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Development of cellular therapies for the treatment of musculoskeletal pathologies in equine patients - Synovial membrane derived mesenchymal stromal/stem cells– (SM-MSCs)

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- Synovial membrane derived mesenchymal stromal/ stem cells– (SM-MSCs)

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Development of cellular therapies for the treatment of musculoskeletal pathologies in equine patients - Synovial membrane mesenchymal stromal/stem cells - (SM-MSCs)

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"Declaro que a presente tese é de minha autoria e não foi utilizada previamente noutro curso ou unidade curricular, desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras da atribuição, e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referenciação. Tenho consciência de que a prática de plágio e auto-plágio constitui um ilícito académico".

List of publications

Based on "nº 2, alínea a, do Art.º 31º do Decreto-lei nº 230/2009", is declared that the author afforded a major contribution to the technical execution of the work, interpretation of the results and manuscript preparation of the published articles included in this project. According to "Artigo nº 31, do Decreto-Lei nº 115/2013", the following results, published or submitted for publication, are an integral part of this thesis.

Scientific publications

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Therapeutical Perspectives—A Review. *Veterinary Sciences*, *11*(5), 190. doi.org/10.3390/vetsci11050190

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Patent

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). https://patentscope.wipo.int/search/pt/detail.jsf?docId=WO2019175773&_cid=P12-KFO3NP-33325-1

À minha família, o meu colo e o meu alento.
Aos “meus” cavalos, a minha motivação!

Do not judge me by my success, judge me by how many times I fell down and got back up again

Nelson Mandela

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“A whole village is needed to raise a child!”

I say: “A whole village is needed to raise a project!”

Having this in mind, I must be grateful to some people who accompanied me during this process or just made part of the process…

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To you Dad, I know that you are always around to protect me. I´m sure you are proud of me! You have always believed in me! “The W…”!

To Tobias and Tommy, my loyal four- legged friends!

PREFACE

Regenera, Regeneratio

Regeneration has its etymological origin in the Latin word “Regeneratio”, which means, “to form again after loss or injury, to replace by regeneration or spiritual rebirth”.

The concept of regeneration can be traced back to Greek mythology, embodied in the figure of Prometheus. Prometheus is renowned for his intelligence and for defying Zeus for the benefit of humanity. He is known for stealing fire from Mount Olympus and giving it to humans, symbolising the gift of knowledge and enlightenment. As punishment, Prometheus was bound to a rock, where his liver was consumed daily by an eagle, which would then regenerate overnight. The liver, believed by ancient Greeks to house human emotions, undergoes perpetual renewal, implying Prometheus's enduring suffering and his solid dedication to humanity. Prometheus' regeneration also alludes to the notion of life's resilience and renewal in the face of adversity.

Tissue regeneration, which naturally occurs in certain animal species, has long captivated scientists and inspired the pursuit of analogous technologies for medical applications. Species such as salamanders and some marine organisms exhibit remarkable regenerative capabilities, enabling them to restore lost appendages and even essential organs.

The term "regenerative therapy," however, is fairly recent. This concept took shape in the mid-20th century, driven by advances in tissue engineering, stem cell research, and biomaterials science. As the field progressed, the late 20th and early 21st centuries saw significant developments in stem cell therapy, tissue engineering, and gene editing technologies. Presently, regenerative medicine adopts a multidisciplinary approach, striving to repair, replace, or regenerate damaged tissues and organs. This field holds considerable promise for treating a diverse array of medical conditions and injuries.

When diving in these lines I realize this theme has been capturing my attention long before I was aware. Working in this project of “regenerative medicine”, allowed me to embrace a new path to help recover one of my favourite animals – the horse.

All along my professional life I have been working with horses and fallen passionate with them... Some have even left part of them with me… From the common leisure horse, to the ones I have fed when they were born, to the one that always came to me for a caress whenever I was around, to the foals I have seen growing up with all the illusions they create on us, to the Grand-Prix ones, to the Garrano in Gerês, to the Olympic and renowned horses. With all of them I have learned! I admire their power, their elegance, their *noblesse*,

their free spirit, their beauty, their *souplesse*, their dedication, their willingness to please us, their empathy and the way we communicate.

