg/kg) of flunixin meglumine intravenously after injection of ACS. The limbs were not bandaged after injection.
Rest, Follow-Up Examination, and Retreatment Protocol

All horses were discharged to the owner/trainers care immediately after the injection was completed. The owner or trainer was instructed to confine the horse to a stall and to walk the horse in hand for 20 minutes daily for 14 days. The horses were reexamined for lameness at 14 days, and degree of the lameness was recorded. The limb(s) affected with PSD were re-injected with ACS in similar fashion, the horse was discharged, and the owner or trainer was instructed to confine the horse to a stall and to walk the horse in hand for 20 minutes daily for another 14 days. At 28 days after the initial injection, the horse was reexamined for lameness, and structures palmar/plantar to the Mc/Mt III were examined ultrasonographically. The horse was then administered a third treatment with ACS. If the horse was judged to be sound during examination and sono graphic quality of the PSL was within normal limits or if the edema and/or cross sectional area of the PSL were reduced, the owner or trainer was instructed to continue confining the horse to a stall but to walk the horse in hand for 20 minutes daily for 5 days and then, on the sixth day, to ride the horse using a regime of gradually increasing, controlled exercise. The owner or trainer of those horses that did not meet the criteria to enter a controlled, ridden exercise program was instructed to continue to confine the horse and to walk it in hand until the horse became sound and had no sono graphic abnormalities, or an alternate treatment plan was instituted.

Rehabilitation Exercise Program

During the first week of controlled, ridden exercise, the horse was walked daily for 15 minutes and then trotted for 5 minutes. For each subsequent week, the daily trotting time was increased by 5 minutes until the horse was able to trot without lameness for 15 minutes for 14 consecutive days (2 weeks). Beginning the fifth week, if the horse was not lame, loping/cantering was added to the exercise regimen. The time of loping/cantering initially was 2 ½ minutes daily, and increased weekly by increments of 2 ½ minutes. After the horse was able to walk for 15 minutes, trot for 15 minutes, and lope for 15 minutes daily for 2 weeks without lameness, the horse was reintroduced to its normal training regimen at the same level of training it had been receiving prior to injury. The treatment protocol was considered successful if the horse entered controlled, ridden exercise at approximately 33 days from initial injection, maintained soundness without deviation from the described protocol of controlled, ridden exercise, entered full training by at least 110 days, and trained at least 1 month without recurrence of lameness.

3. Results

Two hundred seventy-one horses met the criteria required for inclusion in the study, and of these, 47% (127/271) were lame because of PSD of one or both fore limbs, and 53% (144/271) were lame because of PSD of one or both hind limbs. A total of 352 limbs were injected with ACS. No horse suffered an adverse reaction to any treatment with ACS.

Horses Treated for PSD of One or Both Fore Limbs (127/271)

Of horses lame because of PSD of one or both fore limbs, 83% (105/127) were affected unilaterally, and 17% (22/127) were affected bilaterally. Failure of a horse affected bilaterally to return to soundness in either limb was considered a failure of treatment.

Horses Treated for PSD of One Hind Limb (105/127/271)

Of the horses treated for PSD of one fore limb (105), 85% (89/105) were judged to have become sound, able to complete the exercise schedule prescribed, and to have maintained soundness for at least 30 days after reentering full training.

Horses Treated for PSD of Both Fore Limbs (22/127/271)

Of the horses treated for PSD of both fore limbs (22), 82% (18/22) were judged to have become sound, able to complete the exercise schedule prescribed, and to have maintained soundness for at least 30 days after reentering full training.

Horses Treated for PSD of One or Both Hind Limbs (144/271 Total)

Of horses lame because of PSD of one or both hind limbs, 59% (85/144) were affected unilaterally, and 41% (59/144) were affected bilaterally. Failure of a horse affected bilaterally to return to soundness in either limb was considered a failure of treatment.

Horses Treated for PSD of One Hind Limb (85/144/271)

Of the horses treated for PSD of one hind limb (85), 78% (66/85) were judged to have become sound, able to complete the exercise schedule prescribed, and to have maintained soundness for at least 30 days after reentering full training.

Horses Treated for PSD of Both Hind Limbs (59/144/271 Total)

Of the horses treated for PSD of both hind limbs (59), 71% (42/59) were judged to have become sound, able to complete the exercise schedule prescribed, and to have maintained soundness for at least 30 days after reentering full training.

