

Tenogenesis of Equine Peripheral Blood-Derived Mesenchymal Stem Cells: *In vitro* Versus *In vivo*

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Abstract

Background: Tendon injuries are a major cause of orthopaedic injuries, and often compromise the return to the same performance level. Therefore, different regenerative therapies, such as Mesenchymal Stem Cells (MSCs) and Platelet-rich Plasma (PRP) have been explored to improve tendon healing in horses. However, ectopic bone formation of undifferentiated cells is a major concern, because of reports of this phenomenon, after intralesional injections of MSCs in rabbit Achilles tendons.

Methods: After MSC and PRP isolation of the Peripheral Blood (PB) of a donor horse, tenogenic induction of the MSCs (Tendo-Cell[®]) was confirmed through morphological changes and immunohistochemistry stainings. Secondly, the safety and clinical effects (ultrasound imaging) of Tendo-Cell[®], in combination with PRP, was evaluated after a single intralesional injection in the lateral edge of the Superficial Digital flexor tendon (SDFT) (n=10) and the lateral branch of the suspensory ligament (SL) (n=15) in 25 horses. Different independent veterinary practitioners were asked to give a score from 0-5, at approximately 6 weeks after treatment (0=no ultrasonic improvement and 5=no ultrasonic abnormalities, the tendon has the same consistency and fiber orientation as the contralateral tendon).

Results: In 96% of the patients, the same positive evolution was noticed on the ultrasound images, and this was translated to a score 3 or more ($\geq 60\%$ improvement or better than a successful conservative therapy). In addition, for both tendons (SDFT & SL), 40% of the horses received a score 5.

Conclusion: In conclusion, the present study is the first to describe the treatment of lesions in the SDFT and SL, with allogenic tenogenic induced PB-derived MSCs, in combination with PRP, with a positive outcome in 24 out of the 25 horses.

Keywords: Allogenic; Stem cells; Blood; Horse; SDFT; SL

Introduction

Tendon injuries are a major cause of orthopaedic injuries, and often compromise the return to the same performance level. In thoroughbreds, tendon and ligament injuries are the most common orthopaedic injuries [1,2], and a large number of event, dressage and show jumping horses [3,4], suffer from tendon pathologies, as well. After an injury, tendons heal with the formation of scar tissue. Although it has been reported in early chronic tendinopathies that tendon structure may normalize in some cases [5], in other cases, scar tissue may have important consequences for the individual, in terms of reduced performance and a substantial risk of re-injury [6]. Therefore, there is a need to restore tendon functionality and this has encouraged the development of regenerative therapies [7]. In this regard, it has been reported that 82% (n=168) of the horses suffering from tendinosis of the Superficial Digital Flexor Tendon (SDFT), treated with bone marrow-derived Mesenchymal Stem Cells (MSCs), performed at their original level without re-injury in the next year [8], whereas 42-44% of the horses with SDFT tendinosis treated with conservative therapy, with hyaluronic acid and polysulphated glycosaminoglycans re-injured [9]. Moreover, a 2 year follow-up study of 141 horses with SDFT tendinosis confirmed a significantly lower re-injury rate of 27%, after bone marrow-derived MSC treatment [10]. In addition, the use of adipose tissue-derived MSCs has been described for the treatment of several clinical cases of acute and chronic tendinopathy, as well [11,12]. Because autologous MSC treatment is not always feasible under field circumstances, sample sterility depends on the practitioner, donor influences are difficult to control, and the isolation protocol takes

approximately 1 month, we decided to use allogenic MSCs as a basis for this study.

Although different human and equine studies report no ectopic bone formation after the clinical application of MSCs [8,10,11,13-16], in rabbits, ectopic bone formation has been reported in 28% of the animals (n=34), after injecting their bone marrow-derived MSCs into acute tendon lesions [5]. Indeed, animals with a high tendency for the formation of calcifications have a higher risk of ectopic bone formation after MSC injection in their tendons, because of the disturbed micro-environment or niche. In order to avoid this, MSCs could be tenogenic induced, before clinical application.

