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Article

Treating horse chronic laminitis with allogeneic bone marrow mesenchymal stem cells

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Abstract:

Chronic laminitis is a disabling condition that affects the laminar *corium* of the horse's hooves. Commonly, it develops as a collateral injury of numerous primary systemic diseases. It is believed that the critical physiopathological event that renders a hoof laminitic is the loss of mesenchymal stem cells. This loss greatly impairs the ability of the laminar *corium* to regenerate. Although previous work provides credibility to this notion, there remain unsettled issues that must be addressed before accepting it as a well-founded fact. Here, it was reexamined the central tenet of the physiopathological model of laminitis by infusing allogeneic bone marrow-derived mesenchymal stem cells (ABM-MSCs), through the digital palmar vein, into the hooves of horses afflicted by chronic laminitis. Horses were clinically monitored during 6 mo by evaluating them monthly using the lameness-modified Obel-Glasgow's scale and hooves thermography. Venograms and lamellar biopsies were taken at the beginning and at the end of the study period to gathered evidence on vascular remodeling and laminar *corium* regeneration. The results showed that ABM-MSCs infusion promotes vascular remodeling and laminar *corium* regeneration, further supporting that the loss of stem cells is the critical event leading to chronic laminitis. This work also demonstrated that the infusion of ABM-MSCs is safe since the treated horses did not develop local or systemic, negative clinical manifestations attuned with rejection reactions, at least during the 6-mo period they were follow up and under the therapeutic scheme proposed.

Key words: Horses, Laminitis, ABM-MSCs, aMSCs, MMP, MSCs, PRP, Platelet rich plasma.

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Introduction

Chronic laminitis is an incapacitating podal condition in horses. Its prevalence ranges 7 to 14 % worldwide[1]. Laminitis is the negative collateral outcome of numerous primary digestives, respiratory, urinary, metabolic, orthopedic and reproductive diseases[2-4]. Faleiros *et al*[5] and Leise *et al*[6] have elaborated a three-phase model to understand the progression of laminitis: the developmental phase, the acute phase, and the chronic phase.

It has been proposed that the laminar *corium* in hooves of horses afflicted by chronic laminitis loses its abilities to sustain regenerative processes[3]. Since mesenchymal stem cells are responsible for carrying out this task, a central piece of the puzzle to understand the physiopathology of chronic laminitis is therefore the loss of mesenchymal stem cells

during the inflammatory process[3]. This notion is not incongruous since mesenchymal stem cells keep under tight control the homeostasis, remodeling and repair of the laminar *corium*[7-8], promote tissue regeneration by secreting growth factors[9-12]; and anti-inflammatory cytokines[11,13,14]. They also protect against oxidative damage and against hypoxia / reperfusion injury by preserving endothelial integrity and promoting angiogenesis[3].

In 2017, Angelone *et al*[3] pioneered an experimental work aimed at assessing whether the loss of mesenchymal stem cells was indeed a fundamental piece of the physiopathological mechanism leading to chronic laminitis. Their results answered positively their enquiry, only to some extent. Unfortunately, they could not irrefutably ascribe the clinical improvement and the venographic evidence of angiogenesis observed in the treated horses solely by the infused adipose tissue-derived mesenchymal stem cells because the cells were co-infused with platelet-rich plasma. In addition, even though previous evidence has revealed that allogeneic stem cells have no deleterious effects on the horses' health even if they may trigger immune responses in the treated horse against foreign antigens[15-21], they opted to first infused allogeneic and then infused autologous adipose tissue-derived mesenchymal stem cells, thus confounding the interpretation of their results. Lastly, even though treated horses improved significantly when clinically assessed and despite the evidence presented supporting ongoing angiogenesis in the infused laminitic hooves, the cytological regeneration within the hoof can only be inferred. The present work was designed to overcome some of the technical pitfalls of the pioneer experimental assessment conducted by Angelone, while evaluating the role of mesenchymal stem cells in the pathophysiological process underlying laminitis. To successfully do this, it was infused allogeneic bone marrow-derived mesenchymal cells (ABM-MSCs), suspended in culture media, into both front hooves of horses chronically affected by laminitis, monitored the clinical progress of infused horses through clinical and venographic assessments. In addition, the study included hooves biopsies to directly evaluate laminar *corium* regeneration and hooves thermography to keep track of hooves temperature. Overall, the results showed that ABM-MSCs infusions: 1) restore to a significant extent the cytoarchitecture of the laminar *corium*, 2) regenerate segments of the vascular bed and 3) notoriously improves the clinical stance of the treated horses, while having no local nor systemic deleterious effects on the horses' health at least for up to the time the horses were followed and under the treatment protocol devised here. Hence, our observations support that the loss of mesenchymal stem cells play a fundamental role in the pathogenesis of chronic laminitis; their restitution may well reverse the condition. As subsidiary corollaries, clinicians must investigate measures aimed at preventing mesenchymal stem cell loss through inducing hooves protective immune tolerance or by encouraging hooves beneficial auto-immunity in horses coursing with primary, pro-laminitis diseases. Lastly, it is worth to emphasize the need to investigate the possibility of using mesenchymal stem cells to manage pain in horses.