Confinement coupled with controlled exercise was continued for many of the horses for which treatment was considered to have failed (i.e., those horses unable to sustain the scheduled rehabilitation and train without lameness for at least month), and although many of these horses eventually became sound and were trained successfully, we have not determined the percentage that did.

4. Discussion

Proximal suspensory desmitis is a common cause of lameness of performance horses of many disciplines.
and other categories of horses as well. In the practice of one of the authors, PSD is the number one cause of fore limb lameness of young, Western performance horses that precludes a horse from competing in a major event for which it has trained and rank in the top 3 causes of fore limb lameness overall. In the same practice, PSD is also one of the 3 most common causes of lameness in the hind limb of the Western performance horse.

Treatment of horses for PSD typically includes a long, costly period of confinement and inactivity (i.e., 3–12 months) and is often the cause of missing a major event for which they were bought and trained. When a young, Western performance horse misses a major event such as the discipline's major futurity, the worth of the horse is reduced. Horses with PSD of one or both hind limbs have a guarded prognosis for return to full athletic function at all. Treatment of horses for acute or chronic PSD has encompassed a vast array of therapies, perhaps because no single therapy is effective for most horses affected with PSD. Almost every therapy used to combat pain and inflammation or stimulate healing in any other portion of the body has been used to treat horses for PSD. The authors believe that although some traditional therapies, such as regional injections of a corticosteroid, initially reduce pain, swelling, and inflammation, they inhibit healing by slowing metabolism, growth, and replication of fibroblasts important in repair of the injured ligament. Some therapies, such as internal blisters of 2% iodine in almond oil or intrasynovial injection of extracellular matrix may lead to production of non-elastic scar tissue within or around the PSL. Other therapies, such as extracorporeal shock wave therapy, may only reduce ligamentous pain, rather than stimulate healing, thereby potentially perpetuating damage to the ligament when the horse is returned to training.

Our goal for using ACS to treat horses lame because of PSD was to not only rapidly reduce pain, and therefore lameness, by reducing inflammation in the ligament but also to augment healing to return the ligament to its original form and function. Treatment with ACS shortened the time of lay-up of horses lame because of PSD as compared to other published reports, thereby allowing many horses to compete in events they otherwise would have missed, thus preserving the horse's worth and reducing expense to the owner. Numerous cases that the authors considered failures for this report continued to have further treatments of ACS and controlled exercise and many of them too eventually reentered training.

Autologous conditioned serum has historically been considered to be an anti-catabolic treatment by virtue of its ability to block the inflammatory cascade initiated by IL-1. By blocking inflammation, ACS might have an indirect, anabolic, pro-healing effect. However, ACS also contains the growth factors listed above, and perhaps others, that are released by platelets activated during clotting while whole blood is incubated with the glass beads. These growth factors may be directly beneficial in healing, and therefore, ACS may also have a direct, anabolic effect on healing. Numerous studies have documented the beneficial effects of individual growth factors found in ACS, such as vascular endothelial growth factor (VEGF), TGF-β, platelet-derived growth factor (PDGF), growth and differentiation factors, IGF-1, basic fibroblast growth factor (FGF-2, also bFGF), and bone morphogenetic proteins (BMP-12 and BMP-13), on tendon healing using animal models. Exposure, in vitro, of patellar tendon fibroblasts to bFGF was found to affect cellular proliferation and collagen type III expression, both early events in tendon healing.

Majewski, et al showed that ACS, when injected into the rat Achilles tendon, accelerated the rate of organization of repair tissue. Specifically, it increased collagen mRNA expression, collagen deposition, as reflected by tendon thickness, and accelerated collagen fiber maturation, an aspect the authors believe may be important in treating horses for PSD. Majewski, et al also showed that stiffness of the ACS-treated tendons improved significantly over that of controls by week 4 post-treatment and theorized that this effect was likely due to reduction in time required for collagen cross-linking and remodeling of the repair site. This time frame corresponded with our time frame of implementing controlled work in horses post ACS treatment of PSD. Majewski, et al noted that remodeling of tendinous and ligamentous tissue is a slow process as evidenced by their finding that tendon thickness remained elevated even by week 8, but PSD of the horses in our study was accompanied by minimal
loss of fiber. Majewski, et al observed that ACS treatment might shorten recovery time because ACS-aided tendon repair is characterized by repair with well-organized, strong collagen fibers, suggesting that the healed tendon should contain fewer imperfections than in repair tissue formed after spontaneous healing. They believed such tissue would be less prone to re-injury, and they acknowledged that the biologically active component(s) in ACS that were responsible for the accelerated healing of the injured Achilles tendons remained to be identified.18