In addition, positive effects of PRP on MSC tenogenesis could be attributable to the various endogenous Growth Factors (GFs) PRP contains: Platelet-derived GF (PDGF), Transforming GF-beta (TGF- β), Insulin-like GF (IGF) and Vascular Endothelial GF (VEGF) [17]. Therefore, PRP may stimulate cell proliferation and the synthesis

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of Extracellular Matrix (ECM) components after intralesional injection in a tendon, in combination with MSCs [18]. The addition of PRP to human tenocytes *in vitro* resulted in cell proliferation, total collagen deposition, and improved gene expression for matrix degrading enzymes and endogenous GFs [19]. Moreover, *in vitro* studies with Superficial Digital Flexor Tendon (SDFT) explant cultures have shown that PRP increases the expression of tendon matrix genes in these explants [20]. In this regard, the successful use of autologous Platelet-rich Plasma (PRP) to treat chronic desmitis of the suspensory ligament (n=3) has been reported [21]. Also, another group described the *in vivo* effects of PRP injection 1 week after the surgical induction of SDFT defects, using several different parameters. In those placebo-controlled experimental studies, they found an increased neovascularization, as well as improved histological, biochemical and biomechanical properties of the tendons [22,23], suggesting that adding PRP to MSC might be beneficial in the treatment of tendon lesions. Indeed, it has been reported that using PRP in combination with bone marrow-derived mononuclear cells for the treatment of chronic equine tendinosis, would enhance equine tendon regeneration in clinical cases [24].

For all the aforementioned reasons, the first goal of the present study was to tenogenic induce allogenic Peripheral Blood (PB)-derived MSCs (Tendo-Cell®). Secondly, the safety and clinical effects (ultrasonography) of Tendo-Cell®, in combination with PRP, was evaluated after a single intralesional injection in the lateral edge of the SDFT (n=10), and the lateral branch of the suspensory ligament (n=15) of 25 horses, in total.

Materials and Methods

Isolation and tenogenic induction of Mesenchymal Stem Cells (MSCs)

In total, 50 ml of blood was collected in sterile EDTA tubes from the *vena jugularis* of the 6-years-old donor gelding for Mesenchymal Stem Cell (MSC) isolation. At the same time, serum was collected and sent to Böse laboratory (Harsum, Germany), for testing on the following transmittable diseases: Equine Infectious Anemia (EIA), Equine Rhinopneumonia (EHV-1, EHV-4), Equine Viral Arteritis (EVA), West-Nile Virus (WNV), African Horse Sickness (AHS), Dourine (*Trypanosoma*), Piropalmsosis, Malleus, Glanders, Equine Influenza A (equi I and II, American and European type) and Borreliosis (*Borrelia burgdorferi*, the Lyme disease). Three weeks later, a second blood sample was sent to Böse again, in order to confirm if antibody production was due to vaccination. After arriving in the lab, the 50 ml of blood was centrifuged at 1000 G for 20 minutes, and the buffy coat was collected and diluted 1:2 with Phosphate-buffered Saline (PBS). Afterwards, this suspension was gently layered on an equal amount of percoll (GE Healthcare). The further isolation was performed, as previously described [25]. Then, 20 million Peripheral Blood Mononuclear Cells (PBMCs) were seeded per T₇₅ flask in 3 flasks and cultured in culture medium, as previously described [25]. The medium was refreshed twice a week and the cells were maintained at 37°C and 5% CO₂. At 60% confluency, the cells were trypsinized with 0.25% trypsin-EDTA and subcultured in tenogenic inducing medium for 3 days. At the next confluency, the cells were trypsinized and resuspended in 1 ml of Dulbecco's Modified Eagle Medium (DMEM) low glucose, with 10% of Dimethyl Sulfoxide (DMSO, Sigma). At this point, the Tendo-Cell® was frozen overnight in isopropanol at -80°C. The samples were then stored in -80°C and shipped on dry-ice before clinical application.

Preparation of Platelet-rich Plasma (PRP)

In total, 300 ml of peripheral blood was taken in a Citrate Phosphate Dextrose Adenine-1 (CPDA-1) single blood bag (Terumo®) for Platelet-rich Plasma (PRP) preparation. From this donor horse, 25 samples of 1 ml PRP were prepared, as previously described [26]. Each sample contained approximately 200×10⁶ platelets, and was frozen and stored at -80°C, before clinical application.

