Case Report

Treatment of Equine Tarsus Long Medial Collateral Desmitis with Allogenic Synovial Membrane Mesenchymal Stem Cells Enhanced by Umbilical Cord Mesenchymal Stem Cell-Derived Conditioned Medium: Case Report

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Treatment of Equine Tarsus Long Medial Collateral Desmitis with Allogenic Synovial Membrane Mesenchymal Stem Cells Enhanced by Umbilical Cord Mesenchymal Stem Cell-Derived Conditioned Medium: Case Report

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Abstract: Horses are high performance athletes prone to sportive injuries such as tendonitis and desmitis. Fibrous tissue formation with loss of mechanical properties occurs in tendon repair, becoming a therapeutic challenge to overcome. This impels regenerative medicine to develop innovative therapies that enhance tissue regeneration retrieving original tissue properties. Mesenchymal stem cells (MSCs) have been successfully used to develop therapeutic products. They secrete a variety of bioactive molecules that play a pivotal role in tissue regeneration. These factors are released in culture media producing conditioned media (CM). The aforementioned assumptions impelled us to formulate equine synovial membrane stem cells (eSM-MSCs) – the cellular pool that naturally regenerates joint tissue, combined with medium enriched in immunomodulatory factors produced by umbilical cord stroma-derived MSCs (eUC-MSCs), that naturally contribute to suppress the immune rejection in the maternal-fetal frontier. A clinical case of an equine acute desmitis, treated with the abovementioned formulation is presented. Ligament regeneration occurred in a reduced time frame, reducing stoppage time, allowing return to unrestricted competition after completion of a physical rehabilitation program. This study focus was determination of the formulation therapeutic potential and the evaluation of its synergistic effect in an equine desmitis treatment, utilizing the cells themselves and its secretome.

Keywords: allogenic; case study; ligament; mesenchymal stem cells; MSC-based therapies; secretome; sport horses; synovial membrane mesenchymal stem cell; umbilical cord conditioned medium

1. Introduction

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Tendon and ligament injuries account for a large proportion of sport horse's wastage and early retirement. After tendon/ligament injury, the scar tissue that replaces the damaged tendon, reduces athletic performance and has a high risk of reinjury. Most injuries are overstrained injuries as tendons and ligaments operate near their functional limits in sport horses. Tendons and ligaments are highly organized tissues that depend on the strength and structure of the extracellular matrix (ECM) to function [1]. Overloading can lead to physical damage and degeneration [1,2]. Although tendons and ligaments can heal spontaneously with time, the fibrous scar tissue formed is biomechanically inferior leading to recurrent reinjuries and lameness.

1.1. Tarsocrural desmitis

Tarsocrural desmitis, an inflammatory event affecting the ligaments of the tarsocrural joint, is a cause of severe hindlimb lameness in horses, often clinically underdiagnosed because it is uncommon and has a guarded prognosis for athletic soundness [3]. Tarsus pathologies are a complex puzzle to solve because of an intricate relationship between structures: synovial structures, bone, bursae, ligaments, tendons and tendon sheaths that are susceptible to a considerable incidence of pathology [4]. An overview of kinematic and a good knowledge of anatomy are essential for a better understanding of tarsus pathology [5] – Figure 1.

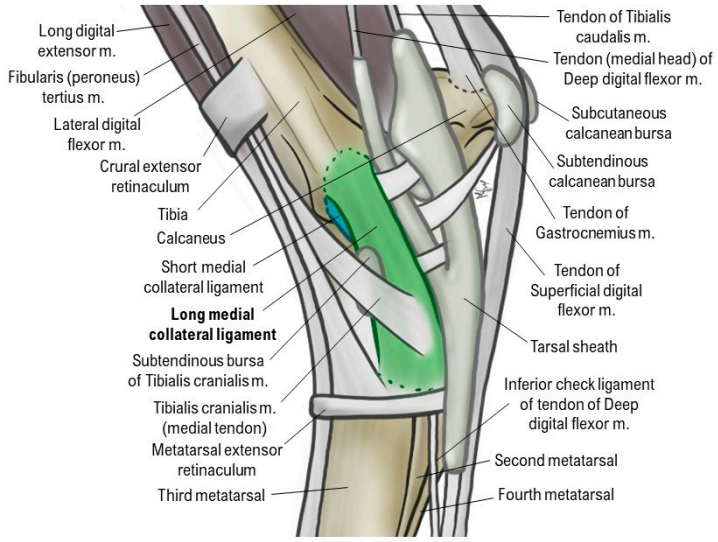

Figure 1. Anatomy of medial aspect of right tarsocrural joint. This image evidences the complexity of the tarsocrural joint structures. Long medial collateral ligament is highlighted in soft green.

1.1.1. Anatomy and Biomechanics

Tarsus movement is linked with stifle movement, coordinating due to reciprocal apparatus [6]. This reciprocal mechanism is responsible for the long standing position of equines, keeping their hindlimbs straight without muscular effort [7]. This is an anatomical feature of the equine hindlimb characterized by simultaneous stifle, hock and fetlock extension and flexion, due to interaction of ligaments and muscles. This mechanism is also important for counteraction and absorption of concussive forces.

The tarsus is a ginglymus joint, a uniaxial joint in which a transversely cylindric convexity on one bone – cochlea of the tibia - fits into a corresponding concavity on the other – trochlea of the talus, forming the tibiotarsal joint - allowing motion in one plane only.

When the joint is flexed, the distal limb is flexed and is pulled slightly to one side as the trochlear ridges slant outwards. The tibiotarsal joint is the joint of highest motion, accounting for 90% of motion

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range. The three lower joint below are responsible for the remaining 10% of the motion range [7,8]. To ensure the distal tarsal bones have minimal movement there is a system of collateral ligaments.

There are four collateral ligaments in the tarsus. Long lateral collateral ligament (LLCL): arises from the lateral malleolus of the tibia and inserts on the proximal end of metatarsal IV (lateral splint bone). Between these points it attaches to the lateral tarsal bones. Short lateral collateral ligament (SLCL): deep to the long lateral collateral, it arises from the lateral malleolus of the tibia and attaches via two branches, one to the calcaneus and one to the talus. Long medial collateral ligament (LMCL): arises from the medial malleolus and inserts on the proximal end of metatarsal II (medial splint bone). Between these points it attaches to the medial tarsal bones. Short medial collateral ligament (SMCL): deep to the long medial collateral, it arises from the medial malleolus and attaches via two branches, one on the calcaneus and one on the talus.

From a functional perspective, the long CLs are taut during extension and loose during flexion of the tarsocrural joint. The short CLs show a more complex behavior during the range of motion of the tarsocrural joint but most parts are tense in flexion and loose in extension [5].

1.1.2. Clinical signs

Intra-articular soft tissue injuries of the tarsus are poorly documented because the clinical signs are very unspecific and may include edema over the injured collateral ligament and/or effusion within the tarsocrural joint and mild to non-weight bearing lameness with positive joint flexion[9,10]. Chronic pain and osteoarthritis (OA) may develop secondary to joint instability leading to cartilage damage in horses with collateral ligament injuries of the tarsocrural joint. *Blaik et al.*, 2000, reported tarsal pain is responsible for 80% of chronic, low-grade hind limb lameness in horses, with bone spavin, osteochondrosis, tendon and ligament injury and synovial disease being frequently diagnosed [11]. Hemarthrosis may also develop and is documented as a major factor of pain in both horses and humans [4,9,12–17].

1.1.3. Diagnostic complementary exams

As a result of nonspecific clinical signs, definitive diagnosis is often difficult to achieve, and implies resorting to diagnostic imaging techniques such as radiology, ultrasonography, computed tomography (CT) or magnetic resonance imaging (MRI) [11,18–20].

Radiology is a noninvasive and less expensive technique but has limitations when evaluating complex joints once it gives a two-dimensional (2D) image of three-dimensional (3D) structures. Multiple soft tissues and bony structures are superimposed being difficult to evaluate them, making radiography an unappealing method for soft tissue evaluation [21,22]. However, in cases of tarsus distension, radiography should be advised as an initial diagnostic procedure. It is useful in the diagnosis of avulsion fractures and their configuration. In acute cases of CL desmopathy or enthesopathy without fragmentation of the insertional surface, it may reveal only soft tissue swelling [23].

Diagnostic ultrasound is also a noninvasive and inexpensive technique, excellent for soft tissue evaluations and provides a dynamic evaluation. A thorough ultrasound assessment of tarsal CL injuries is very important to correctly diagnose these lesions [23]. The disadvantage is that it does not allow visualization of structures deep to bone [13,22,24].

Computed Tomography allows visualization of bones and soft tissue, avoiding superimposition of structures, enabling a 3D image reconstruction [20]. However, it implies general anesthesia.

Magnetic Resonance provides cross sectional images which allow accurate and detailed visualization of soft tissues and bones [20]. The other advantage is that is possible to do standing MRI's with an open U-shaped MRI, that is designed to enable imaging up to the level of carpus and tarsus [25]. Closed MRI's have the same disadvantage as CT scans, the need of general anesthesia [20,25].

Diagnosis of tarsus collateral ligaments desmitis is often made by ruling out other causes of articular injury in combination with diagnostic imaging specific for soft tissue such as ultrasound and MRI [18].

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Garrett, 2014, explains that desmitis of any of the collateral ligaments can occur, it is most commonly seen in the long medial collateral ligament [13]. Horses with collateral ligament desmitis typically demonstrate moderate to severe lameness but may present an obscure lameness accompanied by synovial effusion and minimal radiographic change [26]. Ultrasonographic signs of desmitis are similar to those in any ligament and include increased size, decreased echogenicity, and abnormal fiber pattern [13].

Medial tarsal colateral desmitis has been few described in veterinary literature (*Lamb et al*, 2012; *Sherlock*, 2011; *Tokkatelof et al*,2011; *Raes et al*, 2009; *Bramlage et al*, 2006; *Withcomb*, 2006; *Rose and Moore*, 2003; *Dik*, 1993), however is one of the most common soft tissue injuries of the tarsus [13]. *Whitcomb*, 2006, corroborated this fact by presenting a study where collateral desmitis was the most common tarsal tendon or ligament injury (26/128 horses), being medial collateral desmitis of tarsus the most common ultrasonographic diagnosed injury (18/26 horses) [27].

1.1.4. Treatment: current regenerative therapies

Regenerative medicine aims to replace or regenerate cells and tissues and to restore normal structure and function of injured tissue [1,3]. Nowadays there are different approaches of regenerative therapies, namely stem cell therapies, immunomodulatory therapies or paracrine therapies, gene therapies and tissue engineering.

Among these, the most used in equine musculoskeletal medicine are stem cell therapies and immunomodulatory/paracrine therapies. They are gaining increased interest due to their anti-inflammatory and immunomodulatory properties, regenerative potential, and high tolerability [7].

Stem cell therapies consist in the injection of mesenchymal stem cells (MSC's) or induced pluripotent stem cells (iPSCs) [4–6]. As immunomodulatory or paracrine therapies, we may consider hemoderivative therapies such as platelet rich plasma (PRP) [1,3,9–12], autologous conditioned medium (ACS) [13–17], autologous protein solution (APS) [18,19] and, more recently, receiving more attention from the scientific community – extracellular vesicles (EVs). PRP's gained a special attention in equine and human medicine due to their ability of angiogenesis, proliferation and migration of fibroblasts, collagen synthesis and chemotaxis of macrophages, which are necessary for tissue healing. Platelets are a rich source of growth factors, cytokines and chemokines, released during the early stages of tissue healing. PRP is produced after a centrifugation process of whole blood in which red blood cells and buffy coat are separated from plasma, which is rich in platelets. Platelets release a range of growth factors responsible for healing processes, such as IGF-I and IGF-II, TGF-β1, FGF, VEGF, PDGF and Platelet Derived Epidermal Growth Factor (PDEGF), after degranulation of alpha granules in the platelet cytoplasm following activation with citrate [7].