As a veterinarian dedicated to equine sports medicine, encountering limitations in therapeutic outcomes for injuries in my patients was profoundly frustrating. Traditional treatments often failed to restore full functionality and athletic performance in sport horses. The investment of substantial time and effort into researching and implementing novel regenerative therapies developed by our team has yielded promising results, which is immensely fulfilling. These advanced treatments have significantly enhanced the recovery process, allowing many horses to not only regain their previous quality of life but also to resume and excel in their athletic activities, surpassing the results achievable with conventional therapies.

Additionally, during this process, me, myself and I also experienced a renewal… I have also performed a “rebirth” … beside having literally given birth twice and being reborn as a Woman-Mother… by the same token, “giving birth” to this thesis work, and all it encompasses, made me more scientifically and technically skilled, resilient, stronger and richer. This project awakened in me a passion that has already existed for a long time and has now reached its maximum expression. Regenerative Medicine allowed me to recover patients, athletes or not, breaking classic paradigms by offering new and more appropriate tools to achieve positive therapeutical outcomes. With this in mind, I became another “me” ... I grew up as a person, as an engaged professional and, a small part of me that I thought didn’t exist was also reborn… as a scientist.

So many joys, so much anguish, so many challenges, so many enlightenments, but first and foremost ... so much Growth!

Now, when I may gaze toward the conclusion of this journey, I can confidently affirm that this is truly a work of regeneration in all its forms!

RESUMO

Quando se trata de aplicações de medicina regenerativa em ortopedia, a medicina equina destaca-se por estar no topo da investigação e uso clínico. Os equinos são atletas de alto desempenho, em várias modalidades, que são expostos a desafios crescentes, rigorosos treinos para competição, mudança frequente de ambiente, ao que acresce a exigência de se manterem competitivos ao mais alto nível. As patologias musculoesqueléticas em equinos estão entre as principais razões para cuidados veterinários e são a principal causa de perda de desempenho, cessação atlética, declínio funcional e, em último caso, ocisão. Os tratamentos médicos e cirúrgicos convencionais atualmente não conseguem satisfazer as exigências da vida desportiva, pois reduzem a dor e a inflamação, mas não proporcionam reparação e regeneração das estruturas afetadas.

Nas últimas décadas, as terapias regenerativas no domínio da medicina equina avançaram significativamente, ultrapassando as disponíveis nas práticas médicas humanas. Muita investigação tem sido feita numa tentativa de ser vanguardista e pioneira em novas terapias regenerativas adaptadas para a espécie equina, com o objetivo de aumentar a longevidade atlética e as capacidades de desempenho.

Esta tese centra-se no desenvolvimento e no efeito de formulações ortobiológicas únicas na área da medicina regenerativa equina. Estas formulações baseiam-se no uso de células estaminais/estromais mesenquimatosas de membrana sinovial e no uso de meio condicionado de células estaminais/estromais mesenquimatosas de cordão umbilical. Com base no conhecimento do potencial de cada tipo celular, desenvolveu-se uma formulação com o objectivo de potenciar as suas propriedades complementares, com o objectivo final de regeneração de estruturas musculo-esqueléticas e de imunomodulação/imunossupressão para viabilizar a existência de um dador universal.

Nesta pesquisa, foram meticulosamente desenvolvidos, produzidos e aplicados dois novos produtos terapêuticos regenerativos em casos clínicos reais de pacientes equinos afetados por patologias musculoesqueléticas, num ambiente de clínica ambulatória. O resultado do tratamento foi feito através de avaliações clínicas e imagiológicas, bem como as repercussões na qualidade de vida do paciente e até mesmo no seu desempenho desportivo.

Os estudos clínicos descritos aqui são baseados em evidências e os resultados alcançados revelaram interações biológicas complexas, cruciais para refinar as terapias médicas regenerativas e preencher a lacuna que atualmente existe na medicina ortopédica

equina. As melhorias obtidas de forma gradual e clinicamente relevantes, contribuirão para a construção contínua e personalizada da medicina regenerativa equina.

Este trabalho culminou no pedido de patente para este tipo de produtos terapêuticos - REGENERA® (PCT/IB2019/052006, WO2019175773) e na publicação de quatro artigos.