Immunohistochemistry

Immunohistochemistry (IHC) was performed to evaluate the expression of markers present on tenogenic induced MSCs. Cells were fixed for 10 minutes with 4% PF and permeabilized for 2 minutes with 0.1% Triton X at room temperature. Subsequently, cells were incubated with hydrogen peroxide (0.03%) for 5 minutes at room temperature and after washing with PBS, incubated for 2 hours at room temperature with the primary mouse IgG2a monoclonal anti-human Smooth Muscle Actin (SMA) antibody (Dako, 1:200) and the rabbit IgG polyclonal anti-human collagen type IA2 (Col IA2) antibody (Abcam, 1:100). After washing with PBS, secondary ready to use goat anti-mouse and anti-rabbit Peroxidase (PO)-linked antibodies (Dako) were added and incubated for 30 minutes at room temperature. Finally, 3,3'-Diaminobenzidine (DAB) was added for 2-10 minutes, and a counter staining with hematoxylin was performed to visualize the surrounding cells. As controls, identical stainings were performed on undifferentiated MSCs, and background staining was assessed by using the proper isotype-specific IgG's. All isotypes were matched to the immunoglobulin subtype and used at the same protein concentration as the corresponding antibodies.

Injecting Mesenchymal Stem Cells (MSCs) and scoring system

After thawing, the Tendo-Cell® (1 ml) and PRP (1 ml) were aspirated in the same syringe and intralesionally administered by means of ultrasound guidance. Twenty five acceptor horses were selected based on their injuries: a clear lesion had to be visible on the ultrasound, at the lateral edge of the superficial digital flexor tendon (SDFT, n=10), or at the lateral branch of the suspensory ligament (SL, n=15). Clinical lameness was also noticeable in most of the cases. Afterwards, the horses were closely monitored for 1 week, by means of a daily examination of their tendons, and by observing possible adverse effects or hypersensitivity reactions (wheal formation, sweating, strong respirations, or even fever). Subsequently, the tendons were evaluated at approximately 6 weeks post injection through ultrasound imaging, and by lameness evaluation. Four veterinary practitioners were asked to give a score between 0 and 5 for their ultrasound images, at approximately 6 weeks after the treatment. A score of 0 corresponded with 0% improvement, or no ultrasonic improvement; 1=20% improvement or little ultrasonic improvement, but less than after conservative treatment; 2=40% improvement, or greater ultrasonic improvement, as is usually seen after a successful conservative treatment; 3=60% improvement, or better ultrasonic improvement than after successful conservative therapy; 4=80% improvement or very good ultrasonic improvement, much better than after successful conservative therapy, but not yet fully recovered; 5=100% improvement or no ultrasonic abnormalities, the tendon has the same consistency and fiber orientation as the contralateral tendon.

Results

Isolation and tenogenic induction of Mesenchymal Stem Cells (MSCs)

After 17 days, the first spindle shaped cells were noticed in the culture flasks, and at 21 days after isolation, the cells were trypsinized at approximately 60% confluency. Light Microscopic images (LM) of the isolated cells, as well as after tenogenic induction, are depicted in figure 1. Undifferentiated MSCs had a stellate/spindle-shaped morphology and grew in colonies. After tenogenic induction, the cells showed a stretched morphology and fiber orientation could be noticed after 3 days of culturing in the tenogenic inducing medium.

Immunohistochemistry

After 3 days of tenogenic induction, the cells were all positive for Smooth Muscle Actin (SMA), which strongly indicates that they gained in elasticity (Figure 2). Moreover, some of the cells started to produce collagen type I, which is the functional tendon collagen (Figure 3). These results implicate that tenogenic induction was successful, but that the cells were not terminally differentiated towards tenocytes, because the extracellular matrix production remained limited. This was in fact one of our objectives, since we were aiming to induce the MSCs towards tenocytes, without producing all the extracellular matrix components *in vitro*, before their *in vivo* application.