The authors believe the horses selected to treat with ACS for PSD suffered from acute or chronic SL strains rather than major tearing of the SL. Strain is damage to elastic fibers from stretching of the fibers beyond their elastic limit without visual disruption of fibers. This results in pain, swelling, and inflammation within the strained structure as well as potentially causing compressive damage to adjacent nerves.21 Tóth, et al21 clearly showed that horses with PSD of the hind limbs could also have pathologic changes of the deep branch of the lateral plantar nerve caused by compression from swelling of the PSL. They theorized that this nerve damage could be one potential reason for the poor long-term prognosis of PSD in the hind limbs of horses.21

While ACS could have an indirect effect on nerve pathology by reducing compression on the nerve by reducing inflammation and thus swelling of the PSL, there is evidence that ACS can have a direct effect on pain from compressed nerves. Becker, et al demonstrated that epidural injections of ACS to treat lumbar radicular compression in humans were associated with clinically remarkable positive outcomes, potentially superior to the same treatment method using triamcinolone.20

The authors believe the acutely lame horses in this study were ideal candidates for treatment with ACS because the ligaments of these horses had no ultrasonographically discernible major disruption of fibers. Lameness of some horses may go undiagnosed causing it to become chronic if lameness of a fore limb is mild, if the lameness involves both fore limbs, or if the lameness affects one or both hind limbs. The authors believe that as long as major disruption of fibers is not ultrasonographically evident, even horses chronically lame because of PSD are good candidates for treatment with ACS as it may be possible to return the tissue to normal size and function and heal or treat pain from potential neuropathies. Periligamentous injection, as described in this report, rather than intrasional injection, is indicated for horses without a core lesion. Forcing ACS into ligamentous or tendinous tissue in which there is no tissue void forces fibers apart, disrupting important cross-linking, this may worsen lameness and lengthen time of healing.

Short-comings of this study are the short follow-up time and lack of control groups. However, it has been the experience of one of the authors7, in many cases of PSD in the young Western performance horse, that if we can get a horse to train a month without immediately getting lame, we have a chance of successfully keeping that horse in training long-term. With that stated, however, many of the horses will get lame again at some point from the same cause albeit usually less severely. When this occurs, we reevaluate sonographically, and unless we have tissue disruption of some sort (core lesion, etc.), we have been able to re-inject most of those and only give them short lay-offs of 5 to 7 days and resume training. Many do this several times in their careers and get into a routine with a fairly consistent interval. As these young Western performance horses mature, however, the intervals between flare-up usually widens until they reach 6 years of age in which many seem to mature out of the syndrome. We hypothesize that this is partly due to a reduced training schedule and amount of work needed to stay in top form (i.e., the horse knows its job) but also because the horse begins adaptive remodeling, which becomes complete by the time it is 5 to 6 years old. Occasionally, it was observed that the ligament begins to fail requiring that the horse be removed from training and subjected to a different treatment type or prolonged rest period.

An important consideration when using regenerative products, such as ACS, is that differences may occur between commercially available products. The concentration of IL-1 receptor antagonist protein and IL-1 itself, as well as anabolic growth factor concentrations, may vary among manufacturers. The concentrations may also vary both between and within patients. Hraha, et al6 tested 2 different commercially available veterinary systems, irapc,d and Arthrex IRAP II, using whole blood in a clot tube as a control. They compared each system using the blood of 5 horses and found that IL-1ra was up-regulated by both systemsc,d and within the control, clot tube, but that the IL-1 to IL-1ra ratio was only increased compared to serum in Arthrex IRAP II,du thus illustrating differences. The authors used one of the systems5 and did not measure IL-1ra or other factors of interest. The authors believe caution should be used when extrapolating this data to other systems used to produce ACS.