Palavras-chave: Células estromais/estaminais mesenquimatosas de membrana sinovial e de cordão umbilical, equinos, meio condicionado, músculo-esquelético, regeneração.

ABSTRACT

When it comes to the applications of regenerative medicine in orthopaedics, equine medicine stands in the topline of the research and clinical use.

Equines are high performance athletes in various disciplines, prone to increasingly challenges, experiencing rigorous competition trainings, new environments and the demand of staying competitive at the highest level. Musculoskeletal disorders in equines are among the primary reasons of veterinary care and are the leading cause for performance loss, athletic cessation, functional decline, and ultimately, culling. Conventional medical and surgical treatments currently fail to meet the demands of sports life, as they reduce pain and inflammation but do not provide repair and regeneration of the affected structures.

In the last decades, regenerative therapies in the domain of equine medicine have advanced significantly, surpassing those available in human medical practices. Extensive research endeavours have been undertaken to pioneer novel regenerative therapies tailored for equine species, aiming to enhance their athletic longevity and performance capabilities.

This thesis focuses on the development and effects of unique orthobiological formulations in the field of equine regenerative medicine. These formulations are based on the use of mesenchymal stem/stromal cells from synovial membrane and conditioned medium from umbilical cord mesenchymal stem/stromal cells. Leveraging the known potential of each cell type, a formulation was developed to enhance their complementary properties. The ultimate goal is to achieve regeneration of musculoskeletal structures and provide immunomodulation/immunosuppression to enable the existence of a universal donor.

In this research, two new regenerative therapeutic products have been meticulously developed, produced and applied in real clinical cases of equine patients affected by musculoskeletal pathologies, in an ambulatory clinic setting. The treatment outcome was evaluated through clinical and imaging assessments, as well as the effect on the patient's quality of life and even sportive performance.

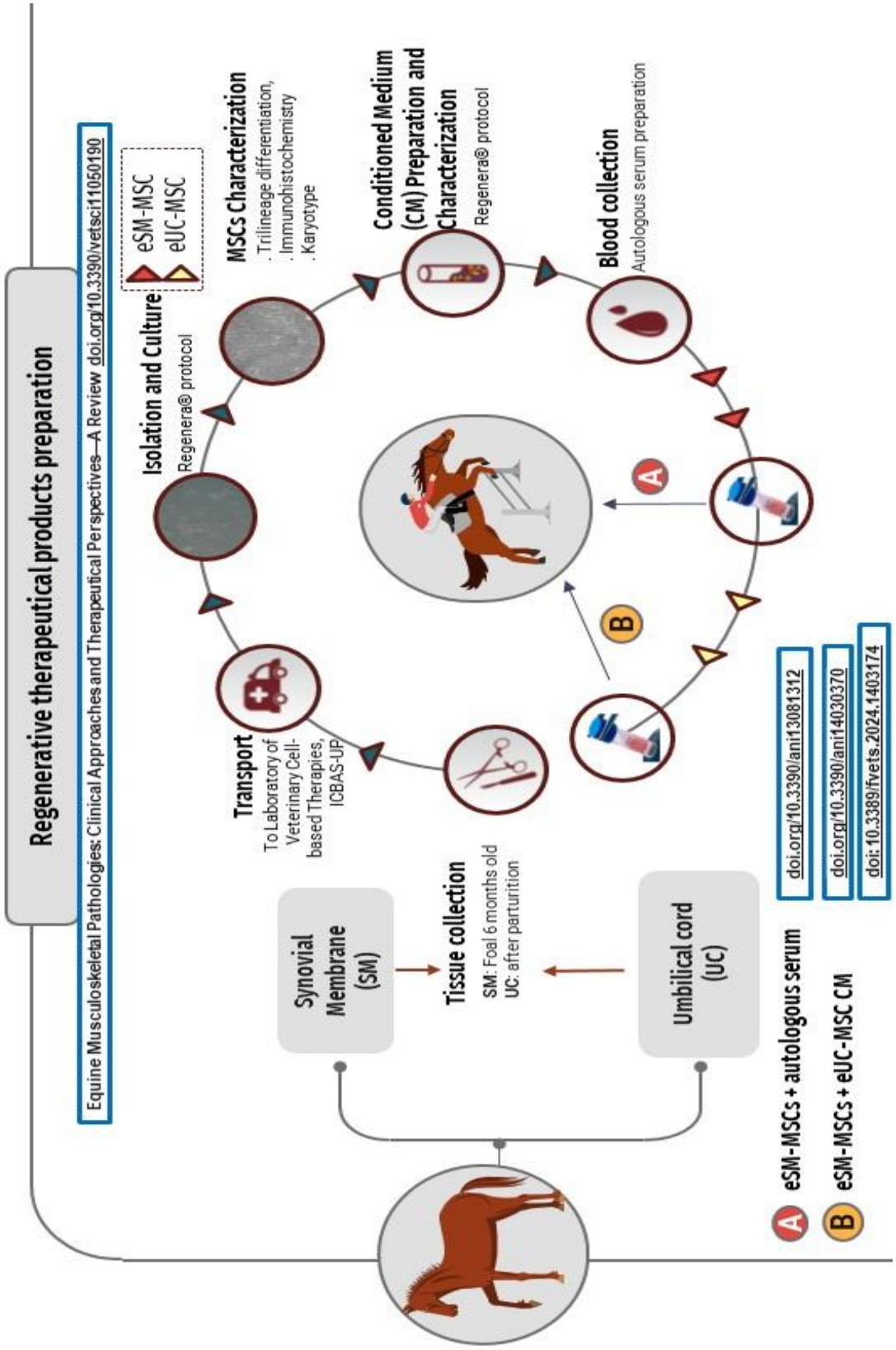
The clinical studies described here are evidence-based and the results achieved revealed complex biological interactions crucial for refining regenerative medical therapies and to fill the gap that currently exists in equine orthopaedic medicine.