Scoring of the ultrasound images

It has been reported that frozen equine PB-derived MSCs do not lose their stem cell characteristics [27]. In this regard, the use of frozen tenogenic induced samples was defensible. After the treatment with allogenic tenogenic induced MSCs (Tendo-Cell®), the horses were closely monitored and no adverse effects could be noticed by the attending veterinarian, or by the owners. After approximately 6 weeks, in all 10 horses with a lesion in the lateral edge of the Superficial Digital Flexor Tendon (SDFT), a score 3 or more was given (60% improvement, or better ultrasonic improvement than after successful conservative therapy) (Figure 4). Indeed, the ultrasound images of figure 5 show that there is a substantial improvement, 39 days after the treatment on the longitudinal (left images), as well as the transversal ultrasound images (right images). In addition, it should be mentioned that 4 out of the 10 horses (40%) even had a score 5 (100% improvement or no ultrasonic abnormalities, the tendon has the same consistency and fiber orientation as the contralateral tendon) at 6 weeks after the treatment (Figure 4). After approximately 6 weeks, in 14 out of 15 horses with a lesion in the lateral branch of the Suspensory Ligament (SL), a score 3 or more was given as well (Figure 4). Moreover, as for the SDFT lesions, 40% of the horses with a SL lesion (6 out of 15) received a score 5 (Figure 4). Also here, ultrasound images revealed promising results as shown in figure 6. In addition, it should be noticed that even a calcification (black arrow) did not hinder the tenogenic induced MSCs to fill up the defect without further calcifying the tendon, which was the goal of the present study.

Discussion

In the present study, we have performed a tenogenic induction of Peripheral Blood (PB)-derived Mesenchymal Stem Cells (MSCs) (Tendo-Cell®), with clinical application in combination with Platelet-rich Plasma (PRP) in 25 horses. In 96% of the cases (24 out of 25), the same positive evolution was noticed on the ultrasound images, which was translated in a score 3 or more (better than a successful conservative

therapy). Since the MSCs used in this study were tenogenic induced, no ectopic bone formation was expected after injection. Indeed, in all the cases, no ectopic bone formation was noticed, and even a calcified micro-environment (black arrow, Figure 6) did not induce any supplementary calcifications in one case. However, further research might provide more insights in the exact mechanisms of ectopic bone formation and inhibition after cell therapy. The use of allogenic tenogenic induced MSCs and PRP did not induce any other adverse reactions. In this regard, it has been reported that MSCs inhibit the innate immune activation, by suppressing monocyte and T-cell activity with a significant reduction of inflammation activators, such as Tumor Necrosis Factor- α (TNF- α) and Interleukin (IL)-6 [28-32]. Moreover, MSCs increase the *in vitro* production of inflammation reducing agents,

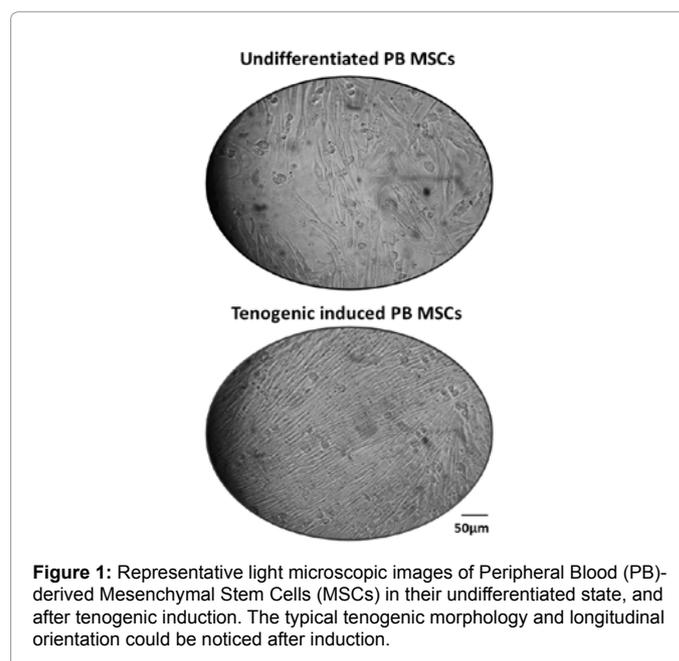


Figure 1: Representative light microscopic images of Peripheral Blood (PB)-derived Mesenchymal Stem Cells (MSCs) in their undifferentiated state, and after tenogenic induction. The typical tenogenic morphology and longitudinal orientation could be noticed after induction.