Material and methods

Animals

Female (n= 5) and male (n= 5) horses of different breeds, age and different activities afflicted by chronic laminitis were used to conduct this study (Table 1), none of the horses were under any current treatment. All of the animals displayed distal phalanx rotation but were not sinkers. Blood cell count (CBC) was drawn from the jugular vein was used to appraise the initial inflammatory state. All horses were kept and treated in their corresponding regular stalls. Animals had free access to water and were fed with oat hay twice a day. Informed consent forms were signed by the owners. Horse clinical and experimental procedural and handling protocols were approved by the Veterinary College (FMVZ-UNAM) Institutional Animal Care and Use of Animal Committee (No. MC-2016/2-5; "Evaluación terapéutica del uso de células troncales mesenquimales alogénicas, en el control del dolor, inflamación y estructura lamelar en caballos con lamintis crónica").

Table 1: Horses of different breeds, age and different activities afflicted by chronic laminitis

No.	Breed	Sex	Age (years)	Activity	Laminitis cause	Laminitis duration	Initial prognosis	Initial weight	Final weight	Initial Obel-Glasgow	Final Obel-Glasgow	Shoeing
1	Quarter Horse	Mare	7	Mexican sport	Colic	13 months	Poor	480	408	4	1	Orthopaedic
2	Appendix	Mare	12	Police service	Abortion	+24 months	Bad	430	450	13	4	None
3	Thoroughbred	Male	8	Jumper	Diet	1 month	Poor	450	465	2	1	Orthopaedic
4	Quarter horse	Gelding	3	Pet	Trimming	12 months	Poor	320	360	7	2	None
5	Quarter horse	Male	14	Mexican sport	Colic	24 months	Bad	360	370	13	2	Orthopaedic
6	Quarter horse	Mare	19	Police service	Placental retention	+24 months	Bad	460	470	12	4	None
7	Thoroughbred	Mare	5	Race	Unknown	12 months	Bad	496	490	11	4	Orthopaedic
8	Quarter horse	Gelding	16	Mexican sport	Diet change	+24 months	Bad	365	330	13	8	Standard
9	Santa Gertrudis	Mare	8	Military service	Colon displacement	2 months	Poor	455	460	2	0	None
10	Thoroughbred	Male	3	Race	Running	4 months	Poor	400	470	4	0	Orthopaedic

Lameness assessment

An orthopedic evaluation was made by observing the static and dynamic postures of each horse. It was used the modified multifactorial evaluation pain score adapted for laminitic horses that rates signs of pain and altered behavior by combining, respectively, Obel's and Glasgow's criteria[4]. The highest the score (maximum 14), the greatest the pain.

Hooves temperature assessment

Lamellar inflammation leads to increments in hooves temperature[20]. Infrared thermography may be used to estimate the change in temperature through hoof imaging. It was utilized the infrared FLIR XX5 camera (Flir™, USA) to image real time front limb hooves of horses once a month for six months. The temperature of the coronary band was recorded *in situ* and graphed individually. It was decided to use the coronary band as an anatomical reference to measure the temperature because it lacks hoof wall. This feature makes this region amenable for obtaining reliable temperature readings; the anatomy of hoof wall in chronic laminitic horses may become greatly deformed. Imaging sessions were conducted at the same hour (≈ 8 AM), while keeping horses stood in its stall and before being hand-walked. Hooves were always cleaned before imaging. Lateral and dorsal views were taken at a distance of 30 centimeters away from the hoof. Color coded images were obtained based on a calibrated linear scale built up on the camera acquisition program.

Venography

Horses were sedated with 10% xylazine hydrochloride injected through the jugular vein. The lateral and medial palmar digital nerve were blocked using 2% lidocaine at level of proximal sesamoids; previously the area was shaved and cleaned with a solution containing 20% chlorhexidine gluconate. A tourniquet was placed with an Esmarch bandage at the fetlock. The lateral digital vein was then punctured using a butterfly catheter 23G and the contrast medium Iopamidol (Scanlux, San Chemia, México) injected through it slowly. To assure an adequate distribution of Iopamidol throughout the entire horse's digit vascular bed, the knee dorsal aspect was flexed dorsally, and the heels lifted from the ground. At the end of the administration, the heels were weight bearing, the syringe removed, and the catheter clamped with a Halsted tweezers. Latero-medial and dorso-palmar views were taken at 60 sec from the contrast medium[21].