To sum up, the PRP provides a growth factor concentrate that enhances cellular repair of musculoskeletal lesions. The main advantages of PRP as a regenerative therapy are its autologous nature, rapid preparation and non-invasive collection process [8].

Autologous conditioned serum (ACS) is another hemoderivative prepared with the use of a commercial kit, by the incubation of the horse own whole blood with medical-grade glass spheres, resulting in serum enrichment in interleukin-1 receptor antagonist (IL-1Ra), anti-inflammatory cytokines (IL-4, IL-10, and IL-13), and high concentrations of growth factors including insulin-like growth factor-1, platelet-derived growth factor, and transforming growth factor-beta, in the liquid blood phase [7,9]. In equine medicine, ACS is commercially known by the name - Interleukin Receptor Antagonist Protein (IRAP®)- is a natural anti-inflammatory product used for treatment of many joint injuries and lameness [7]. The interleukyne-1 (IL-1) is one of the major mediators responsible in the pathogenesis of osteoarthritis as it activates an inflammatory response leading for cartilage degradation. Blood derived cytokines and proteins have an important anti-inflammatory action through a competitive blocking of IL-1 receptors [7,10].

Autologous protein solution – APS - was developed almost a decade after initial investigation of ACS [11] and intends to increase the anti-inflammatory and anabolic concentrations of hemoderivatives of clinical use. APS consists in the preparation of a PRP that is processed in a special commercial kit intended to stimulate white blood cells (WBC) to produce anti-inflammatory cytokines

concentrating its content in a smaller volume of plasma. In equines, APS is commercially available as Prostride®, and presents significantly more white cells, platelets and less erythrocytes compared with whole blood [12]. Cytokines such as Tumor Necrosis Factor-α (TNF-α), Transforming Growth Factor-β (TGF-β), IL-1β, IL-6, IL-10, Matrix Metalloproteinase-3 (MMP-3), and IL-1Ra among others [12,13] were detected in APS. In fact, a positive ratio of IL1Ra:IL-1β was observed in APS [14]. Horses with naturally occurring OA, APS significantly improved lameness, pain-in-flexion, gait analysis and range of motion up to 14 days after treatment compared with baseline and controls [13]. In equine joint fluid, there was a significant decrease in protein concentration in treated horses compared to untreated controls [12–14].

Mesenchymal stem cells exert their anti-inflammatory and pro-regenerative action through the secretion of soluble factors with paracrine action. Recent studies reveal that part of this action is mediated by extracellular vesicles which are soluble factors from MSCs secretome. EV's are important mediators in cell-to-cell communication and have various subtypes as exosomes and microvesicles [15]. They carry certain proteins, glycoproteins, lipids, and ribonucleic acids that transmit biological information to support healing in injured tissues. EV's can be used naturally or engineered in order to provide superior biocompatibility and biostability, representing a big therapeutical promise in regenerative medicine as they are considered useful for stimulating regeneration with comparable effectiveness to MSC's [7], this way they are considered one of the best candidates to replace cells in regenerative and immunomodulating medicine field [16]. Generally, they also have low immunogenicity, non-cytotoxicity, and non-mutagenicity, these natural nano-sized carriers hold the superior potential to liposomes and polymeric nano formulations in the drug delivery research areas [16].

1.2. Regenerative medicine in desmitis: Cell- based and Cell-free therapies

MSCs are adult multipotent progenitor cells found in many organs and tissue types, that are able to self-renew, migrate to injury sites (homing), multilineage differentiation and secrete bioactive factors, providing immunomodulation, increasing proliferation and migration of tendon stem/progenitor cells via paracrine signaling and increasing regeneration ability of tissues with poor aptitude [28–33]. MSCs hold immense promise for use in diverse cell-based therapies [34]. Originally considered as whole-cell therapy, whereby injected MSCs migrate to the site of tissue damage (homing) and differentiate into cells needed for repair or regeneration, it is now accepted that transplanted MSCs do not survive for long and that the effects of MSC-based therapies are due to a broad array of secreted bioactive factors, collectively referred to as the secretome [35–37]

The recognition that MSC secreted factors are responsible for the positive effects of MSCs on tissue repair is significant, as it spurs the design of MSC-based therapies that do not require administration of the cells , thus avoiding negative side effects that might be correlated with cell administration such as unwanted differentiation of engrafted MSCs, immune reactions or unwanted tumor growth [38,39]. MSC-sourced secretome is immediately available for treatment of acute conditions and could be massively produced from commercially available cell lines avoiding invasive cell collection procedure [38]. Cell-free products have demonstrated preclinical efficacy without significant safety issue, as they appear to be non-cytotoxicity, non-mutagenicity and have low immunogenicity. They also have the advantage of overcoming the challenge of cell viability maintenance and potency throughout the manufacture, storage, and delivery, maintaining the advantages of therapeutic ability [40]. Several cell-free preparations have shown encouraging outcomes in early stage clinical trials [41].

The secretome of cells is a commixture of soluble factors (cytokines, chemokines and growth factors) as well as non-soluble factors - molecules associated with extracellular vesicles - lipid bilayer delimited particles of various sizes and complexities containing proteins and nucleic acids released from cells into the extracellular space. These particles can be found in secretome at various concentrations and activity levels determined by cell type and environment [42,43]. They may also activate the resident stem cells and hence mediating the endogenous regeneration [44].

Though MSCs isolated from various tissues display a variety of common appearances, their biological functions, and some markers are dissimilar depending on the their origins [45]. MSCs

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derived from diverse origins are phenotypically heterogeneous and demonstrate varied differentiation possibilities and release of bioactive factors related to tissue origin [45].

This study focused on the determination of the therapeutic potential of eSM-MSCs and eUC-MSCs populations and the evaluation of their synergistic effect in the treatment of an equine ligament desmitis case, utilizing the cells themselves and cell-derived secretome. For such the therapeutic potential of eSM-MSCs and eUC-MSCs was assessed through the determination of bioactive factors of both secretome as a prospect of its beneficial contributes and therapeutic properties.

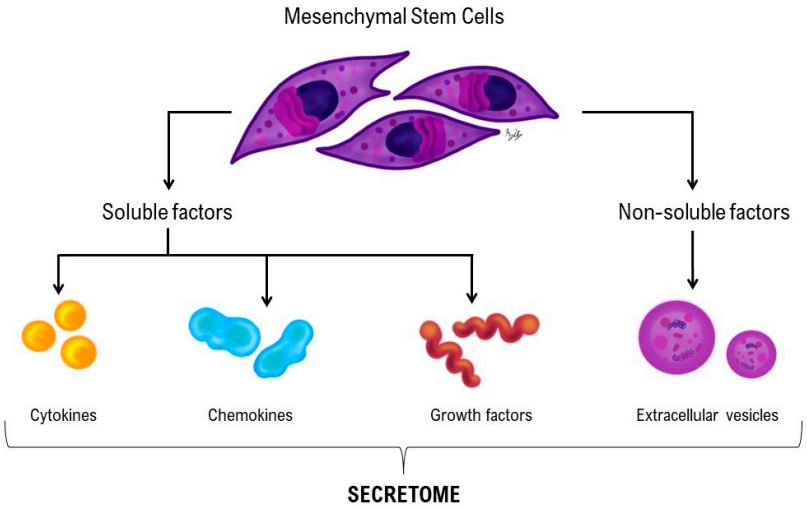

Figure 2. Secretome. Mesenchymal stem cells release a variety of factors, -soluble and -non soluble in the microenvironment which modulate the biology and tissue response of the cells, such as cytokines, chemokines, growth factors and extracellular vesicles.

Further, a clinical case of a male show jumping horse with seven years old, who sustained an injury of the long medial collateral ligament (LMCL) of the right hindlimb tarsus during a training session, receiving eSM-MSCs + eUC-MSCs CM treatment is presented. After diagnosis of a desmitis of the above-mentioned structure, he was treated with the therapeutic combination - two administrations intralesionally, 15 days apart. The treatment was accompanied by a physical rehabilitation program of 8 weeks and returned to work.

Pre- and post-treatment evaluations consisted of clinical and orthopedic evaluation, tarsus radiographs and ultrasound exams. The patient did not receive any biological treatment before this medical approach. Clinical and sportive follow-up of this case presented very positive outcomes, with complete regeneration of the structure and good return to sportive career,

2. Materials and Methods

2.1. Ethics and Regulation

This study was carried out in accordance with *Organismo Responsável pelo Bem Estar Animal* (ORBEA) from ICBAS-UP, project number: P289/ORBEA/2018 recommendations and authorization. Treatments were performed with permission and signature of an informed consent from the patient's legal tutor, following a thorough explanation on the procedure itself and possible risks and associated effects, in accordance with national regulations and project approval from the competent authorities.

No animals were euthanized for this study.

2.2. Patient identification

In this clinical case report, the patient is a seven-year-old stallion, show jumper with an acute lesion of the right hindlimb tarsus.

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A prospective study was designed to understand the potential benefits of the association of UC-MSCs CM to eSM-MSCs in the treatment of tendonitis and/or desmitis. The following inclusion criteria were determined: horse with acute or chronic lameness, diagnostic of tendonitis and/or desmitis and no signs of systemic disease. No other medical treatment (including nonsteroidal anti-inflammatory drugs, intra-articular corticosteroids, hyaluronan, glycosaminoglycans, hemoderivative treatments and other MSC's preparations) should have been administered at least 2 months before allogenic eSM-MSCs + UC-MSC CM treatment and for at least 2 months *post* the cell-based treatment. The patient presented these criteria and was treated in an acute stage of disease.

2.3. Patient clinical evaluation

A seven-year-old stallion was examined for a complaint of a swollen right tarsus. Upon examination, the right tarsus presented significant effusion of the tarsocrural joint- (Figure 3). Patient undergone identification, anamnesis, physical examination (cardiac and respiratory frequency, body temperature, mucous membrane examination, inspection of the whole body and palpation), orthopedic examination (evaluation of the limbs, gait inspection and movements – walk, trot and gallop, and flexion test of the main joints for 60 seconds followed of trot). Lameness was evaluated at walk and trot on hard surface and scored in a scale of 0 to 5, according to American Association of Equine Practitioners (AAEP) parameters. Palpation, manipulation, flexion test, and pain to pressure were performed as described in [46,47]. Complementary diagnostic exams included radiographs and ultrasound image, as reported in other studies [59,63–70]. Radiographs and ultrasound examination were also performed. Lameness was evaluated and scored accordingly with AAEP lameness grading scale as described in Table 1 [47,48].

Table 1. Score systems used to assess lameness, response to flexion test and pain to pressure [47].