The authors believe that the large number of horses with PSD treated with ACS demonstrates the safety and effectiveness of this treatment when horses are selected appropriately. There were no control groups of horses with PSD that received other therapies to which can be compared to the results of treatment with ACS, but this experience with other treatments of horses for PSD as well as the published results of other clinicians8 indicates that horses treated with ACS are able to be returned successfully to training much sooner than horses receiving other treatment. Treatment with ACS has allowed many young Western performance horses to resume training in time to complete their
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schedule of competitions. Further, the authors believe that this treatment should be evaluated in blinded clinical trials and that work should be done to define the exact mechanism of action.

Acknowledgments

Conflict of Interest

The Authors declare no conflicts of interest.

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Review of Vascular Administration of Mesenchymal Stem Cells in the Equine Distal Limb

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Intravenous regional limb perfusions result in heterogeneous and asymmetric distribution of stem cells. Furthermore, serious limitations related to poor foot perfusion and tourniquet failure are identified in standing horses. Intra-arterial injections should not be performed with a tourniquet due to a risk of arterial thrombosis. Intra-arterial injections without a tourniquet lead to homogeneous and diffuse distribution and are considered the preferred technique. Although more challenging, intra-arterial injections can be performed in the standing horse. Authors' address: School of Veterinary Medicine, University of California, Davis, One Shields Ave, Davis, CA 95616; e-mail: mspriet@ucdavis.edu. *Corresponding and presenting author. © 2014 AAEP.

1. Introduction
Vascular administration of mesenchymal stem cells (MSCs) to the equine distal limb has been proposed as an alternative to direct injection into lesions. The main advantages of such techniques are the treatment of lesions that cannot be reached directly with a needle (for example, lesions within the hoof) or diffuse lesions that would require multiple injection sites. Another advantage of the technique is to avoid iatrogenic damage created by needles in the structures adjacent to lesions. Both intravenous and intra-arterial injection techniques have been used in laboratory animals1,2 and in human patients.3 The intra-arterial technique seems beneficial regarding the perfusion of peripheral tissues.1-3 In the horse, techniques of vascular administration can be subdivided according to the type of vessel used (vein or artery), the exact site of injection, and the use or not of a tourniquet. The outcome might also be affected by the use of standing sedation or general anesthesia.

The use of an intracellular radioactive label (99mTc-HMPAO) allows scintigraphic tracking of the MSCs after injection. Different injection techniques have recently been evaluated with scintigraphy. The goal of this review is to summarize the pros and cons of each technique and issue a recommendation regarding the clinical use of vascular administration of MSCs. The different techniques that have been investigated include cephalic vein and palmar digital vein regional limb perfusion (RLP) and median artery injection. The arterial injections have been assessed both with and without the use of a tourniquet. All techniques have been performed both in anesthetized and in standing horses.

NOTES
2. Cephalic Vein Regional Limb Perfusion in Anesthetized Horses

Cephalic vein RLP under general anesthesia was the first technique investigated. A pneumatic tourniquet inflated to 400 to 500 mm Hg was placed on the forearm just proximal to the cephalic vein catheter for 30 min. Twenty-five to 45 million radiolabeled MSCs suspended in 2 mL of saline were injected and flushed with 20 mL of saline. All of the radiolabeled MSCs remained in the limb while the tourniquet was in place and no major loss of radioactive signal was observed immediately after removal of the tourniquet. Only 3 of the 6 injected limbs had radioactive signal in the entire distal limb, whereas the other 3 limbs only had uptake in the carpal and proximal metacarpal areas. The maximal signal intensity was mostly in the area of the larger vessels (Fig. 1A). In horses with induced lesions in the superficial digital flexor tendon, the distribution and quantification of the radioactive signal was not different from the control horses.

3. Lateral Palmar Digital Vein Regional Limb Perfusion in Anesthetized Horses

The lateral palmar digital vein RLP was assessed in a later study with the main objective to improve distribution of MSCs to the foot when compared with cephalic vein RLP. A pneumatic tourniquet inflated at 450 mm Hg was placed for 30 min in the metacarpal region, proximal to the injection site. Approximately 35 million MSCs suspended in 10 mL of saline and flushed with an additional 10 mL of saline were injected. Similar to the cephalic vein RLP in the anesthetized patient, the tourniquet effectively retained all radioactivity within the limb. The radioactive signal was present within the foot in all 6 injected limbs; however, a marked asymmetry was identified with low to absent radioactive signal at the medial aspect of the foot and pastern. Most of the radioactive signal was located in the area of the lateral palmar digital vein and coronary band vascular plexus (Fig. 1C).