The relevant and gradual clinical improvements and advances obtained will contribute to the continuous construction and personalization of equine regenerative medicine.

This work culminated in the filing of a patent for these types of therapeutic products - REGENERA® (PCT/IB2019/052006, WO2019175773) and in the publication of four articles.

Keywords: conditioned medium, equines, musculo-skeletal, regenerative, synovial membrane and umbilical cord mesenchymal stromal/stem cells.

GRAPHICAL ABSTRACT



ABBREVIATIONS

3D three-dimensional

AAEP American Association of Equine Practitioners

ACI Autologous chondrocyte implantation

ACS autologous conditioned serum

AE1/AE3 Pan-Cytokeratin

AFS-MSC amniotic fluid mesenchymal stromal/stem cell

AINEs non-steroidal anti-inflammatories

APS autologous protein serum

AT-MSCs Adipose Tissue mesenchymal stromal cells

BM-MSC Bone Marrow mesenchymal stem cell

CD Cluster differentiation

CD complementary diagnostic exams

CDET common digital extensor tendon

c-Kit Proto-oncogene receptor tyrosine kinase or stem cell factor receptor

CL Collateral ligament

CM Conditioned Medium

cm centimetre

CM Conditioned Medium

cm2 square centimetre

Coll-II Collagen type II

COMP Cartilage oligomeric matrix protein

COX cycloxigenase

CPD Cumulative population doublings

CSA cross sectional area

CT Computed Tomography

CT Computed Tomography

CTA Computed Tomography arthrography

D Day

d Days

DDFT deep digital flexor tendon

DLPMO Oblique Dorsolateral-plantaromedial

DMEM Dulbecco’s Modified Eagle Medium

DMPLO Oblique dorsomedial-plantarolateral

DMSO Dimethylsulphoxide

DNA desoxyribonucleic acid

DP Dorso plantar

DPBS Dulbecco′s Phosphate Buffered Saline

DP-MSC dental pulp mesenchymal stromal/stem cell

ECM extracellular matrix

ESC embryonic stem cell

eSM-MSC Equine synovial membrane mesenchymal stem/stroma cell

ESWT extracorporeal shock wave therapy

eUC-CM Equine Umbilical cord conditioned medium

eUC-MSC CM Equine Umbilical cord mesenchymal stem/stromal cell conditioned medium