Parameter	Score	Clinical implication
AAEP Grading	0	No Lameness
	1	Lameness not consistent
	2	Lameness consistent under certain circumstances
	3	Lameness consistently observable on a straight line.
	4	Obvious lameness at walk: marked nodding or shortened stride
	5	Minimal weight bearing lameness in motion or at rest
Flexion Test	0	No flexion response
	1	Mild flexion response
	2	Moderate flexion response
	3	Severe flexion response
Pain to pressure	0	No pain to pressure
	1	Mild pain to pressure
	2	Moderate pain to pressure
	3	Severe pain to pressure

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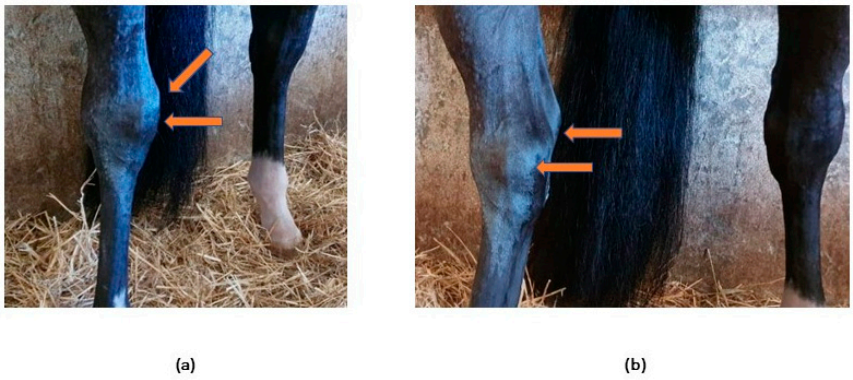

Figure 3. Horse clinical inspection. Evidence of increased volume of the right tarsocrural joint. (a) frontal view and (b) medial view.

2.4. Diagnostic Complementary exams

2.4.1. Radiological examination

Radiological examination (X ray) of the right tarsocrural joint was performed with a digital system - CareRay Cw series®, radiological constants: 72Kv, 0.8mA. Four standard views – lateromedial, dorsoplantar, dorsolateral-plantaromedial and dorsomedial-plantarolateral – were obtained.

2.4.2. Ultrasound examination

Ultrasound examination (U/S) of the right and left tarsocrural joint was performed with and ultrasound machine - Sonoscape A6®, probe 7,5 MHz.

The contralateral limb was considered normal and used as control. Echogenicity, fiber pattern, and cross-sectional area were evaluated in each collateral ligament. The synovial fluid was evaluated for signs of hemarthrosis (increase in echogenicity and/or a swirling echogenic pattern). The synovial lining was evaluated for thickening and fibrinous loculations in the tarsocrural joint. The medial and lateral long and short collateral ligaments of the tarsus were examined in longitudinal and transverse planes, from proximal to distal.

2.5. Donor selection and SM collection

eSM-MSCs' donor was a young and healthy foal who died accidentally. Briefly, the tutor authorized synovial membrane collection from hocks, knees and fetlocks. Skin covering the incisional field was surgically cleaned with chlorohexidine and alcohol. Skin and subcutaneous tissue were incised, and debrided, articular capsule was opened, and synovial membrane was isolated and extracted into a Dulbecco's Phosphate Buffered Saline (DPBS) container. Samples were transported to the laboratory with ice packs for refrigerated temperatures. These procedures were previously described at *Leal Reis et al*, 2023 [49].

2.6. eSM-MSCs isolation, culture and characterization

After collection, equine synovial membrane, was prepared at the Laboratory of Veterinary Cell-based Therapies - ICBAS-UP. The isolation protocol of eSM-MSCs was developed by patented proprietary technology Regenera® (PCT/IB2019/052006, WO2019175773 – Compositions for use in the treatment of musculoskeletal conditions and methods for producing the same leveraging the synergistic activity of two different types of mesenchymal stromal/ stem cells - Regenera®), previously described [49].

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Equine SM MSC's were characterized through tri-lineage differentiation and immunohistochemistry protocols. Karyotype analysis was also performed and described [49].

2.7. UC-MSC's isolation, culture and characterization

eUC-MSCs were isolated from the equine umbilical cord matrix - Wharton's jelly.

This process was performed and is patented proprietary technology (PCT/IB2019/052006, WO2019175773 – Compositions for use in the treatment of musculoskeletal conditions and methods for producing the same leveraging the synergistic activity of two different types of mesenchymal stromal/ stem cells - Regenera®). Briefly, tissue samples were collected and placed in transport media [supplemented with 3% (v/v) Penicillin-Streptomycin (Gibco®) and 3% Amphotericin B (Gibco®)]. Upon arrival, umbilical cord tissues were decontaminated and dissected for the isolation of the stromal tissue, which was digested using Colagenase I (Gibco®) and Dispase II (Sigma®). Single cell suspension of the digested tissues was obtained through a 70 µm cell strainer (Falcon®) and cultured in DMED-HG (Gibco®), 20% (v/v) FBS (Gibco®), 1,5% (v/v) Penicillin-Streptomycin (Gibco®) and 1,5% Amphotericin B (Gibco®), for the first 24 hours. Non-adherent cells were discarded after 24 hours and remaining cells further expanded in DMEM-LG (Gibco®), 10% (v/v) FBS (Gibco®), 1% (v/v) Penicillin-Streptomycin (Gibco®) and 1% Amphotericin B (Gibco®), to form culture of adherent cells with fibroblastic morphology. This process was performed and is patented proprietary technology Regenera® (PCT/IB2019/052006, WO2019175773 – Compositions for use in the treatment of musculoskeletal conditions and methods for producing the same leveraging the synergistic activity of two different types of mesenchymal stromal/ stem cells - Regenera®)

2.8. Secretome – Conditioned Medium preparation and analysis

Conditioned Medium of eSM-MSCs and eUC-MSCs in passage 4 and 6, respectively, was analyzed to identify cytokines and chemokines secreted after conditioning. When in culture, after reaching a confluence of around 70–80%, the culture medium was removed, and the culture flasks were gently washed with DPBS two to three times (2 to 3x). Then, the culture flasks were further washed two to three times with the basal culture medium of each cell type, without any supplementation. To begin the conditioning, non-supplemented DMEM/F12 GlutaMAX™ (10565018, Gibco®, Thermo Fisher Scientific®, Waltham, MA, USA) culture medium was added to the culture flasks, which were then incubated under standard conditions. The culture medium rich in factors secreted by the cells (CM) was collected after 48 h. The collected CM was then concentrated five times (5×). After collection, it was centrifuged for 10 min at 1600 rpm, its supernatant collected and filtered with a 0.2 µm Syringe filter (Filtropur S, PES, Sarstedt®, Nümbrecht, Germany). For the concentration procedure, Pierce™ Protein Concentrator, 3k MWCO, 5–20 mL tubes (88525, Thermo Scientific®, Waltham, MA, USA) were used. Initially, the concentrators were sterilized following the manufacturer's instructions. Briefly, the upper compartment of each concentrator tube was filled with 70% ethanol (v/v) and centrifuged at 300× g for 10 min. At the end of the centrifugation, the ethanol was discarded, and the same procedure was carried out with DPBS. Each concentrator tube was subjected to two such centrifugation cycles, followed by a 10-min period in the laminar flow hood for complete drying. Finally, the upper compartment of the concentrator tubes was filled with plain CM (1× concentration) and subjected to new centrifugation cycle, under the conditions described above, for the number of cycles necessary to obtain the desired CM concentration (5×). The concentrated CM was stored at -20°C and subsequently subjected to a Multiplexing LASER Bead analysis (Eve Technologies, Calgary, AB, Canada) to identify a set of biomarkers present in the Equine Cytokine 8-Plex Assay (EQCYT-08-501).

UC-MCS CM secretome was performed in an early phase of our study and two biomarkers were searched: Interleukins (IL) IL-6 and IL-8. Biomarkers used at secretome characterization of eSM-MSCs were: Basic Fibroblast Growth Factor (FGF-2), Granulocyte Colony Stimulating Factor (G-CSF), Granulocyte-macrophage Colony Stimulating Factor (GM-CSF), Monocyte Chemoattractant Protein-1 (MCP-1), Interleukins (IL) IL-6, IL-8, IL-17A and Human Growth-regulated oncogene/Keratinocyte Chemoattractant (KC/GRO). Secretome characterization is previously described [49]. All samples were analyzed in duplicate.

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2.9. eSM-MSCs + eUC-MSC CM solution preparation

The eSM-MSCs solution for intra-ligamental clinical application, was a combination of allogenic eSM-MSCs suspended in eUC-MSCs CM. Prior to preparation of the final therapeutic combination, eSM-MSCs and UC-MSCs CM were produced and preserved as described above.

Cryopreserved P3 eSM-MSCs batches were suspended in treated animal's autologous serum. For this purpose, 10 mL of whole blood was collected into two dry blood collection tubes and allowed to clot. The tubes were then centrifuged at 3200 rpm for 10 minutes and the supernatant (autologous serum) was collected into a 15ml Falcon tube. The serum sample was heat inactivated for 20 minutes at 56ºC (water bath), quickly cooled down in an ice bath and sterile filtered with a syringe (0,22 um) into a new 15ml Falcon tube. For one $9x10^6$ eSM-MSCs dose, 3x1ml eSM-MSCs vials containing $3x10^6$ cells each were thawed in a 37ºC water bath and the cell suspension of the 3 vials were mixed into one 15ml Falcon tube. 2-3ml of autologous serum were slowly added to the tube (drop-wise) and the suspension was gently mixed. 5mL of PBS were slowly added into the tube and the suspension gently mixed and centrifuged at *1600 rpm* for 10 minutes. The supernatant was discarded and the cell pellet resuspended in a mixture of autologous serum in a ratio of 0,8:1. Cell counting and viability was determined by the Trypan Blue exclusion dye assay (Invitrogen ™) using an automatic counter (Countess II FL Automated Cell Counter, Thermo Fisher Scientific®). Cell number was then adjusted to $10x10^6$ cells/ ml. At this point the conditioned medium from UC-MSCs was thawed and added to the suspension to a final 1:1 concentration. 2 ml of the solution of eSM-MSCs suspended in UC-MSCs CM were transferred to a perforable capped vial and preserved on ice until the moment of administration.

2.10. Treatment Protocol

The injured structure – LMCL - was treated with the mixture of allogenic eSM-MSCs and UC-MSCs CM. The animal did not receive any treatment before or after the administration of the therapeutic, except for those foreseen in this treatment protocol.

Patient was monitored for 48 hours after treatment and any occurrence was registered. Following the treatment, patient was assessed periodically to control swelling of the joint, lameness and ultrasonographic changes (echogenicity, cross sectional area and fiber alignment). Corrective asymmetrical shoeing with more support (wider branch) on the medial side was performed – "Denoix asymmetric shoe".

2.10.1. Intralesional eSM-MSCs + eUC-MSCs CM administration

Patient was sedated with detomidine (Domosedan®, 0.02 mg/kg, IV), the right tarsus trichotomized and skin was surgically disinfected with chlorohexidine and alcohol. The prepared therapeutic combination was aspired to a 2ml syringe and homogenized. Ultrasound was used to identify the lesion site, and an ultrasound guided injection was performed at the lesion site. Patient received a single administration of phenylbutazone (2.2 mg/kg, IV) at the end of the treatment. The stablished protocol included a second eSM-MSCs + eUC-MSCs CM administration 15 days after the first treatment using the same protocol.