4. Cephalic Vein Regional Limb Perfusion in Standing Sedated Horses

For this study, horses were sedated with 0.01 mg/kg detomidine IV and 0.01 mg/kg butorphanol IV and a pneumatic tourniquet inflated at 225 mm Hg was placed on the forearm proximal to the injection site for 30 min. Approximately 40 million radiolabeled MSCs suspended in 2 mL of saline were injected and flushed with 20 mL of saline. Only 3 of 6 horses showed a radioactive signal distal to the proximal metacarpus (Fig. 1B). The quantification of the signal revealed a much lower uptake than on the initial study under general anesthesia, suggesting failure of the tourniquet.

5. Lateral Palmar Digital Vein Regional Limb Perfusion in Standing Sedated Horses

Horses were sedated similarly as for the cephalic vein RLP study. A pneumatic tourniquet inflated to 450 mm Hg was placed on the metacarpus for 20 min. Forty million radiolabeled MSCs in 10 mL of saline and flushed with an additional 10 mL of saline were used. Despite all horses tolerating the tourniquet well, very little radioactive signal was present distal to the injection site in 3 of the horses. The quantification of the signal revealed a wide range of uptake. The dynamic acquisition at the time of injection demonstrated failure of the tourniquet with the radioactive signal proximal to the tourniquet. In the 3 horses with a better distal radioactive signal, uptake within the hoof was lower than in the anesthetized horses, suggesting poor perfusion related to the weight-bearing position (Fig. 1D).
6. Median Artery Regional Limb Perfusion in Anesthetized Horses

Ultrasound guidance was used to place an 18 gauge catheter in the median artery at the level of the chestnut. A pneumatic tourniquet inflated to 400 to 500 mm Hg was placed on the forearm just proximal to the catheter for 30 min. Twenty-five to 45 million radiolabeled MSCs suspended in 2 mL of saline were injected in the median artery without the use of a tourniquet. A diffuse homogeneous distribution of the radioactive signal was observed through the entire distal limb (Fig. 2C). Quantification of the signal suggested that about half of the radiolabeled MSCs initially remained in the distal limb despite the absence of a tourniquet. No evidence of thrombosis was observed.

7. Median Artery Injection Without a Tourniquet in Anesthetized Horses

A catheter was placed similarly as for the median artery RLP. Thirty-five million radiolabeled MSCs suspended in 2 mL of saline were injected in the median artery without the use of a tourniquet. A diffuse homogeneous distribution of the radioactive signal was observed through the entire distal limb (Fig. 2C). Quantification of the signal suggested that about half of the radiolabeled MSCs initially remained in the distal limb despite the absence of a tourniquet. No evidence of thrombosis was observed.

8. Median Artery Injection Without a Tourniquet in Standing Sedated Horses

The feasibility of injecting the median artery in standing horses was first assessed using radiographic contrast material. The use of a catheter versus direct needle injection was assessed in 6 horses. Ultrasound guidance was used for both techniques. Catheter placement was more challenging and induced a higher risk of arterial spasm, precluding injection. Furthermore, due to the deep location of the artery, motion of the limb after catheter placement led to retraction of the catheter from the artery. For these reasons, despite a risk of peri-arterial extravasation, direct needle injection was considered the preferred technique.

Approximately 35 million radiolabeled MSCs were administered in the median arteries of 3 horses via direct needle injection. Five out of 6 of the injections were successful; one of the injection led to partial peri-arterial injection. Diffuse symmetric uptake was present in all distal limbs with a strong radioactive signal in the hoof (Fig. 2D). No arterial thrombosis was observed; however, partial venous thrombosis was present in 3 of the horses. No lameness was associated with the partial venous thrombosis.