Lamellar biopsy

The fetlock and pastern were washed and dried. Horses were sedated and abaxial lateral and medial nerves blocked following the guidelines provided above. A tourniquet was placed in the fetlock using an Esmarch bandage to reduce bleeding. The dorsal hoof wall was drilled with a 4.8 mm diameter polishing stone (Dremel, Bosch, USA), 2 cm distal from the coronary band. The cavity made (9 mm diameter) traversed the external and medium strata of the hoof's wall until reaching the white line, an anatomical landmark that indicates proximity to the lamellar *corium*. Saline solution was constantly irrigated to avoid overheating during drilling. Once in the white line, the laminar *corium* was incised with a scalpel armed with a No. 11 blade, oriented perpendicular to the hoof wall. The scalpel was moved clockwise following the perimeter of the cavity until reaching the third phalanx. Then, with a No. 4 Frahm's scaler, the laminar tissue was removed from third phalanx[22]. A cylindrical-shaped laminar sample, measuring approximately 7 mm at the base and 7 mm in height, was obtained. The biopsy was fixed in buffered paraformaldehyde (4%) for 24 h, then submerged into a buffered sucrose solution (15 %) for 24 h, and finally transferred to a buffered 30% sucrose solution at 4 °C until evaluation. The surgical area was dried with gauzes and sealed with methyl-methacrylate. The tourniquet was removed after methyl-methacrylate dried.

Histology

Biopsies were embedded in Tissue-Tek (Neg-50™, Richard-Allan Scientific, USA), frozen in dry ice and cut longitudinally (30 µm thick) in a cryostat (SLEE), at -25 °C. Serial sections were mounted on to gelatin subbed slides and stored at -4 °C until used. A total of 15 slides were obtained per sample. A slide was taken every third to be stained with Cresyl violet, Periodic Acid-Schiff's base and Masson's Trichromic stain. Slides were cover slipped with Cytoseal™ 60 (Richard-Allan Scientific) and observed through an Olympus BX51-WI microscope. The tissue cytological integrity and degree of inflammatory infiltration was estimated qualitatively, and representative fields of this material were digitally photomicrographed, under bright-field microscopy, by using the Stereo Investigator™ Software (10X).

Transmission electronic microscopy

Samples of the *lamellar corium* obtained from a healthy and a laminitic horses were fixed in a tampon solution containing 4% paraformaldehyde / 2.5% glutaraldehyde (Electron Microscopy Sciences) for 72 h. After three gentle washes with phosphate buffer-saline

(0.1M; pH 7.2), the samples were post-fixed with osmium tetroxide (1%) for 1 h, then dehydrated and embedded in Epon 812. Polymerized at 60 °C for 24 h. Semi-thin sections (150 nm) were mounted in slides and dyed with Toluidine blue, the interest area was chosen and Ultra-thin sections (70 nm) were mounted on cupper grids, contrasted with uranyl acetate (2%) and lead citrate (50%) (Electron Microscopy Sciences) and observed through a Jeol 1010 transmission electron microscope (60kV). The tissue ultrastructural integrity and degree of inflammatory infiltration was estimated qualitatively, and representative fields of this material were digitally photomicrographed.

Bone marrow sampling and mesenchymal stem cell collection, isolation and expansion

Bone marrow (BM) samples (15 mL) were collected (using 20 mL syringes containing 1,000 UI heparin/ml BM) through a sternal puncture from male and female healthy horses (3-8 yr-old) of different breeds. Samples were stored at 4 – 8 °C and transferred in aseptic conditions to the cell culture facility within an hour from collection. Bone marrow was transferred to sterile tubes filled with Ficoll Hypaque at a 1:2 proportion. The samples were centrifuged at 400 xg for 30 min and the mononuclear fraction was isolated in a sterile tube at 4°C. The fraction was centrifuged at 300 xg for 10 minutes at 4 °C. The supernatant was discarded and the cells were re-suspended and seeded, at a density of $5x10^4$ cells/cm^2, in 25 cm^3 flasks containing DMEM:F12 (1:1) supplemented with 20% fetal bovine Serum, 500 U/mL of penicillin, 50 μg/mL of streptomycin and 2.5 μg/mL amphotericin B. The cells were incubated at 37 °C and 5% CO_2 and the culture media were changed every three days until reaching an 80% confluence[23].