2.10.2. Post-treatment monitoring - clinical evaluations

Tissue regeneration was indirectly estimated through lameness evaluation, pain to pressure, limb inflammation, limb sensitivity and ultrasound image. For each assessment, a complete examination of the structure was conducted by means of longitudinal and transverse scans for three parameters: lesion echogenicity, lesion longitudinal fiber alignment (FA) and cross-sectional area. The contralateral healthy limb was used as a control. Ultrasonographic evaluation was performed on assessment day, treatment day (day 1 – T0) and on days 15 (T1 – second administration), 30 (T2), 45 (T3), 60 (T4) and 90 (T5) *post*-treatment. According to the classification proposed by *Guest et al*, this is a short term period study [50].

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Rehabilitation program consisted of an exercise-controlled program including stall confinement an increasing time of exercise, as presented on Table 2 [1,51–54]. Exercise with simple movements, for most injuries, can begin within 3 days if careful protocols are followed. For severe ligament and tendon injuries, exercise can begin at 3 weeks, with initiation of low-level movements at 3 days. Early movements should include weight-bearing, strengthening and flexibility activities, whereas stall rest alone should be used as infrequently as possible [52].

Regular ultrasound evaluations were also performed at T1(second administration) and T2. For the following 60 days, physical rehabilitation program was maintained, and three additional ultrasound examinations were performed. Veterinary assessment at day 90 (T5) determined if the horse could return to regular work based on lesion regeneration evidenced by normal echogenicity, good fiber alignment and normal cross-sectional area of the ligament when compared with contralateral limb (Figure 4). Limb sensitivity and lameness were also evaluated.

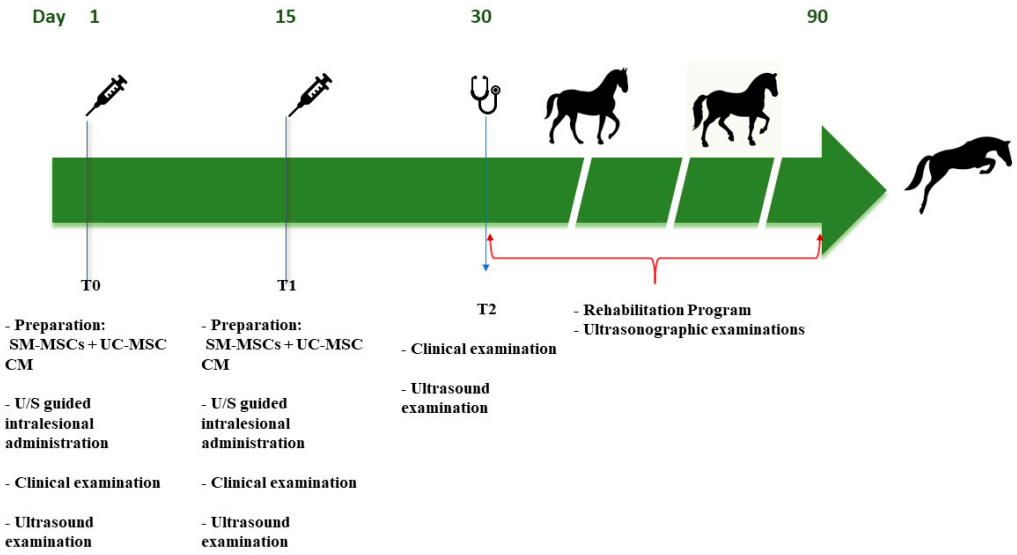

Figure 4. Timeline of eSM-MSCs treatment protocol and rehabilitation program. T0 is the day of the first treatment with the administration of eSM-MSCs + UC-MSCs CM combination. Beside the intralesional application of the therapeutic combination, a clinical and ultrasound examinations were also performed. T1 refers to the second application of the composition 15 days after T0, when the same procedure was repeated. At day 30 (T2), a clinical and ultrasound examination was performed and if a favorable outcome was identified, the animal progressed to a physical rehabilitation program.

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Table 2. Physical rehabilitation program. After eSM-MSCs+eUC-MSCs CM treatment, patient underwent a rehabilitation program consisting of two days of box rest followed by 13 days of 10 minutes hand-walk. Bandage applied on treatment day was removed 24h after treatment. At day 15 the second treatment was performed followed by the same day 15 rehabilitation program, until day 30. Between day 30 and day 45 the work consisted of 20 min hand-walking, between day 45 and day 60 the work was 30 minutes of hand-walking, between day 60 and day 75, 30 minutes of hand walking plus 5 minutes trot and finally between day 75 and day 90, patient underwent 30 minutes of hand-walking plus 10 minutes of trot. After this the patient could return to full work.

Week	Exercise
0-2	2 days: stall confinement
	Handwalk: 10 min
	Day 15: new treatment
3-4	2 days: stall confinement
	Handwalk: 10 min
	VET-CHECK + U/S
5	Handwalk: 15 min
6	Handwalk: 20 min
	VET-CHECK + U/S
7	Handwalk: 25 min
8	Handwalk: 30 min
	VET-CHECK + U/S
9-10	Handwalk: 30 min + 5 min trot
11-12	Handwalk: 30 min + 10 min trot
	VET-CHECK + U/S

3. Results

3.1. Clinical evaluation

On assessment day, right tarsus was severely swollen with significant effusion of the tarsocrural joint (Figure 4). Palpation and manipulation were not resented and no swelling in the lower limb was observed. The horse was not lame at the walk or trot in a straight line but was a little reluctant to fully bear weight on the right hind leg when turned to the right (grade 2/5 according to AAEP lameness grading scale). Flexion test and pain to pressure were also evaluated and no flexion response and no pain to pressure were identified [55].

3.1.1. Radiological examination

The horse had no significant articular abnormalities within the tarsocrural joint during the radiological exam performed at the first visit. There was tarsocrural joint distension, soft tissue distension and slight evidence of tissue thickening at injured long medial collateral ligament. Radiographs are presented in Figure 5.

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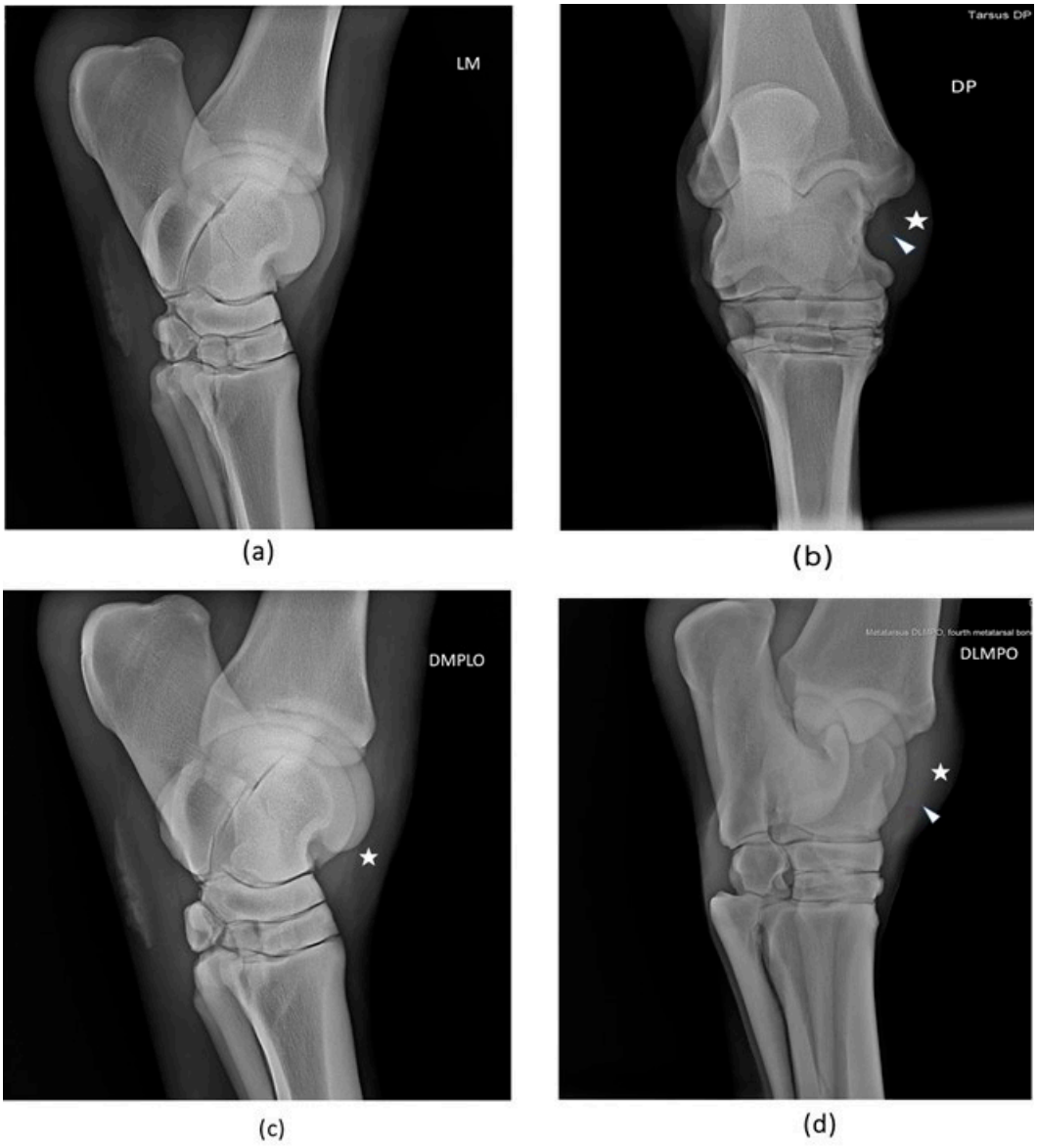

Figure 5. Patient right tarsus radiographs. Four projections were taken: (a) Lateromedial (LM), (b) Dorsoplantar (DP), (c) Oblique dorsomedial-plantarolateral (DMPLO), (d) Oblique dorsolateral-plantaromedial (DLPMO). The white head of the arrow ( ) points to increased radiopacity of the long medial collateral ligament and the star ( ) signals soft tissue swelling and joint distension. There are no significant radiological alterations of articular surfaces.

3.1.2. Ultrasound examination

On assessment day, ultrasonographic exam evidenced moderate fiber pattern disruption – hipoechoic region - of LMCL at its insertion at medial maleolus as well as an increased cross-sectional area - Figure 6a. Increased amount of hypoechoic fluid containing areas of increased swirling (heterogeneous echogenicity), as shown on Figure 6b, suggestive of an organized hematoma and/or fibrin within the joint – hemartrosis. Cartilage's surface was normal.