EV extracellular vesicles

FBS Fetal bovine serum

FEI Federation Equestre Internationale

FGF fibroblast growth factor

FGF-2 Basic Fibroblast Growth Factor

FL forelimbs

Flex flexed

FSE Fast scan echo

G Gauge

GAG glycosaminoglicans

G-CSF Granulocyte Colony Stimulating Factor

GF growth factor

GFAP Glial fibrillary acidic protein

GM-CSF Granulocyte-macrophage Colony Stimulating Factor

GRO/KC Human Growth-regulated oncogene/Keratinocyte Chemoattractant

h hour

HILT high intensity laser therapy

HL hindlimbs

HLA human leucocyte antigen

hPSCs human Pluripotent Stromal/Stem Cell

HSC hematopoietic stem cell

HyA hyaluronic acid

Hz Hertz

IA Intra-articular

ICBAS-UP Instituto de Ciências Biomédicas Abel Salazar – Universidade do Porto

IGF insulin growth factor

IL Interleukins

IL-1Ra Interleukin one receptor antagonist

ILGRE Gradient recalled echo

IM intramuscular

IPSC induced pluripotent stem cell

IRAP Interleukin receptor antagonist protein

IRU radioisotope uptake

ISCT International Society for Cellular Therapy

IV Endovenous

KC/GRO Human Growth-regulated oncogene/Keratinocyte Chemoattractant

kg Kilogram

KT Kynesiotaping

Kv Kilovolt

LF Left forelimb

LLCL Long lateral collateral ligament

LLLT low level laser therapy

LM Lateromedial

LMCL Long medial collateral ligament

mA milliampere

MC3 Third Metacarpus

MCB Master Cell Banks

MCj Metacarpophalangeal joint

MCP-1 Monocyte Chemoattractant Protein-1

mg milligram

MHC-II Major Histocompatibility Complex

MHz Megahertz

min minutes

mL millilitre

MMP-3 Matrix metaloproteinase-3

MRI Magnetic Resonance Image

MSC mesenchymal stem/stromal cells

MT-MSC muscle tissue mesenchymal stromal/stem cell

mW milliwatts

NEMS Neural electrical muscle stimulation

nm nanometres

NSAIDs non-steroidal anti-inflammatory drugs

OA Osteoarthritis

ºC Celsius degree

OCT-4 Octamer-binding transcription factor 4

ORBEA Organismo Responsável pelo Bem-estar Animal

P Passage

P1 First phalanx

PBS Phosphate-buffered saline

PDEGF platelet derived epidermal growth factor

PDGF platelet derived growth factor

PEMF pulsed electromagnetic frequency

PGE2 Prostaglandin E2

PHyd Polyacrylamide hydrogel

PMDLO Oblique palmaromedialdorsolateral

Po -MSC periosteum mesenchymal stromal/stem cell

PO Per os

PRP Platelet-rich plasma

PSAG Polysulfated glycosaminoglycan

RNA ribonucleic acid

ROM range of motion

rpm Rotations per minute

RT-PCR Reverse transcription polymerase chain reaction

SAIDs steroidal anti-inflammatories

SDFT superficial digital flexor tendon

SID Once a day

SL suspensory ligament

SM Synovial membrane

SM-MSC Synovial membrane mesenchymal stromal/stem cell

STIR Short Tau Inversion Recovery

TENS transcutaneous electrical nerve stimulation

TGF Transforming growth factor

TW Time weighted image

U/S Ultrasound

UC Umbilical cord

UC-MSC MC Umbilical cord mesenchymal stem/stromal cell conditioned medium

UC-MSC umbilical cord derived Whartons jelly mesenchymal stromal/stem cell

UDP Uridine Diphosphate

UDPGD Uridine Diphosphate Glucose Dehydrogenase UDPGD

VEGF vascular endothelial growth factor

VEGF-R1 Vascular endothelial growth factor

Vet-check Veterinary check-up

W Watts

W weight

WJ Wharton Jelly

WT water treadmill

X-ray Radiograph

α2M Alfa-2 macroglobulin

TABLE OF CONTENTS

CHAPTER 1
……1

Paper I: Equine musculoskeletal pathologies: clinical approaches and therapeutical perspectives - a
review……3