9. Discussion

The intravenous RLP techniques performed under general anesthesia demonstrated good efficiency of the tourniquet and retention of MSCs in the limb after removal of the tourniquet. However, the distribution pattern was not optimal. With cephalic vein RLP, the inconsistent perfusion of the distal limb was questioned as being related to too small of a perfusion volume. In a later study, we investigated cephalic vein RLP using 22 mL of iodinated contrast material for radiographic evaluation of the distribution. The authors observed radiographic contrast in the entire distal limb (including the foot) in all cases (unpublished data).
It was concluded that the incomplete perfusion of the distal limb with radiolabeled MSCs was potentially due to obstruction of the venous valves by the MSCs. The lateral palmar digital vein RLP improved the perfusion of the foot compared to the cephalic vein RLP but the asymmetric distribution, with lower or absent radioactive signal medially, suggested that both the lateral and medial palmar digital veins should be injected in order to perfuse the entire foot.6

When performed in the standing horse, an additional limitation was the much lower signal observed in the limb.7 Failure of the tourniquet was clearly demonstrated with the lateral palmar digital vein RLP when the radioactive signal was seen migrating proximal to the tourniquet on the dynamic acquisition at the time of injection.7 Although similar dynamic data were not available for the cephalic vein RLP study, based on the limited distribution and poor signal observed on static acquisitions while the tourniquet was still in place, failure of the tourniquet likely also happened. The lower pressure (225 mm Hg) used in standing horses for the cephalic vein RLP can be blamed for tourniquet failure; however, for the lateral palmar digital vein RLP, the same pressure (450 mm Hg) was used for the tourniquet in anesthetized and standing horses. The tension in the tendons in the weight-bearing limbs likely explains the lower efficiency of the tourniquet in standing horses.7

The lower uptake within the hoof in standing horses compared with anesthetized horses is another significant limitation of the standing technique. This is likely related to the difference in pressure in the venous system in the weight-bearing foot.7

Another limitation to all intravenous RLP techniques was the heterogeneity of the signal distribution with higher uptake matching the location of the larger vessels, suggesting that the MSCs mostly remain in these larger vessels.5,6

The intra-arterial RLP (with a tourniquet) was advantageous compared with the intravenous RLP in terms of distribution and uptake in lesion areas; however, due to the severe arterial thrombosis that occurred in two out of twelve horses, this technique could not be recommended for clinical use.4,5 Intra-arterial injection without a tourniquet resolved the thrombosis issue.6 The retention of MSCs within the distal limb can be explained by trapping of the cells in the capillary beds. Some MSCs are lost to the general circulation due to the absence of the tourniquet but better perfusion and more homogeneous distribution of the MSCs can be appreciated. For these reasons, the intra-arterial technique was considered the preferred technique for vascular administration of MSCs. A limitation of this technique was the technical difficulty of placing a catheter in the median artery in a standing horse. The use of direct needle injection of the median artery under ultrasound guidance appeared to be an interesting alternative to the use of a catheter to facilitate the use of this procedure in the standing sedated horse.8 Peri-arterial injection remains an inherent risk of this technique; however, this risk can be minimized in the hands of an operator experienced with ultrasound guidance. We have now performed over 30 such injections and peri-arterial leakage occurred in only 2 occurrences.

The difference in the pattern of distribution between intra-arterial injections in anesthetized and standing horses with a higher accumulation of MSCs in the foot in standing horses was an unexpected finding.9 Interestingly, intravenous RLP led to a lower accumulation of MSCs in the foot of standing horses compared with anesthetized horses.6 These findings are most likely related to the difference in pressure and perfusion at the capillaries of the lamina between weight-bearing and non-weight-bearing positions. The presence of partial subclinical thrombosis of the veins following arterial injections in standing horses was surprising since this did not happen in anesthetized patients. Again, differences in the capillary circulation with probable higher stress on the MSCs in the standing horses might explain this phenomenon. Partial venous thrombosis observed in the scintigraphic study9 might also be related to higher stress on the MSCs from the labeling manipulation. We have now performed this technique with non-labeled MSCs in over 30 limbs and have only occasionally observed mild transient swelling of the distal limb without further complications.

10. Conclusion

Scintigraphic tracking has been extremely useful in comparing different vascular injection techniques for MSC administration to the equine distal limbs. As could be expected, the results in the anesthetized horses were different from the standing horses. As performed in these studies, the intravenous RLPs were not reliable in the standing horses. The use of different tourniquets could be considered to try to improve the intravenous RLP; however, the intra-arterial injection technique without a tourniquet is our preferred technique as it provides better distribution of the MSCs.

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Conflict of Interest

The Authors declare no conflicts of interest.

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