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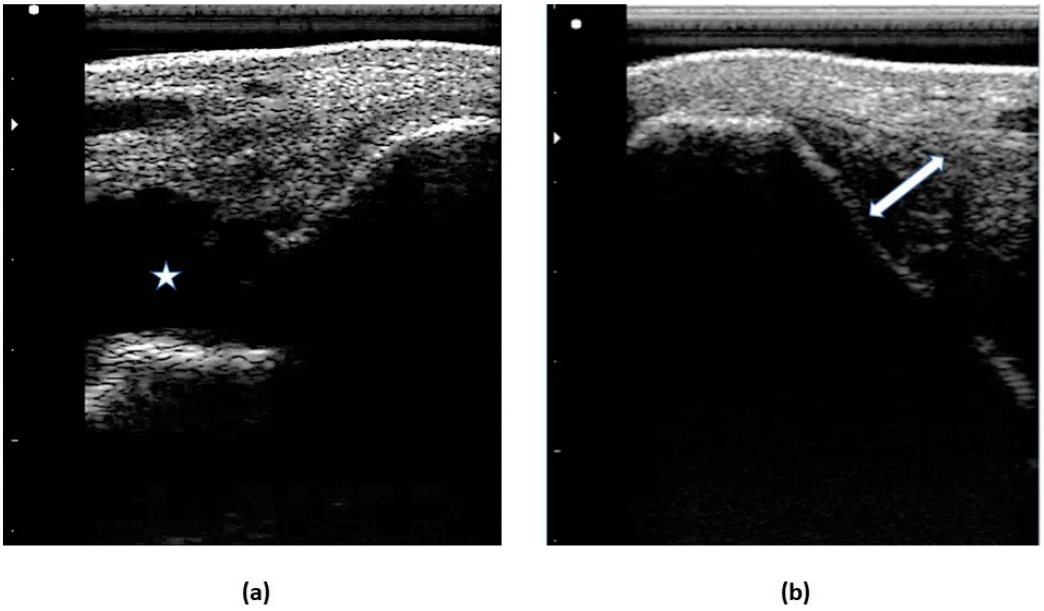

Figure 6. Images of the first ultrasonographic assessment. Desmitis of LMCL's insertion at the medial malleolus: (a) increased amount of hypoechoic fluid within the joint, signaled with the star (*) (b) disruption of the fibers at the insertion, signaled with the double arrow (↔).

3.2. MSCs isolation and characterization

Equine SM-MSCs were successfully isolated and expanded from the donor. Cells observed radiating from the explants and those identified in culture showed clear plastic adhesion and mostly fibroblast-like morphology, an essential feature to characterize cells as MSCs [49]. eSM-MSC's characterization results were previously presented at *Leal Reis et al* [49].

Equine UC-MSCs have been successfully isolated from UC tissue. Cells in culture presented clear plastic-adhesion and fibroblast-like morphology – Figure 7.

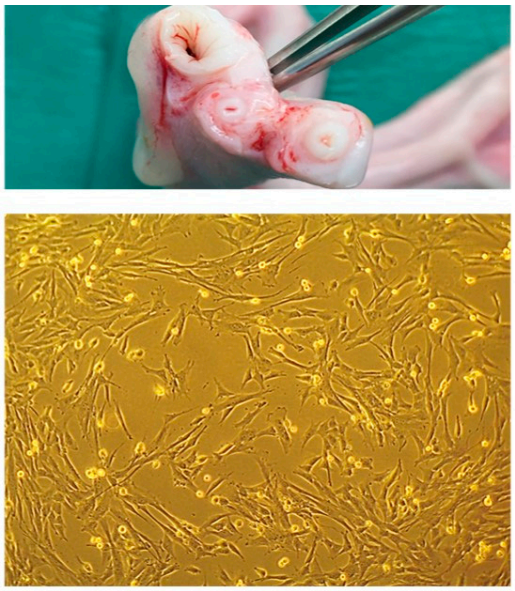

Figure 7. – Isolation of MSC from equine umbilical cord tissue. In the upper image umbilical cord tissue. In the lower image, isolated population of eUC-MSCs at P3 – plastic adhesion, monolayer, and fibroblast-like shape of eUC-MSCs may be observed.

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3.3. Secretome: conditioned medium analysis

Equine SM-MSC secretome was previously performed and described [49]. Equine UC-MSCs CM was concentrated 5x and assessed for the production of IL-6 and IL-8. Results indicate these two interleukins are produced in high levels. Comparing IL-6 and IL-8 production between eSM-MSCs and eUC-MSCs, was concluded eUC-MSCs produces almost three times more IL-6 than IL-8 and that eSM-MSCs produces a higher amount of IL8 than eUC-MSCs. There are significative differences in the production of IL-6 between eSM-MSCs and UC-MSCs, between the production of IL-6 and IL-8 by eUC-MSC's and less significative, the difference in the production of IL-8 between eSM-MSC's and e UC-MSC's. – Figure 8 and Table 3.

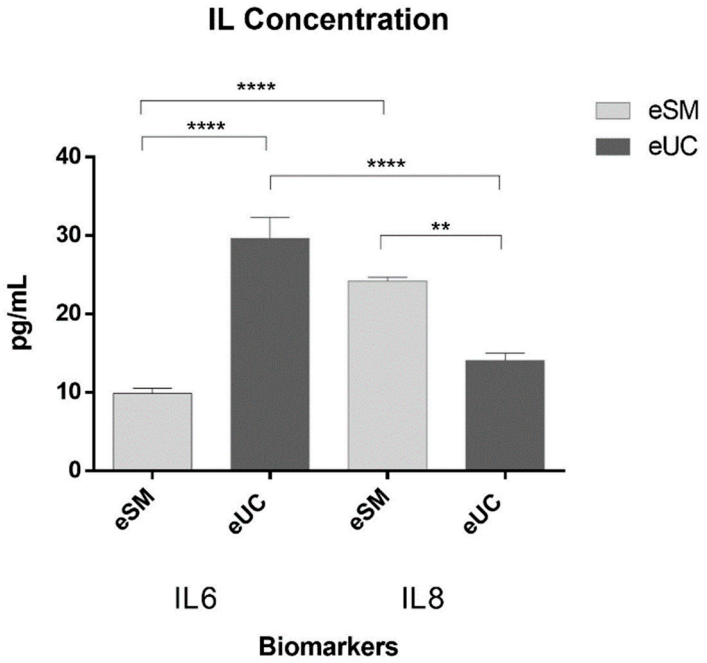

Figure 8. Bioactive molecules in secretome - IL-6 and IL-8: Differences of production by eSM-MSC's and eUC-MSC's. Results presented as (mean ± SEM). * Corresponds to $0.01 \leq p < 0.05$, ** to $0.001 \leq p < 0.01$, *** to $0.0001 \leq p < 0.001$, and **** to $p < 0.0001$.

Table 3. Normalized mean concentration of each biomarker in the CM of eSM-MSCs and eUC-MSCs in pg/mL (mean ± standard error mean (SEM)).

Biomarker	Mean normalized concentration
IL-6 eSM-MSC's	9,855 ± 0,6460
IL-6 eUC-MSC's	29,66 ± 2,636
IL-8 eSM-MSC's	24,17 ± 0,4965
IL-8 eUC-MSC's	14,05 ± 0,9557

3.4. Treatment Results

The patient did not present any adverse event that required study cessation, unplanned procedures, or additional treatments. The two intra-ligamentar administrations and follow-up procedures had no adverse reactions (inflammation, infection, deterioration of the lesion, increased lameness), as reported by *Godwin et al*, 2012 [56], neither at treatment time (T0 and T1) nor at the following weeks.

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At day 30 (T2), increased echogenicity of the lesion was evidenced as well as reduction of cross-sectional area with good fiber alignment. There was also no evidence of pain and lameness. Fluid within the joint reduced as well as joint swelling. Nevertheless, compared with the contralateral limb, the right tarsocrural joint diameter was larger than the left.

After eSM-MSCs treatment, the patient complied a rehabilitation program as explained in Table 2.

Over the course of the follow-up ultrasonographic examinations, it was evidenced an increasing echogenicity of the lesion, reduction of the cross-sectional area, good fiber alignment and reduction of the abnormal synovial fluid. At day 60 (T4), two months after the first treatment, there was a complete regeneration of the ligament - lesion completely fulfilled, good echogenicity, good fiber alignment and normal cross-sectional area. No pain and no lameness were present, as well as no signs of cartilage remodeling. Despite this achievement, physical rehabilitation program was followed as stated until day 90 (T5).

Ultrasound images at day 1 (T0), 15 (T1), 30 (T2), 45 (T3) and 60 (T4) are presented at Figure 9. Lesion regeneration through the follow-up period evidenced by progressive increased echogenicity and fiber alignment, decrease of ligament cross-sectional area and synovial fluid accumulation within the joint space.

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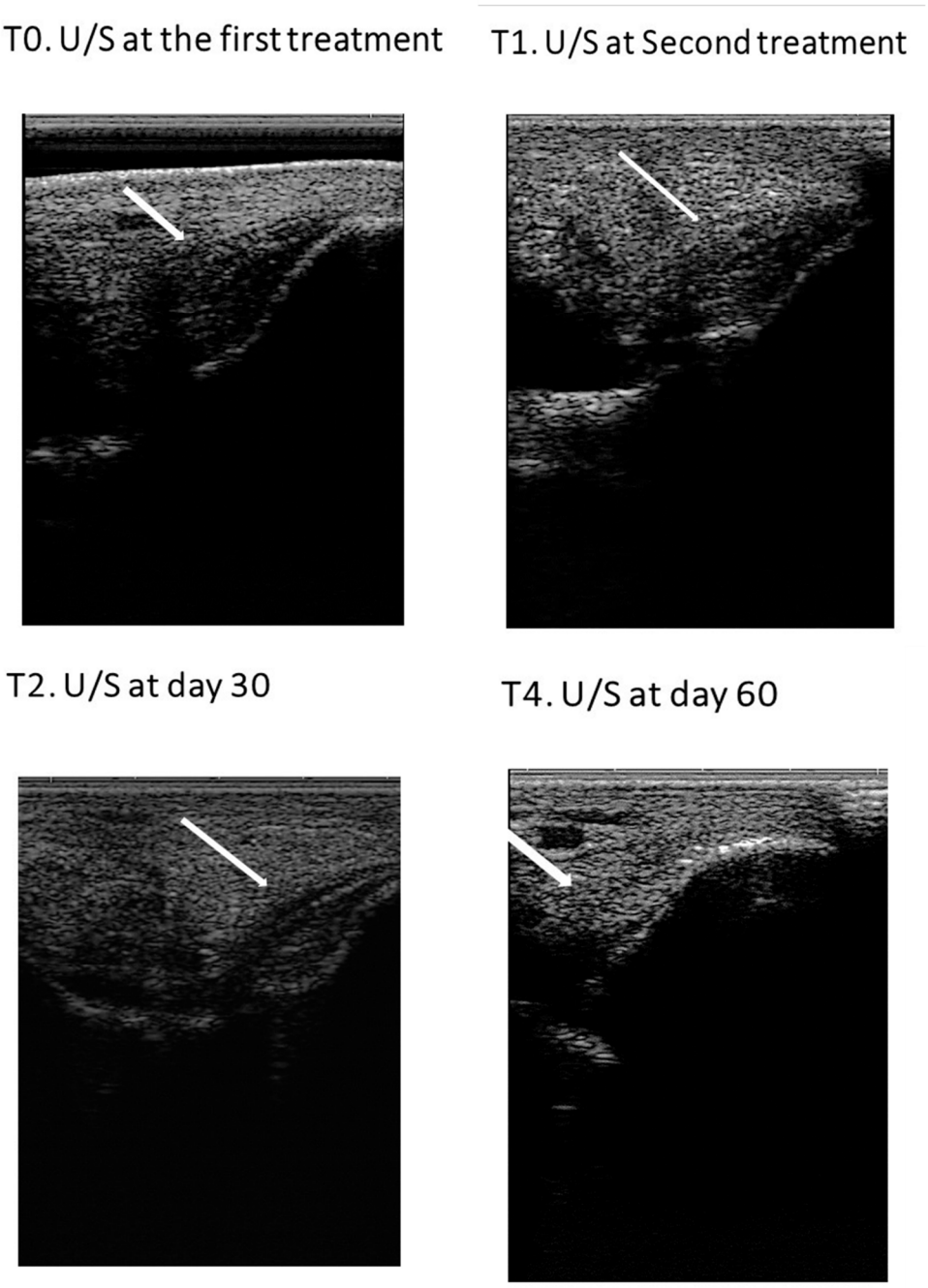

Figure 9. Images of ultrasonographic follow-up. (a) Day 1 (T0), (b) day 15 (T1), (c) day 30 (T2) and (d) day 60 (T3). Evidence of ligamentar regeneration: increased echogenicity and fiber alignment, decrease of cross-sectional area and synovial fluid accumulation within the joint space. At day 90 (T5), patient returned to regular work with no lesion relapse reported to eighteen months after injury. Additional information reports patient is already doing competition in a higher level than before injury.