Equine mesenchymal stem cell subculture and scaling

After the 80 % confluence was reached, the cells were sub-cultured weekly as follows: the monolayer was washed with DPBS and the cells were detached using HBSS with 7.5 mg of porcine trypsin and 0.6 mg of EDTA. The cells were seeded at a density of $5x10^4$ cells/cm^2 in 75 cm^3 flasks (first subculture) and then in 175 cm^3 (subsequent subcultures) with DMEM F12 (1:1) supplemented with 20% Fetal Bovine Serum.

Flow-cytometry

Allogenic, bone marrow mesenchymal stem cells were characterized through flow-cytometry. Three sets of antibodies were used to immunophenotype them. ABM-MSC

used for infusion were positive to the surface markers CD73, CD90, CD105, CD28 and CD44 (CD28/CD44 specific for equine MSC), negative to CD45, CD34, CD14 and CD79. Flow cytometry was conducted based upon protocols previously reported[24-28].

Administration of ABM-MSC's

Horses were sedated and prepared for administration of ABM-MSC's as previously described (see venography section). ABM-MSCs were diluted in physiologic saline solution and administered (10-30 x 10^6) into the lateral (or medial) digital palmar vein under sterile conditions via a 21-gauge butterfly catheter, attached to a 20 mL syringe, in the standing horse. Tourniquet was removed 20 min after administration of cells. The digit was kept bandaged for one day. ABM-MSCs were injected three times at one-month intervals.

Results

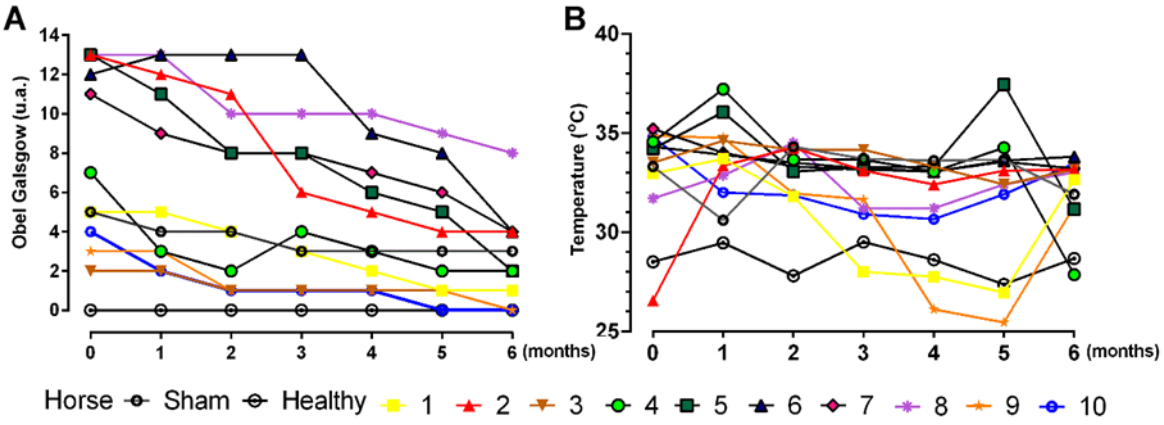
Clinical condition of horses treated with ABM-MSCs

Horses with chronic laminitis had Obel-Glasgow's scores that range between 2 to 14 before ABM-MSCs treatment onset (Figure 1A). In contrast, the healthy horse displayed neither lameness or pain, nor hooves temperature increments at any time along the period evaluated. As the study progressed, all ABM-MSCs treated horses improved their clinical condition after the second or third month of having received ABM-MSCs venous infusions. By the end of the six months' period, all ABM-MSCs treated horses decreased their Obel-Glasgow scores and significantly improved their mobility and clinical condition. Overall, the horses with the worst initial condition, improved the most (Figure 1A). All horses had an improvement in mobility, quality and shape of the hoof, they also improved their behavior and the factions of their faces (Grimace scale), having a good quality of life. This improvement was attributable to ABM-MSCs infusion, since the sham horse treated only with vehicle did not improve through the course of the experimental time (Figure 1A).

In contrast, hooves' temperature in laminitic horses ranged between 27 °C and 35.5 °C (average 33.26 °C $\pm$ 2.58) before ABM-MSCs treatment started. As a reference, the temperature in the healthy horse's hooves ranged between 27 °C and 30 °C (average 28.56 °C $\pm$ 0.79). ABM-MSCs treatment had no major impact on hooves temperature (Figure 1B). In a handful of treated horses, hoof's temperature tended to decrease slightly (1 to 3 °C) after ABM-MSCs infusions (average 32.27 °C $\pm$ 1.78). In others, temperature shifts

were highly variable throughout the experimental timeline. In all cases, nonetheless, ABM-MSCs treated horses and the sham horse (average 33.01 °C ± 1.29) never reached steady healthy values of hooves' temperature.