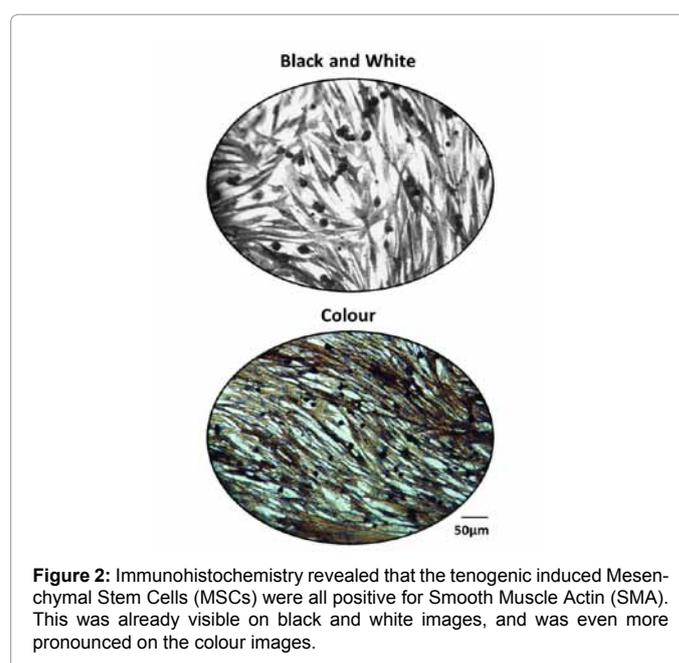
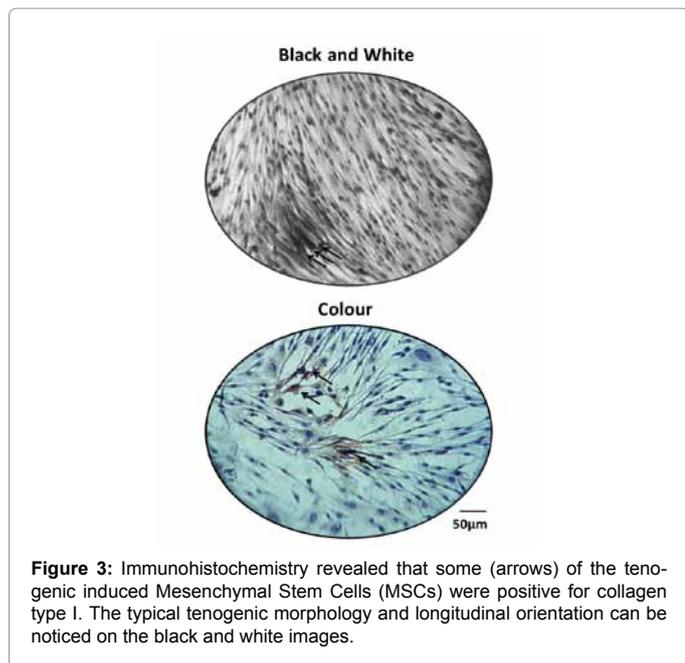


Figure 2: Immunohistochemistry revealed that the tenogenic induced Mesenchymal Stem Cells (MSCs) were all positive for Smooth Muscle Actin (SMA). This was already visible on black and white images, and was even more pronounced on the colour images.