4. Discussion and Conclusions

The focus of this case was to evaluate the synergistic effect of eSM-MSC's and eUC-MSCs CM in the treatment of an equine ligament desmitis. The state of the art concerning regenerative and biological therapies involves the use of mesenchymal stem cells and more recently, becoming widely studied, the resource to its secretion products.

Therefore, the combination of the therapeutic advantages of MSCs administration and cell-free approaches emerges as an innovative strategy, with increased therapeutic potential, currently patented proprietary technology (PCT/IB2019/052006, WO2019175773 – Compositions for use in the

treatment of musculoskeletal conditions and methods for producing the same leveraging the synergistic activity of two different types of mesenchymal stromal/ stem cells - Regenera®).

This case report discloses application of such strategy and details on the evaluation of the synergistic effect of eSM-MSCs and eUC-MSCs populations in the treatment of an equine ligament desmitis, utilizing the cells themselves and cell-derived secretome, respectively.

Equine SM-MSCs are an interesting subject for those who study cellular and cell-based therapies due to their promising ability to promote tissue regeneration with high capacity of regeneration of articular structures, tendon and ligaments. Their osteogenic, myogenic and tenogenic superiority, suggests that SM-MSCs are a good candidate for efforts to regenerate musculoskeletal tissues, as evidenced [57,58] and previously reported by the research group [49]. Additionally, the evaluation of each secretome is important to understand biological potential and their synergistic action with other cell sources.

As previously presented, the latest studies highlight the importance of paracrine action of MSC's through the release of soluble and non-soluble factors, primarily secreted in the extracellular space by stem cells – secretome [59]. Secretome paracrine signaling can be considered as the primary mechanism by which MSCs contribute to healing processes, becoming their study an interesting subject [60,61].

Mocchi et al, agreed that secretome is assuming the center of a new potential therapeutic strategy in different diseases [59,62]. Avoiding the need of living cell implantation, secretome presents itself as a big promise as a pharmaceutical product suitable for regenerative medicine [63,64]. In particular, extracellular vesicles (EV) are considered a new therapeutic tool having a prominent role in musculoskeletal disorders [59].

Al Naem, 2020, reported EVs, resulting from the paracrine action of MSCs, play a key role in the therapeutic mechanisms mediated by stem cells. MSC-EVs are thus largely implicated in the regulation of proliferation, maturation, polarization and migration of various target cells. Evidence that EVs alone represent a complex network involving different soluble factors and could then reflect biophysical characteristics of parent cells, has fuelled the importance of developing highly specific techniques for their isolation and analysis [61]. A considerable number of studies are now being conducted in this area.

At the moment, although these considerations, in veterinary medicine, the clinical use of CM and MSC-EVs is very embryonic and more studies need to be performed.

Equine SM-MSC's secrete high levels of KC/GRO, MCP-1, Il-6, FGF-2, G-CSF, GM-CSF and IL-8 , as previously described [49]. Equine SM-MSCs are responsible for a higher excretion of IL-8 and eUC-MSCs for IL-6, creating an environment of high levels of these two cytokines responsible for anti-inflammatory and regenerative activities.

This profile supports their reported benefits in fibroblast intense activity (KC/GRO) and lesion reperfusion (MCP-1), both essential to successful completion of musculoskeletal tissue after ischemic injury [65]. The production of FGF-2 is also significant and recognized for proliferation of tenogenic stem cells, enhancing cell proliferation and collagen production [66]. Other factors such as G-CSF and GM-CSF also depict potential as skeletal muscle repair mediator, including those with pro-inflammatory functions [67,68]

Pro-inflammatory factors such as those found at these cells secretome (GM-CSF, G-CSF, Il-6, IL-8 and IL-17), are frequently regarded as deleterious, however they are involved in damage signaling and subsequent activation of resident tendon cells for effective healing, stimulating tendon cell proliferation [69,70].

The current analysis focused on the secretion ofIL-6 and IL-8 dueto their known activity in tissue regeneration. Interleukin-6 bares pro-inflammatory and angiogenic functions, capable of increasing the expression of other growth factors (GF). Immunosuppressive properties are also described, which may be prime motors for the success of allogenic MSC implantation [71,72]. This pro-inflammatory nature is associated with the induction of acute-phase proteins, inducing a potent regeneration of various tissues such as liver, kidney, neural tissues and others, supporting their potential as a therapeutic approach for regenerative medicine [73,74].

Previous studies have likewise demonstrated IL-6 is a potent anti-inflammatory cytokine significantly up-regulated in injured tendons [75]. This cytokine has been demonstrated to have an important role in regulating tendon-derived stem cells (TDSC) activity and differentiation, however inhibiting their tenogenic differentiation, *in vitro*[76], while in an *in vivo* model (IL6 −/− mice), it was demonstrated to be involved in the complex mechanisms that contribute to mechanical and organizational properties of injured tendons [77].

Another *in vivo* study demonstrated that human Achilles tendon presented high levels of various growth factors after exercise. From these, IL-6 was present in the largest amount, suggesting this cytokine was responsible of transforming collagen under biomechanical stimulation. An experimental infusion of IL-6 in the peritendinous tissue followed by exercise suggested this ILstimulates collagen synthesis, corroborating the hypothesis that IL-6 is an important growth factor of the connective tissue in healthy human tendons[78].

These observations suggest IL-6 has an important role in tendon regeneration, despite the need for further research to more accurately understand IL-6 real role in vivo.

IL-8 is also a recognized pro-inflammatory mediator anda potent angiogenic factor associated with increase in VEGF concentration. Interleukin-8 was directly related to VEGF stimulation helping revascularization and ligamentization of a grafted tendon [79]. IL-8 has a similar effect to IL-6 but has a longer half-life [80].

Up-regulation of both IL-6 and IL-8 is consistent with tissues healing and its inflammatory phase. A study with human Achilles tendon presented that IL-6, IL-8 and IL-10 were upregulated in a tendon healing phase with absence of inflammation, indicating that these cytokines may be associated with anti-inflammatory and regenerative activity on tendon healing process [75].

Herein, the in vitro production of these bioactive molecules by the MSCs populations under study was assessed. Equine SM-MSCs are responsible for increased excretion of IL-8 and eUC-MSCs for IL-6, suggesting diverse biological potential of both cell types for immunomodulative and regenerative therapy, magnifying their potential benefits, confirming its immunosuppressive, angiogenic and pro-inflammatory profile, thus validating their complementarity and synergistic activity in anti-inflammatory and regenerative events as stated before. [81].

To sum up, eSM-MSCs and eUC-MSC's secretome factors are able to promote tendon/ligament healing by stimulating reperfusion, reactivating growth programs, reducing inflammation and fatty infiltration, stimulating cell proliferation, collagen production and tenogenic differentiation [82].

Clinically, the availability of a bank cell and secretome with well-known mediators with specific beneficial characteristics and recognized capacity of tissue regeneration induction is very relevant and highly appealing. This fact allows an early medical intervention with prompt procedures, in acute cases, enabling tissue regeneration, a better functional outcome and a rapid and sustainable return to sportive career. The other advantage in this study is related to the presence of CM which plays an important role optimizing the effects of the paracrine factors, whose importance was previously described. MSCs derived secretome, in the form of conditioned medium, represent therefore a new class of therapeutics with broad application for the treatment of disease and injury.. The influence of fibroblastic proliferation, angiogenic stimulation and development of mature vascular structures who provide a wide variety of GF, accomplishes not only lesion repair with regenerated tissue but also strengthening of the entire ligament, reducing the risk of lesion recurrence [83]. It is also relevant to note that both eSM-MSCs and eUC-MSCs were obtained from a donor horse, deeming them of allogeneic nature. No adverse or rejection reactions were observed, further supporting their potential as alternatives to autologous therapies, which bare relevant drawbacks to their widespread application, such as the health status of the source tissue (and its impact in their regenerative performance) and the time required for tissue processing and therapeutic dosage production. The allogeneic approach enables a curated selection of tissue donors, as well as the production and validation of both MSCs and secretome, which can be stored and be readily available for acute application in the event of injury.

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Once substantiated the therapeutic potential of the combined use eSM-MSCs and eUC-MSCs CM for the treatment of tendinopathies and desmopathies, the approach presented suitable for the application in the tarsus medial ligament lesion reported.

Tarsus medial collateral ligament lesions are the most prevalent, being the long ligament the most affected. Occasionally lesions of multiple CLs have been found [27,84]. These lesions derive mostly from rotational forces beyond the normal range of joint motion occurring during tight turns or forced asymmetrical movements, increasing strain on the CLs. This was the reason hypothesized for the lesion sustained in the present case, a traumatically induced lesion during dressage exercises with tight turns that caused an abnormal hock extension (LMCL extension). Usually CL injuries present themselves as acute lameness with tibiotarsic joint effusion and therefore should be included in the differential diagnosis of the swollen hock [4]. As stated by, *Sherlock et al*, 2011, prognosis for medial tarsal collateral ligament desmitis appears good for survival but fair for return to previous levels of performance and requires prolonged periods of rest and a controlled exercise program [18]. Literature often refers to treatment of this pathology with rest, oral and/or systemic anti-inflammatories, antibiotherapy local and/or systemic, shock wave, joint lavage, arthroscopy and ligament engraftment [9,14,18,24,26,85]. The outcome of these procedures is not very successful, since there is a guarded prognosis to return to same performance level and degenerative joint disease might even be secondarily associated. There are high percentages of recurrence and lameness is often present. Another study presents the treatment of this type of lesion with Platelet rich plasma (PRP's), achieving a return to the same level work in 180 days, in 81% of the horses [83].

In the presented case, the therapeutic combination of eSM-MSC and eUC-MSC CM was considered very successful as we had a return to full work in 90 days, reducing in 50% the time to return to full work when compared with other therapies in the same type of lesion. The physical and orthopedic outcome of the patient, as well as the ultrasonographic recovery of the ligament was considered complete at day 60 (T4). The horse presented no lameness (Score 0/0, AAEP Lameness Score). Lameness evaluation is a clinically relevant marker of orthopedic injury improvement and was used as our primary outcome as severe lesions in tendons, ligaments, and joints present with lameness as the main clinical sign. In opposition to reference literature descriptions, in the present case we had a significative reduce in rest period. This is a great attainment comparing with recovery times described in literature concerning equine clinical trials of desmitis of collateral ligament of tarsus and equine tendonitis [9,18,85–88] – in 2 days the horse started rehabilitation program *versus* 30 days to 180 days of rest [14] and return to full work after 90 days versus 180 days presented in other studies -reduce in 50% of time recovery [23,83]. In 30 days the ligament's cross-sectional area returned to normal size, good fiber alignment and echogenicity was achieved, *versus* 30-120 days usually described, a recovery that might represent up to 75% of U/S recovery time [9,14]. It must be highlighted that in some cases regeneration is never achieved with conventional treatments, only repair with scar tissue. At day 45 (T3) there was an almost total ultrasonographic recovery, at day 60 (T4) there was a complete ligament recovery, with no scar tissue, good fiber alignment and echogenicity. Only a slight distension of the right tarsus was, and remains, perceptible comparing with contralateral limb. After rehabilitation program, the patient returned to same physical work and, to same performance level. Nowadays he is competing on a higher level. The absence of relapse 18 months after injury is also noteworthy.