Figure 1: Clinical evolution of horses after being treated with allogenic bone marrow, mesenchymal stem cells (ABM-MSCs)



(A) ABM-MSCs treated horses, monitored by the Obel-Glasgow scale and infrared thermography, (B) Hooves temperature.

Hooves vascular pattern and histology of the laminar *corium* after ABM-MSCs treatment

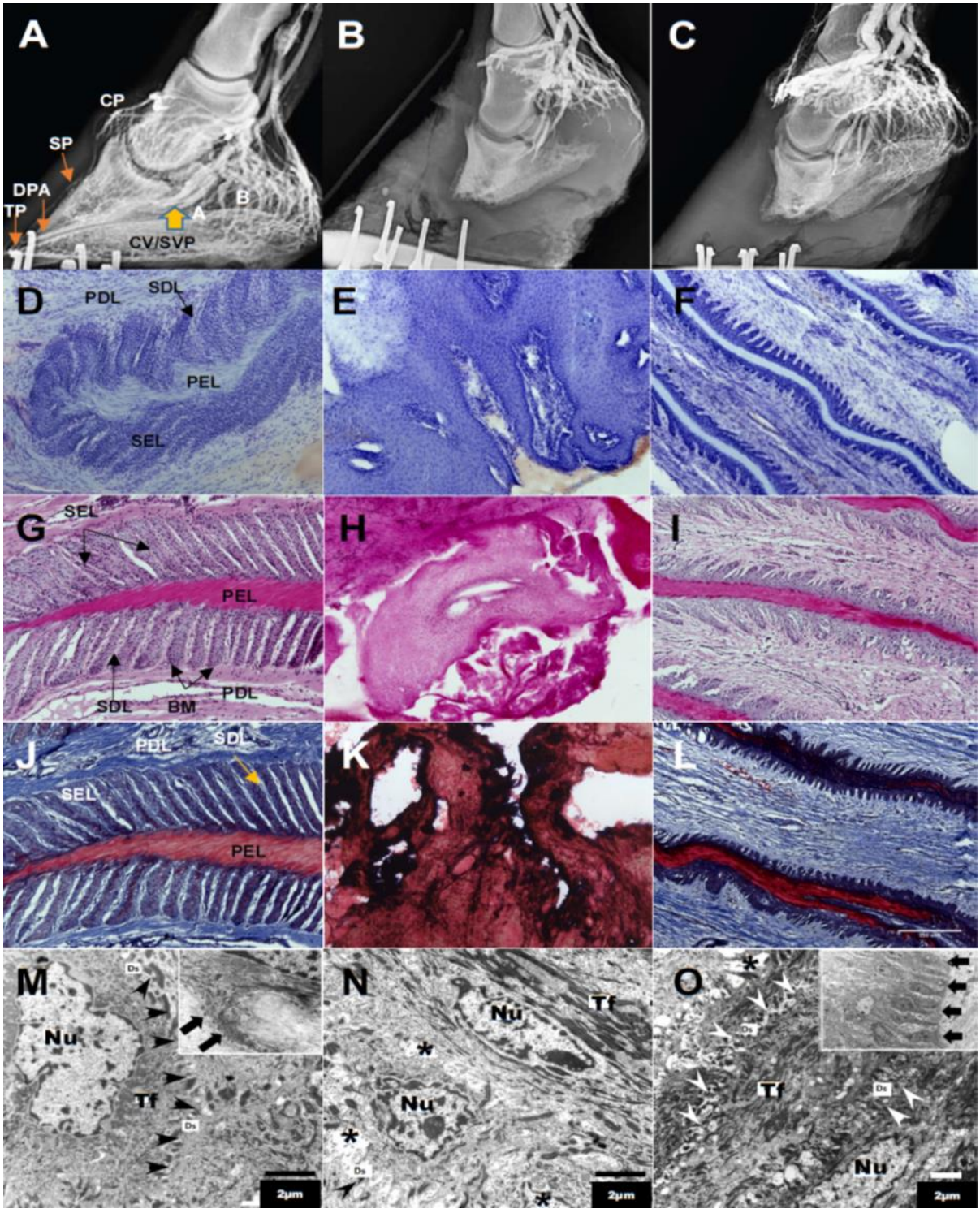
The horse's hooves are irrigated by a highly anastomosed vascular bed formed by the coronary plexus and papillae, the sublamellar venous plexus, the terminal papillae, the circumflex vessels and the sole and heel's venous plexuses (Figure 2A). In laminitic horses, the chronic dislocation stands of the distal phalanx significantly disrupted this neat vascular pattern. As seen in Figure 2B, hooves virtually become avascular; the entire vascular bed showed severe contrast media filling defects except for stem vessels in coronary and heel's plexus where vessels looked irregular and somewhat engorged. In general, the severity of vascular damage correlated with the severity of the symptoms (not shown). In contrast to the venographic findings described for the vascular landscape in laminitic hooves, images from the hooves obtained by the end of the 6th month of having begun ABM-MSC treatment revealed, in all horses regardless of the initial clinical condition and degree of vascular damage, hints of vascular recovery. Commonly, stem vessels of the coronary and heel plexus became widened and images of vascular sprouts were frequently observed as were vessels traversing somewhat "aberrant" trajectories (Figure 2C). In other words, a process of angiogenesis is clearly on its way. None of the horses treated, however, showed a full recovery of the vascular pattern, even though their clinical improvement was evident. Lastly, the sham horse showed no clinical improvement, nor venographic patterns of vascular recovery (not shown).