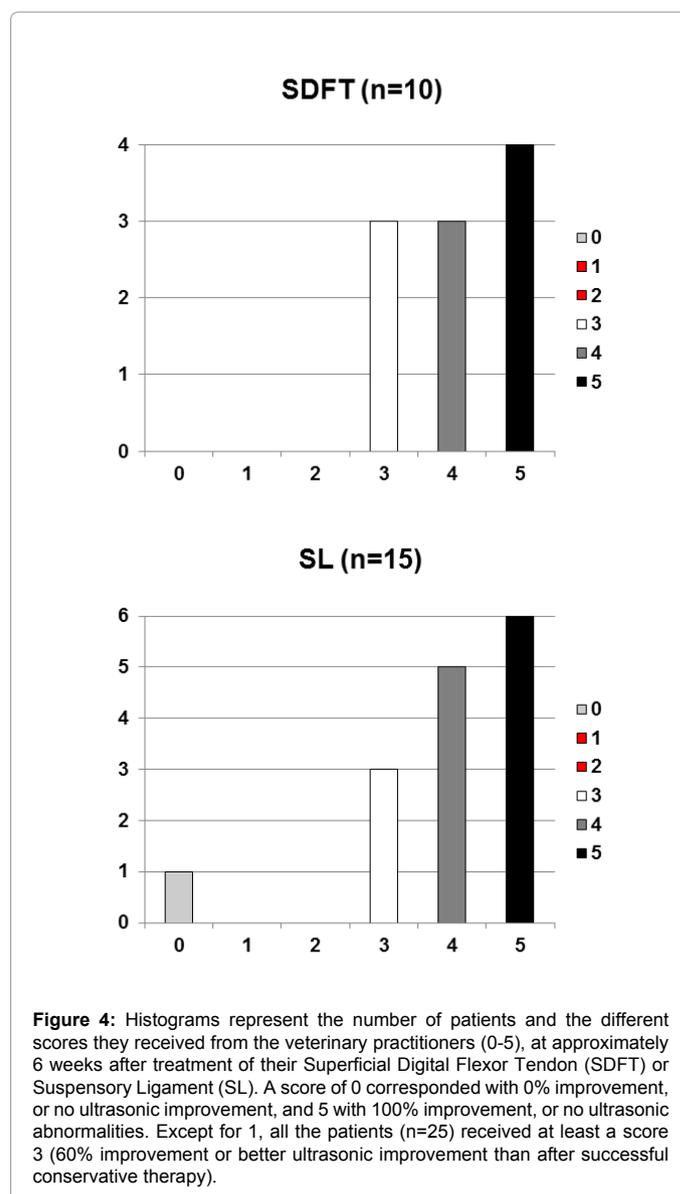


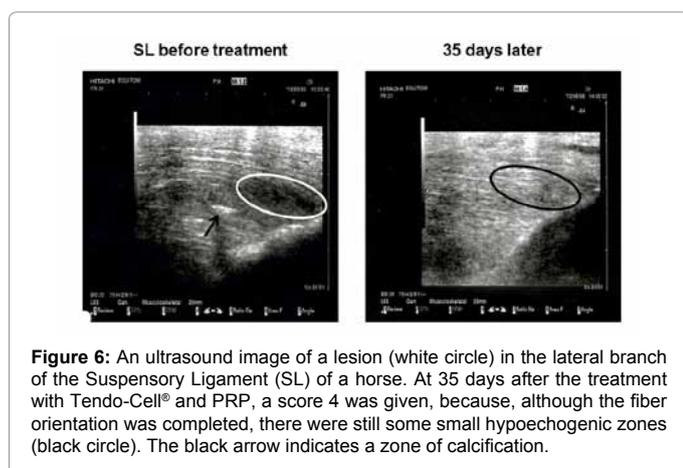
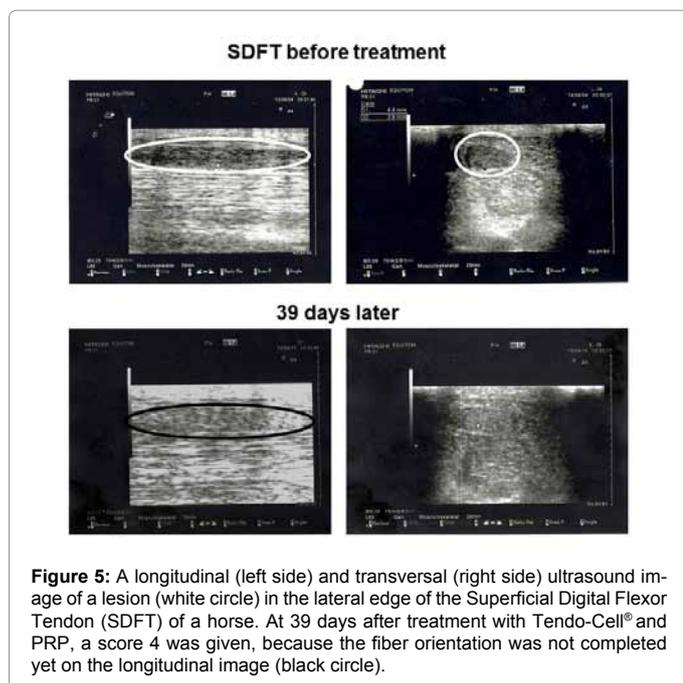
such as IL-1 receptor antagonist [32] and IL-10 [33]. In fact, the safe use of allogenic undifferentiated MSCs has been described before in different human and equine studies [34-37]. However, in 2010, Huang et al. [38] described an *in vitro* increase of Major Histocompatibility Complex (MHC)-Ia and -II (immunogenic) expression and a reduction of MHC-Ib (immunosuppressive) expression, after inducing MSCs towards different cell types with myogenic, endothelial, or smooth muscle characteristics. Whether or not this is the case after *in vivo* tenogenic differentiation, is definitely worth looking into. Concerning the use of allogenic PRP, a safe transfusion of allogenic platelets has been previously reported in humans [39]. Therefore, the combination of these cell types could be safely administered in an allogenic setting. Of course, these cells were derived from one and the same donor. Unfortunately, we have no data on the clinical effects of cells from other or different donors. In this regard, it has been described that older human donors do not only generate stem cells with a significant lower proliferative and migratory potential, but also with a lower differentiation capacity than their younger counterparts [40].