In the presented case, the therapeutic combination of eSM-MSCs + eUC-MSCs CM was considered successful, presenting a short rest period, an earlier return to exercise and to full work with a regenerated structure and no lesion relapse.

It must be highlighted that clinically injuries of LMCL are very difficult to treat, in part due to their frequent misdiagnosis as well as its long-term recovery, meaning outcomes have frequently poor prognosis in terms to competition return. The fact of having a complete regenerated LMCL in 60 days is a very important positive outcome. Clinical and sportive achievements in this case are very encouraging. The use of this combination in the treatment of complicated musculoskeletal injuries presented itself very promising. Nevertheless, this study reports results of only one patient, and more

extensive clinical trials are required to further validate the approach and confirm the real benefits of this combination.

In a "One-health" perspective, the health of animals and human coexist in a coherent system. Thus, the possibility of translational results from equine to human musculoskeletal pathologies is very important as equines play an important role as model for human musculoskeletal disorders, given the high level of anatomic and physiologic analogy between equine and human structures [89,90]. Preclinical studies using equine models of orthopedic disorders are adequate to screen potential procedures for human clinical use, as methods of assessing putative repair techniques have not been developed in vitro [91,92].

From an ethical perspective, it is also significant to state that, in the particular context of orthopaedic research, many studies can be conducted in naturally occurring disease (without premeditated disease induction) and that the horse often poses as both model as well as final beneficiary of the developed therapies, alleviating the ethical burden of such studies.

The enhancement of this combination medical application, the maintenance of great results and clinical achievements might lead to future medical approaches to human medicine.

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Abbreviations

2D -Bidimensional
3D - Tridimensional
AAEP - American Association of Equine Practitioners
ACS - Autologous conditioned serum
APS - Autologous protein solution
BM - MSC Bone Marrow Mesenchymal Stem Cells
CL - Collateral ligament
CM - Conditioned Medium
cm^2 - Square centimetre

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d - Days
DLPMO - Oblique Dorsolateral-plantaromedial
DMEM - Dulbecco's Modified Eagle Medium
DMPLO - Oblique dorsomedial-plantarolateral
DMSO - Dimethylsulphoxide
DP - Dorso plantar
DPBS - Dulbecco's Phosphate Buffered Saline
ECM - Extracellular matrix
eSM - MSCs Equine synovial membrane derived mesenchymal stem cells
eUC-MSCs Equine Umbilical cord-stroma derived mesenchymal stem cell
EV - Extracellular Vesicles
FBS - Fetal Bovine Serum
FGF-2 - Basic Fibroblast Growth Factor
G-CSF - Granulocyte Colony Stimulating Factor
GM-CSF - Granulocyte-macrophage Colony Stimulating Factor
IL - Interleukins
IL-1Ra - Interleukin one receptor antagonist
IRAP - Interleukin receptor antagonist protein
ISCT - International Society for Cellular Therapy
IV - Endovenous
KC/GRO - Human Growth-regulated oncogene/Keratinocyte Chemoattractant
Kg - Kilogram
Kv - Kilovolts
LLCL - Long lateral collateral ligament
LM - Lateromedial
LMCL - Long medial collateral ligament
mA - miliamperes
MCB - Master Cell Banks
MCP-1 - Monocyte Chemoattractant Protein-1
mg - milligram
MHz - Megahertz
min - minutes
ml - millilitre
MMP-3 - Matrix metaloproteinase-3
MSCs - Mesenchymal Stem Cells
OA - Osteoarthritis
ORBEA - Organismo Responsável pelo Bem-estar Animal
P - Passage
PBS - Phosphate-buffered saline
pg - picograms
PRP - Platelet-rich plasma
rpm - Rotations per minute
SEM - Standard error mean
SLCL - Short lateral collateral ligament
SMCL - Short medial collateral ligament
TGF-β - Transforming Growth factor-β
TNF-α - Tumor Necrosis Factor-α
U/S - Ultrasound
VEGF-R1 - Vascular endothelial growth factor

References

1. Ortved, K.F., *Regenerative medicine and rehabilitation for tendinous and ligamentous injuries in sport horses.* Veterinary Clinics: Equine Practice, 2018. **34**(2): p. 359-373.
2. Duddy, H., M. Schoonover, and B. Hague, *Outcome following local injection of a liquid amnion allograft for treatment of equine tendonitis or desmitis–100 cases.* BMC Veterinary Research, 2022. **18**(1): p. 1-10.
3. Stashak, T.S., *Adams' lameness in horses*. 2008: Verlag M. & H. Schaper.
4. Clegg, P., *Differential diagnosis of a swollen hock in the horse.* In Practice, 2003. **25**(6): p. 328-341.
5. Kümmerle, J.M. and M.R. Kummer, *Arthroscopically accessible anatomy of the tarsal collateral ligaments in the horse.* Veterinary Surgery, 2013. **42**(3): p. 267-274.
6. Nagy, A., *The horse in action: anatomy and biomechanics.* Equine Health, 2016. **2016**(29): p. 15-17.
7. Schuurman, S.O., W. Kersten, and W.A. Weijs, *The equine hind limb is actively stabilized during standing.* Journal of anatomy, 2003. **202**(4): p. 355-362.
8. Lohse, C.L. and D.R. Trout, *Equine limb anatomy: peroneus tertius muscle relationships.* Anatomia, Histologia, Embryologia, 1984. **13**(4): p. 313-318.
9. Lamb, L., et al., *Clinical outcome of collateral ligament injuries of the tarsus.* The Canadian Veterinary Journal, 2012. **53**(5): p. 518.
10. Desmaizières, L.M. and E. Cauvin, *Carpal collateral ligament desmopathy in three horses.* Veterinary record, 2005. **157**(7): p. 197-201.
11. Blaik, M.A., et al., *Low-field magnetic resonance imaging of the equine tarsus: normal anatomy.* Veterinary radiology & ultrasound, 2000. **41**(2): p. 131-141.
12. Barker, W., et al., *Soft tissue injuries of the tarsocrural joint: a retrospective analysis of 30 cases evaluated arthroscopically.* Equine Veterinary Journal, 2013. **45**(4): p. 435-441.
13. Garrett, K.S., *Ultrasonography of the hock.* Atlas of equine ultrasonography, 2022: p. 173-188.
14. Tokateloff, N., J. Carmalt, and S. Manning, *Trauma resulting in hemarthrosis and long medial collateral ligament desmitis of the tarsocrural joint in a horse.* The Canadian Veterinary Journal, 2011. **52**(5): p. 519.
15. DYSON, S., *Lameness associated with recurrent haemarthrosis in a horse.* Equine veterinary journal, 1986. **18**(3): p. 224-226.
16. Judy, C.E. and L.D. Galuppo, *Evaluation of iatrogenic hemarthrosis of the metacarpophalangeal joint as a method of induction of temporary reversible lameness in horses.* American journal of veterinary research, 2005. **66**(6): p. 1084-1089.
17. Kawamura, H., et al., *Spontaneous hemarthrosis of the knee in the elderly: etiology and treatment.* Arthroscopy: The Journal of Arthroscopic & Related Surgery, 1994. **10**(2): p. 171-175.
18. Sherlock, C., et al., *Desmitis of the medial tarsal collateral ligament in 7 horses.* Equine Veterinary Education, 2012. **24**(2): p. 72-80.
19. Branch, M.V., et al., *Magnetic resonance imaging of the equine tarsus.* Clinical Techniques in Equine Practice, 2007. **6**(1): p. 96-102.
20. Vanderperren, K., et al., *Diagnostic imaging of the equine tarsal region using radiography and ultrasonography. Part 1: The soft tissues.* The Veterinary Journal, 2009. **179**(2): p. 179-187.
21. PARK, R.D., T.R. NELSON, and P.J. HOOPES, *Magnetic resonance imaging of the normal equine digit and metacarpophalangeal joint.* Veterinary radiology, 1987. **28**(4): p. 105-116.
22. Kraft, S.L. and P. Gavin, *Physical principles and technical considerations for equine computed tomography and magnetic resonance imaging.* Veterinary Clinics of North America: Equine Practice, 2001. **17**(1): p. 115-130.
23. Fraschetto, C., et al., *Conservative management of equine tarsal collateral ligament injuries may allow return to normal performance.* Journal of the American Veterinary Medical Association, 2023. **261**(7): p. 995-1003.
24. Dik, K.J., *Ultrasonography of the equine tarsus.* Veterinary Radiology & Ultrasound, 1993. **34**(1): p. 36-43.
25. Mair, T., et al., *Magnetic resonance imaging of the distal limb of the standing horse.* Equine Veterinary Education, 2005. **17**(2): p. 74-78.
26. Bramlage, L.R. *Traumatic and developmental lesions of the tarsus*. in *Proc. Am. Assoc. Equine Pract*. 2006.
27. Whitcomb, M.B. *Ultrasonography of the equine tarsus*. in *Proceedings of the 52nd Annual Convention of the American Association of Equine Practitioners, San Antonio, Texas, USA, 2-6 December, 2006*. 2006. American Association of Equine Practitioners (AAEP).
28. Chandra, V., P. Mankuzhy, and T. Sharma G, *Mesenchymal Stem Cells in Veterinary Regenerative Therapy: Basic Physiology and Clinical Applications.* Current Stem Cell Research & Therapy, 2022. **17**(3): p. 237-251.
29. Platonova, S., et al., *Equine Tendinopathy Therapy Using Mesenchymal Stem Cells.* KnE Life Sciences, 2021: p. 533–541-533–541.
30. Ribitsch, I., G.L. Oreff, and F. Jenner, *Regenerative medicine for equine musculoskeletal diseases.* Animals, 2021. **11**(1): p. 234.
31. Tuemmers Apablaza, C., N. Rebolledo, and R. Aguilera, *Effect of the application of stem cells for tendon injuries in sporting horses.* 2021.
32. Yu, H., et al., *Bone marrow mesenchymal stem cell-derived exosomes promote tendon regeneration by facilitating the proliferation and migration of endogenous tendon stem/progenitor cells.* Acta biomaterialia, 2020. **106**: p. 328-341.