Histological features

ABM-MSC-infused, but not the sham, horses improved their clinical condition and their hooves' vascular patterns by the end of the 6th mo of treatment. To evaluate whether this recovery might be associated to hooves' tissue restoration, it was biopsied and histologically evaluated the laminar *corium* of a healthy, sham/laminitic and laminitic horses; the second and the third groups were sampled before and by the end of the treatment, 6 mo later from the first biopsy taken. Representative results are exhibited in Figure 2. In the healthy horse's hoof (Figure 2D), it was observed primary epidermal and dermal lamellae interdigitating regularly. Secondary epidermal and dermal lamellae, on the other hand, ran in opposite directions, but parallel next to each other. Secondary lamellae displayed rounded ends thanks to the basal cell's covering that progressed continuously up to the tip. The epidermal basal cells' nuclei were juxtaposed opposite to the basal membrane (not shown). Finally, the basal membrane formed a thin and continuous layer underneath the epithelia lining (Figure 2G) and the collagen regularly organized in parallel bundles across the dermis (Figure 2J). In sharp contrast, the hooves histology in horses with chronic laminitis showed a virtual loss of the laminar architecture (Figure 2E), basal cell hyperplasia with numerous picnotic cell profiles, (Figure 2H), abundant connective tissue, as well as perivascular mononuclear cell infiltration (not shown). No basal membrane (Figure 2H) or collagen (Figure 2K) was observed. Finally, ABM-MSC-infused, but not the sham, horses' hooves regained the laminar arrangement, the integrity of the epithelial monolayer upon a regular, continuous basal membrane and the organization and presence of collagen (Figure 2F, I and L). No evidence of significant perivascular mononuclear infiltration was detected.

Lastly, it was conducted electron microscopic observations to further evaluate the epithelial integrity in hooves of ABM-MSC-infused horses. In the healthy horse (Figure 2M), basal cells join one another by numerous desmosomes. Their eu-chromatic nuclei displayed irregular shapes characterized by indentations. They also presented a conspicuous cytoplasmic arrangement of tonofilaments. The electron dense basal lamina they were attached on was thin, regular and continuous. Laminitic hooves (Figure 2N), on the other hand, showed scarce desmosomes, abundant electro-lucid intercellular spaces. Basal cells tended to show nuclei with chromatin clumps. Cytoplasmic tono-filament aggregates were also frequent, and no evidence of basal membrane was observed. Hooves in ABM-MSC-infused horses (Figure 2O) showed a marked reduction of electro-lucid intercellular spaces. Desmosomes become a regular finding and cell nuclei return to their regular shape and chromatin arrangement.

Figure 2: Vascular pattern and histology of the laminar *corium* after ABM-MSCs treatment



A-C. Representative hoof venographies of a healthy (A), laminitic (B) and ABM-MSCs infused horses (C). Notice the vascular response in hooves of ABM-MSCs-infused horses. CP, Coronary Plexus; SP: Sublamellar Plexus; DPA: Distal Phalanx Apex; TP: Terminal Plexus; CV: Circumflex Vessels; A: Terminal Arch of the palmar digital vessels; B: Heel plexus; SVP: Soleal Venous Plexus. D-L. Representative photomicrographs of sagittal sections of the laminar *corium* of a healthy (D, G, J), laminitic (E, H, K) and ABM-MSCs infused horses (F, I, L) stained with Cresyl Violet (cell nuclei; D-F), PAS (Basal membrane; G-I) or Masson's (Collagen; J-L) histochemical techniques. Notice the restoration