In addition, it has to be mentioned that the scoring veterinary practitioners were not blinded and no control groups were included in the present study. Nevertheless, the present study reports a score 3 or more in 100% (10<10) of the SDFT tendinitis cases, at approximately 6 weeks after treatment. Communication with the veterinary practitioners revealed that 70% of the horses were able to start cantering exercises at 4 months after the treatment. In this regard, it has to be mentioned that literature reports cantering exercises of 57% of the horses, at approximately 1 year after treatment with conservative therapy (hyaluronic acid and polysulphated glycosaminoglycans) [9], and 82% of the horses at 8 months after bone marrow-derived MSC treatment [8]. In the study, 40% of the horses with SDFT lesions showed a 100% filling and fiber alignment (score 5) at only 6 weeks after treatment, whereas Dyson described a fiber alignment score of 0 (75-100% parallel fiber alignment) at 4 months after conservative treatment in 50% of the horses (n=68) [9]. For collagenase-induced SDFT lesions treated with bone marrow-derived MSCs or PBS, Schnabel et al. [41] reported no significant differences in ultrasound parameters after their

8 week study in 12 horses (24 tendons), whereas in the present study, a considerable ultrasonic improvement was noticed at 6 weeks after the treatment.

Concerning Suspensory Ligament (SL) desmitis, less information is available to date. Nevertheless, Herthel [42] described a return to full work of 84% of the horses at 1 year after treatment with bone marrow-derived MSCs, in comparison to 15.2% of a control group. In the present study, 93% (14<15) of the horses treated with tenogenic induced MSCs in combination with PRP, received a score 3 or more, and 73% started cantering exercises at 4 months after the treatment. For all the aforementioned reasons, we may conclude that the present study contains promising preliminary results, for the treatment of both SDFT and SL lesions, but further research should focus on the use of control groups and a double-blinded set-up. Moreover, a long-term follow-up study could provide valuable data, concerning the total number of horses that reach their previous performance level and the sustainability of their tendons.





In conclusion, the present study is the first to describe a successful treatment of lesions in the lateral edge of the SDFT, and the lateral branch of the SL with allogenic tenogenic induced equine PB-derived MSCs, in combination with PRP in 25 horses.

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Author Disclosure Statement

The authors declare competing financial interests in Global Stem cell Technology.

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