CHAPTER 2
……45

Introduction ……..... 49

Paper II: Allogenic synovia-derived mesenchymal stem cells for treatment of equine tendinopathies and desmopathies—Proof of concept. …… 53

Graphical Paper II …… 85

CHAPTER 3 …… ...87

Introduction
……91

Paper III: Treatment of Equine Tarsus Long Medial Collateral Ligament Desmitis with Allogenic Synovial Membrane Mesenchymal Stem/Stromal Cells Enhanced by Umbilical Cord Mesenchymal Stem/Stromal Cell-Derived Conditioned Medium: Proof of Concept …. 97

Graphical Paper III …… 119

CHAPTER 4 …… 123

Introduction ……..…. 127

Paper IV: Case report: equine metacarpophalangeal joint partial and full thickness defects treated with allogenic equine synovial membrane mesenchymal stem/stromal cell combined with umbilical cord mesenchymal stem/stromal cell conditioned medium …….. 135

Graphical Paper IV …… ...149

CHAPTER 5 ……
153

General Discussion ……..
157

Conclusions and future perspectives .....……..
167

CHAPTER I

Equine musculoskeletal pathologies: clinical approaches and therapeutical perspectives - a review

CHAPTER I

- **PAPER I**

Equine Musculoskeletal Pathologies: Clinical Approaches and Therapeutical Perspectives—A Review

Reis, I. L., Lopes, B., Sousa, P., Sousa, A. C., Caseiro, A. R., Mendonça, C. M., Santos J.M., Atayde L.M., Alvites R.D. & Maurício, A. C. (2024). *Veterinary Sciences*, *11*(5), 190. *DOI:10.3390/vetsci11050190*

PAPER I

Equine Musculoskeletal Pathologies: Clinical Approaches and Therapeutical Perspectives – A review.

veterinary sciences

MDPI

Review

Equine Musculoskeletal Pathologies: Clinical Approaches and Therapeutical Perspectives—A Review

Inês L. Reis [1,2,3,4], Bruna Lopes [1,2,3], Patrícia Sousa [1,2,3], Ana C. Sousa [1,2,3], Ana R. Caseiro [2,3,5,6], Carla M. Mendonça [1,2,3,7], Jorge M. Santos [1,2,3], Luís M. Atayde [1,2,3,7], Rui D. Alvites [1,2,3,4] and Ana C. Maurício [1,2,3,7,*]

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Simple Summary: In the current era, sport horses face the challenge of increasingly rigorous workouts, overtaking, and, at times, certain physiological thresholds, leaving them susceptible to injury. This shift underscores the significance of prioritizing the exercise preparation quality and post-care to proactively mitigate the risk of injuries. Despite these measures, injuries may still occur, thus requiring a nuanced understanding of the most effective therapeutic approaches for various types of lesions. In the dynamic field of equine sports medicine, keeping abreast of the expansive therapeutic options proves challenging, especially when aiming to address injuries comprehensively, restore organ function, and sustain the horse's athletic life. The present endeavor seeks to elucidate the array of available therapies, encompassing both conservative and regenerative methods, for the effective management of musculoskeletal injuries in sport horses.

Abstract: Musculoskeletal injuries such as equine osteoarthritis, osteoarticular defects, tendonitis/desmitis, and muscular disorders are prevalent among sport horses, with a fair prognosis for returning to exercise or previous performance levels. The field of equine medicine has witnessed rapid and fruitful development, resulting in a diverse range of therapeutic options for musculoskeletal problems. Staying abreast of these advancements can be challenging, prompting the need for a comprehensive review of commonly used and recent treatments. The aim is to compile current therapeutic options for managing these injuries, spanning from simple to complex physiotherapy techniques, conservative treatments including steroidal and non-steroidal anti-inflammatory drugs, hyaluronic acid, polysulfated glycosaminoglycans, pentosan polysulfate, and polyacrylamides, to promising regenerative therapies such as hemoderivatives and stem cell-based therapies. Each therapeutic modality is scrutinized for its benefits, limitations, and potential synergistic actions to facilitate their most effective application for the intended healing/regeneration of the injured tissue/organ and subsequent patient recovery. While stem cell-based therapies have emerged as particularly promising

for equine musculoskeletal injuries, a multidisciplinary approach is underscored throughout the discussion, emphasizing the importance of considering various therapeutic modalities in tandem.