of the laminar *corium* in ABM-MSCs infused horses. PEL: Primary Epidermal Lamellae; PDL: Primary Dermal Lamellae; SEL: Secondary Epidermal Lamellae; SDL: Secondary Dermal Lamellae; BM: Basal Membrane. M-O. Representative electron micrographs showing ultrastructural features of basal cells in healthy (M), laminitic (N) and ABM-MSCs infused horses (O). Notice the recovery of the chromatin and tonofilaments arrangement, as well as of desmosomes in ABM-MSCs infused horses. Nu: Nucleus; Tf: Tonofilaments; Ds and arrowheads: Desmosomes; ∗: oedema; Arrows in insets pinpoint the basal membrane. Refer to the text for a detailed description of Figure 2.

Discussion

Chronic laminitis is an inflammatory disease that affects the horse's hooves. Angelone *et al*[3] proposed that the loss of mesenchymal stem cells is the fundamental event that underlies the physiopathology of chronic laminitis. Although their results support their claim, they could not irrefutably ascribe their clinical and venographic findings to the infused adipose tissue-derived mesenchymal stem cells because the cells were co-infused with platelet-rich plasma. In addition, they confound the interpretation of their results because they infused first allogeneic and then autologous adipose tissue-derived mesenchymal stem cells in the laminitic horses. Lastly, they did not provide direct proof of laminar *corium* regeneration in the recovered horses. In this work overcome all these technical issues by infusing only ABM-MSCs, suspended in culture media, into both front hooves of horses chronically affected by laminitis. According to Angelone's prediction, infusion of ABM-MSCs into the lateral digital vein of laminitic hooves significantly improved the clinical conditions of all horses treated, while restoring considerably the cytoarchitectural features of the hooves' laminar *corium*. This was accompanied by a reduction of the inflammatory perivascular infiltration of mononuclear cells, as also predicted by Angelone *et al*[3] based upon their molecular biological results; they found increased mRNA expression of anti-inflammatory cytokines and anti-oxidative proteins. The specificity of these findings is supported by the fact that the sham horse, infused only with culture media, remained symptomatic and his hooves displayed the characteristic histological deterioration of the laminar *corium* by the end of the experimental time. Even though these results circumstantially support that ABM-MSCs promotes hooves tissue regeneration by, in part, reducing local inflammation, in this case, this notion still awaits direct confirmation by estimating pro-inflammatory cytokines in the laminar *corium* of ABM-MSCs-treated and untreated laminitic horses. As far as it is known, up to date, there is no reliable way to conduct such estimations[29,30]. In any event, as commented already, these results back up those reported by Angelone *et al*[3], who suggested that mesenchymal stem cells would promote laminar *corium* regeneration by exerting not only anti-inflammatory actions, but also by inhibiting MMPs-mediated extracellular matrix degradation, buffering ROS damage and recruiting local and circulating stem and endothelial cells. Certainly, the venographic evidence obtained in this research from treated horses also supports Angelone´s call.

A clinically relevant finding was that, even though ABM-MSCs-infused horses improved their clinical condition and restored to a great extent hooves' cytoarchitecture, hooves' temperature remained elevated in treated horses. At first glance, one might think that these findings rule out temperature monitoring as a sensitive procedure to follow up treatment progress. However, the fact that vascular restoration is partial in all treated horses by the end the experimental period suggests otherwise. On one hand, this finding might indicate that there remains some degree of inflammation that promotes temperature to stay high through the relatively avascular tissue in the still regenerating hooves. However, higher than normal hooves temperature is likely unrelated to an enduring inflammation since lameness and pain were found reduced significantly in ABM-MSCs-infused horses. Alternatively, the lack of full recovery of the vascular bed may jeopardize heat dissipation from the hooves. Although, as far as is known, such a function has not been attributed to the horse's hooves vascular network, it is well known that vascular beds may work as cooling devices in other parts of the mammalian body [for the Radiator Theory see Falk, 1990[31]]. In any event, long-time monitoring of hooves vascular bed and temperature in treated horses may help in evaluating this possibility.