33. González-González, A., et al., *Mesenchymal stem cells secretome: The cornerstone of cell-free regenerative medicine.* World journal of stem cells, 2020. **12**(12): p. 1529.
34. Pittenger, M.F., et al., *Mesenchymal stem cell perspective: cell biology to clinical progress.* NPJ Regenerative medicine, 2019. **4**(1): p. 1-15.
35. Maguire, G., *Stem cell therapy without the cells.* communicative & integrative Biology, 2013. **6**(6): p. e26631.
36. Moll, G., M.J. Hoogduijn, and J.A. Ankrum, *Safety, efficacy and mechanisms of action of mesenchymal stem cell therapies*. 2020, Frontiers Media SA. p. 243.
37. Wu, X., et al., *Mesenchymal stromal cell therapies: immunomodulatory properties and clinical progress.* Stem cell research & therapy, 2020. **11**(1): p. 1-16.
38. Harrell, C.R., et al., *Molecular mechanisms responsible for therapeutic potential of mesenchymal stem cell-derived secretome.* Cells, 2019. **8**(5): p. 467.
39. Sun, D.Z., et al., *Harnessing the mesenchymal stem cell secretome for regenerative urology.* Nature Reviews Urology, 2019. **16**(6): p. 363-375.
40. Kwon, S., et al., *Engineering approaches for effective therapeutic applications based on extracellular vesicles.* Journal of Controlled Release, 2021. **330**: p. 15-30.
41. Bogatcheva, N. and M. Coleman, *Conditioned medium of mesenchymal stromal cells: a new class of therapeutics.* Biochemistry (Moscow), 2019. **84**(11): p. 1375-1389.
42. Daneshmandi, L., et al., *Emergence of the stem cell secretome in regenerative engineering.* Trends in biotechnology, 2020. **38**(12): p. 1373-1384.
43. Harman, R.M., C. Marx, and G.R. Van de Walle, *Translational animal models provide insight into mesenchymal stromal cell (MSC) secretome therapy.* Frontiers in Cell and Developmental Biology, 2021. **9**: p. 654885.
44. Raik, S., A. Kumar, and S. Bhattacharyya, *Insights into cell-free therapeutic approach: Role of stem cell "supernatant".* Biotechnology and Applied Biochemistry, 2018. **65**(2): p. 104-118.
45. Markov, A., et al., *Mesenchymal stem/stromal cells as a valuable source for the treatment of immune-mediated disorders.* Stem cell research & therapy, 2021. **12**(1): p. 1-30.
46. Broeckx, S.Y., et al., *Equine allogeneic chondrogenic induced mesenchymal stem cells are an effective treatment for degenerative joint disease in horses.* Stem Cells and Development, 2019. **28**(6): p. 410-422.
47. Committee, A.H.S., *Guide to veterinary services for horse shows.* Lexington: American Association of Equine Practitioners, 1999.
48. Practitioners, A.A.o.E. *Guide for veterinary service and judging of equestrian events*. 1991. AAEP.
49. Leal Reis, I., et al., *Allogenic Synovia-Derived Mesenchymal Stem Cells for Treatment of Equine Tendinopathies and Desmopathies—Proof of Concept.* Animals, 2023. **13**(8): p. 1312.
50. Guest, D.J., et al., *Position Statement: Minimal criteria for reporting veterinary and animal medicine research for mesenchymal stromal/stem cells in orthopaedic applications.* Frontiers in Veterinary Science, 2022: p. 199.
51. Thomopoulos, S., et al., *Mechanisms of tendon injury and repair.* Journal of Orthopaedic Research, 2015. **33**(6): p. 832-839.
52. Schils, S. and T. Turner. *Review of early mobilization of muscle, tendon, and ligament after injury in equine rehabilitation*. in *Proceedings of the 56th Annual Convention of the American Association of Equine Practitioners, Baltimore, Maryland, USA, 4-8 December 2010*. 2010. American Association of Equine Practitioners (AAEP).
53. Kaneps, A.J., *Practical rehabilitation and physical therapy for the general equine practitioner.* Veterinary Clinics: Equine Practice, 2016. **32**(1): p. 167-180.
54. Davidson, E.J., *Controlled exercise in equine rehabilitation.* Veterinary Clinics: Equine Practice, 2016. **32**(1): p. 159-165.
55. Davidson, E.J., *Lameness evaluation of the athletic horse.* Veterinary Clinics: Equine Practice, 2018. **34**(2): p. 181-191.
56. Godwin, E., et al., *Implantation of bone marrow-derived mesenchymal stem cells demonstrates improved outcome in horses with overstrain injury of the superficial digital flexor tendon.* Equine veterinary journal, 2012. **44**(1): p. 25-32.
57. Bami, M., et al., *Superiority of synovial membrane mesenchymal stem cells in chondrogenesis, osteogenesis, myogenesis and tenogenesis in a rabbit model.* Injury, 2020. **51**(12): p. 2855-2865.
58. De Bari, C., et al., *Multipotent mesenchymal stem cells from adult human synovial membrane.* Arthritis & Rheumatism, 2001. **44**(8): p. 1928-1942.
59. Mocchi, M., et al., *Veterinary regenerative medicine for musculoskeletal disorders: can mesenchymal stem/stromal cells and their secretome be the new frontier?* Cells, 2020. **9**(6): p. 1453.
60. Madrigal, M., K.S. Rao, and N.H. Riordan, *A review of therapeutic effects of mesenchymal stem cell secretions and induction of secretory modification by different culture methods.* Journal of translational medicine, 2014. **12**(1): p. 1-14.
61. Al Naem, M., et al., *Therapeutic mesenchymal stromal stem cells: Isolation, characterization and role in equine regenerative medicine and metabolic disorders.* Stem cell reviews and reports, 2020. **16**(2): p. 301-322.
62. Li, J.J., et al., *Stem cell-derived extracellular vesicles for treating joint injury and osteoarthritis.* Nanomaterials, 2019. **9**(2): p. 261.

63. Han, C., et al., *Exosomes and their therapeutic potentials of stem cells.* Stem cells international, 2016. **2016**.
64. Pawitan, J.A., *Prospect of stem cell conditioned medium in regenerative medicine.* BioMed research international, 2014. **2014**.
65. Shireman, P.K., et al., *MCP-1 deficiency causes altered inflammation with impaired skeletal muscle regeneration.* Journal of leukocyte biology, 2007. **81**(3): p. 775-785.
66. Zhang, Y.-J., et al., *Concise review: stem cell fate guided by bioactive molecules for tendon regeneration.* Stem Cells Translational Medicine, 2018. **7**(5): p. 404-414.
67. Shiomi, A., T. Usui, and T. Mimori, *GM-CSF as a therapeutic target in autoimmune diseases.* Inflammation and Regeneration, 2016. **36**(1): p. 1-9.
68. Wright, C.R., A.C. Ward, and A.P. Russell, *Granulocyte colony-stimulating factor and its potential application for skeletal muscle repair and regeneration.* Mediators of inflammation, 2017. **2017**.
69. Paredes, J., et al., *Innate tissue properties drive improved tendon healing in MRL/MpJ and harness cues that enhance behavior of canonical healing cells.* The FASEB Journal, 2020. **34**(6): p. 8341-8356.
70. Al-Sadi, O., et al., *Tenocytes, pro-inflammatory cytokines and leukocytes: a relationship?* Muscles, ligaments and tendons journal, 2011. **1**(3): p. 68.
71. Abumaree, M., et al., *Immunosuppressive properties of mesenchymal stem cells.* Stem Cell Reviews and Reports, 2012. **8**: p. 375-392.
72. Hofer, H.R. and R.S. Tuan, *Secreted trophic factors of mesenchymal stem cells support neurovascular and musculoskeletal therapies.* Stem cell research & therapy, 2016. **7**: p. 1-14.
73. Galun, E. and S. Rose-John, *The regenerative activity of interleukin-6.* Tissue-Protective Cytokines: Methods and Protocols, 2013: p. 59-77.
74. Hirota, H., et al., *Accelerated Nerve Regeneration in Mice by upregulated expression of interleukin (IL) 6 and IL-6 receptor after trauma.* The Journal of experimental medicine, 1996. **183**(6): p. 2627-2634.
75. Ackermann, P.W., et al., *Anti-inflammatory cytokine profile in early human tendon repair.* Knee surgery, sports traumatology, arthroscopy, 2013. **21**: p. 1801-1806.
76. Chen, S., et al., *Interleukin-6 promotes proliferation but inhibits tenogenic differentiation via the Janus kinase/signal transducers and activators of transcription 3 (JAK/STAT3) pathway in tendon-derived stem cells.* Medical science monitor: international medical journal of experimental and clinical research, 2018. **24**: p. 1567.
77. Lin, T.W., et al., *Tendon healing in interleukin-4 and interleukin-6 knockout mice.* Journal of biomechanics, 2006. **39**(1): p. 61-69.
78. Andersen, M.B., et al., *Interleukin-6: a growth factor stimulating collagen synthesis in human tendon.* Journal of applied physiology, 2011. **110**(6): p. 1549-1554.
79. Kim, K.-O., et al., *The effect of interleukin-8 in the early stage after anterior cruciate ligament reconstruction with remnant preservation.* Knee surgery & related research, 2020. **32**(1): p. 1-9.
80. Ohls, R.K. and A. Maheshwari, *Hematology, Immunology and Infectious Disease: Neonatology Questions and Controversies: Expert Consult-Online and Print*. 2012: Elsevier Health Sciences.
81. Amable, P.R., et al., *Protein synthesis and secretion in human mesenchymal cells derived from bone marrow, adipose tissue and Wharton's jelly.* Stem cell research & therapy, 2014. **5**(2): p. 1-13.
82. Wang, H.-N., et al., *Advances in Stem Cell Therapies for Rotator Cuff Injuries.* Frontiers in Bioengineering and Biotechnology, 2022. **10**.
83. Scala, M., et al., *Regenerative medicine for the treatment of Teno-desmic injuries of the equine. A series of 150 horses treated with platelet-derived growth factors.* in vivo, 2014. **28**(6): p. 1119-1123.
84. Raes, E.V., et al., *Ultrasonographic findings in 100 horses with tarsal region disorders.* The Veterinary Journal, 2010. **186**(2): p. 201-209.
85. Bell, C., K. Torske, and B. Lobb, *Collateral ligament reconstruction in two horses following traumatic avulsion fracture using a knotless suture anchor construct.* Equine Veterinary Education, 2018. **30**(7): p. 360-366.
86. Salz, R.O., et al., *Treatment of racehorse superficial digital flexor tendonitis–a comparison of stem cell treatments to controlled exercise rehabilitation in 213 cases.* Equine Veterinary Journal, 2023.
87. Rose, P.L. and I. Moore, *Imaging diagnosis--avulsion of the medial collateral ligament of the tarsus in a horse.* Veterinary Radiology & Ultrasound: The Official Journal of the American College of Veterinary Radiology and the International Veterinary Radiology Association, 2003. **44**(6): p. 657-659.
88. Shokry, M., et al., *Autologous mesenchymal stem cells for treatment of acute superficial digital flexor tendonitis in athletic horses-A clinical study of 1 5 cases.* Pferdeheilkunde, 2020. **36**: p. 43-8.
89. Lui, P., et al., *What are the validated animal models for tendinopathy?* Scandinavian journal of medicine & science in sports, 2011. **21**(1): p. 3-17.
90. Melotti, L., et al., *Case Report: Repeated Intralesional Injections of Autologous Mesenchymal Stem Cells Combined With Platelet-Rich Plasma for Superficial Digital Flexor Tendon Healing in a Show Jumping Horse.* Frontiers in Veterinary Science, 2022. **9**.
91. An, Y.H. and R.J. Friedman, *16 Animal Models of Articular Cartilage Defect.* Animal models in orthopaedic research, 2020.

92. Lönker, N.S., K. Fechner, and A. Abd El Wahed, *Horses as a crucial part of one health.* Veterinary Sciences, 2020. **7**(1): p. 28.