Angiogenesis is a process by which blood vessels are newly formed budding off from extant precursor vessels[32-34]. In agreement with Angelone's results, it was obtained venographic evidence of ongoing angiogenesis from the coronary and heel's plexuses of ABM-MSCs-treated, laminitic horses. These data may be interpreted in three non-mutually excluding ways. First, they may show that damaged hooves' blood vessels retain their ability to produce endothelial cells, themselves capable to recreate functional vessels; so, ABM-MSCs infusion may unleash local vascular stem cells. Second, infused ABM-MSCs commit to the endothelial cell lineage thus propelling angiogenesis in hooves of the treated horses once seeded. Third, the infused ABM-MSCs may favor the recruitment of autologous, circulating stem cells that commit themselves to the endothelial lineage. Although current evidence supports that MSCs promote angiogenesis[34], the other alternatives remain open to investigation. So, future cell lineage labeling studies would surely help in disentangle this conundrum. In either case, what seems to be clear is, under the experimental conditions used, that the coronary and heel's plexuses contain niches that facilitate angiogenesis to proceed given adequate conditions. This process seems to recapitulate the embryonic sequence since vasculogenesis in the embryonic hoof proceeds following a proximal to distal gradient[35].

Another interesting finding in this work is that tissue restoration, up to the point the last biopsy was taken from ABM-MSCs-infused horses, was remarkable, having no evidence of vicious healing, nor scars of any sort, at least in the sampled site of all horses evaluated. Such an observation suggests that horse hooves may retain a biochemical ambient similar to that seen at ontogenetic stages. This possibility gains support from observation in the brain where the adult hypothalamus exhibits tremendous potential of plasticity due to the presence of poly-syalilated glycan molecules that greatly easy tissue remodeling[36,37]. Studies aimed at comparing the molecular composition of the hooves connective tissue at different ages may provide evidence to evaluate the merit of this presumption.

In a previous study[3] it was found that a mixture of aMSCs combined with PRP improves the clinical condition of horses afflicted by chronic laminitis. As they recognized, they could not determine whether the clinical improvement observed in the treated horses was due to aMSCs or PRP or responded to a synergistic effect of both, since they did not test the administration of either one alone. In this work, although stem cells are not of the same class, clearly the clinical improvement and tissue restoration can be better ascribed to the infused ABM-MSCs, since the infusion of the vehicle (i.e., culture media) had no noticeable effect on either of the parameters evaluated in the sham horse (future studies must increase the number of sham horses to strengthen these observations). What this study did not unveiled, however, is how ABM-MSCs promote tissue regeneration since vascular reconstitution was only partial [see Angelone[3], King[32], Gu[38], for theoretical considerations]. In this regard, ABM-MSCs might have migrated from the reconstituting vascular bed and colonize relatively avascular territories. Also, ABM-MSCs may produce and release soluble factors or exosomes[33,39-41], that could invigorate local stem cells to proliferate, differentiate and restitute the damaged tissue. Lastly, ABM-MSCs may promote recruiting of autologous circulating stem cells and their invasion of hooves avascular regions. Linage studies combined with additional biopsies taken in places of the hooves distant to the site of administration would help to evaluate these possibilities. They might also help in ruling out possible bias during biopsy taken since biopsies might have been obtained inadvertently from places where the vasculogenesis front was actively ongoing.

A final consideration with regard of the use of allogenic stem cells must be made. One might think that the possibility of inducing immunological reactions would increase by using allogeneic cells[42]. These results and the ones published by others[3,43,44], support that allogeneic stem cell infusion is sufficiently safe since treated horses had no evidence of rejection up to the time point they were evaluated. In contrast, even though many consider the use of autologous stem cells to be safer, recent studies show that they may develop *de novo* mutations in mitochondrial DNA that produce immunogenic neoepitopes[45], thus increasing the possibility of immunological rejection[46-49]. In addition, it has been shown that adipose-derived stem/stromal cells that recapitulate the expression of aging biomarkers show reduced stem cell plasticity[50], thus precluding their potential use as an autologous source of therapeutic cells; this might also be the case for allogeneic cells.

Conclusions and implications

In conclusion, ABM-MSCs infusions improved the clinical conditions and promote hooves histological restoration in laminitic horses. The procedure was innocuous, sex independent and effective at least for up to a period of 6 mo. Future studies must increase the sample size and the follow up time to really appreciate the long-term benefits of the

treatment and whether additional infusions of ABM-MSCs are needed. A less invasive method for administration must also be evaluated, although previous studies showed that administration through this via does not affect stem cells therapeutic efficiency and efficacy if dosage is correctly calculated. The effects of shoeing and trimming of the foot must also be evaluated. Finally, the use of ABM-MSCs to pain management is other topic that deserved investigation based on current results. In any event, the data support that the loss of mesenchymal stem cells is indeed the critical event leading to chronic laminitis. Hence, measures aimed at inducing immune tolerance against hooves' mesenchymal tissue may help in preventing such a loss. In this regard, the infusion of mesenchymal antigens obtained from laminitic hooves into the eye's anterior chamber may be a path to investigate in the coming years[51].

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Conflict of interes

Authors acknowledge that there are no competing financial interests or interest of any other source, to disclose.

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