Keywords: conservative therapies; equine; hemoderivatives; musculoskeletal; osteoarthritis; physiotherapy; regenerative therapies; sports medicine; stem cell; tendinitis

1. Introduction

In equine sports medicine, musculoskeletal lesions such as tendonitis, desmitis, osteoarticular lesions/defects, and muscular strains stand as significant challenges. These conditions often result in a partial or complete loss of performance, jeopardizing the athlete's sporting career. Beyond the immediate impact on performance, these injuries can have far-reaching consequences, affecting the overall well-being of the horse [1,2]. The repercussions extend to individual health, sporting activity, and carry social and economic implications, making the management and prevention of such musculoskeletal issues crucial in the field of equine sports [3–5].

Each of these injuries presents unique characteristics and are a challenge in terms of diagnosis, treatment, and rehabilitation. Understanding and addressing these issues is vital for maintaining the health and longevity of equine athletes.

The present work provides an overview of prevalent musculoskeletal pathologies, explores complementary diagnostic exams, and reviews the existing therapeutic options for managing pain, inflammation, and the healing or regeneration of affected musculoskeletal structures. The current prospect offers a diverse range of therapeutic options, each varying in ease of use, availability, economic considerations, mode of action, effectiveness, and clinical outcomes.

Initial treatment protocols emphasize conservative interventions aimed at alleviating clinical signs, slowing progression, and potentially facilitating tissue repair through fibrosis or scar formation [6]. These interventions span both non-pharmacological and pharmacological approaches, encompassing a spectrum of exercises and physical modalities (e.g., laser therapy, ultrasound, electrotherapy, and shock waves) as well as pharmacological options including anti-inflammatories, viscosupplementation, and bisphosphonates. Surgical techniques such as tendon splitting are also employed for conditions such as tendonitis to provide relief from the clinical signs and induce tissue repair.

More recently, the focus has shifted toward achieving tissue regeneration rather than mere repair. Several pro-regenerative therapeutic options have emerged, and others are currently in development. Termed orthobiologics, a subdivision of regenerative medicine, with a specific emphasis on addressing musculoskeletal conditions, these therapies are based on biological substances to promote regeneration in the tendons, muscles, and joints [7]. Two major categories within orthobiologics are hemoderivatives and stem cell-based therapies. The composition, distinctions, beneficial contributions, advantages offered by each, and the appropriate scenarios for their application will be elucidated through the article. The overarching objective of these regenerative therapies is to preserve organ function and facilitate the restoration of native physiological capacity in musculoskeletal diseases.

2. Clinical Examination

A complete history and a comprehensive clinical and orthopedic examination hold paramount importance in the field of orthopedic medicine to accurately diagnose musculoskeletal conditions [8]. The findings from a clinical and orthopedic examination guide the formulation of an appropriate plan of complementary diagnostic exams and a tailored treatment strategy. Additionally, the examination aids in identifying any underlying issue contributing to musculoskeletal problems. Orthopedic examination must include inspection, palpation, percussion, hoof testers, and dynamic analysis of the horse. This holistic approach ensures that all relevant factors are considered for comprehensive care [9].

Regular clinical examinations can help identify risk factors or early signs of musculoskeletal issues. This allows for the implementation of preventive measures to mitigate the progression of conditions or prevent future injuries. Follow-up examinations are essential to monitor the progress of treatment and adjusting the interventions as needed. This constant process ensures that the patient's musculoskeletal health is continually assessed and managed effectively. In summary, equine clinical examinations are essential for maintaining the health, well-being, and performance of horses [10].

3. Complementary Diagnostic Exams

To accurately diagnose musculoskeletal pathologies, comprehensive physical and orthopedic examinations, along with detailed complementary diagnostic (CD) tests, are imperative. CD tools encompass a broad-spectrum including flexion tests, perineural nerve blocks, intra-articular anesthesia, and various imaging techniques. The selection of these tools depends on factors such as accuracy, accessibility, cost, and the ability of individuals or professionals to interpret the images. For the diagnosis of musculoskeletal injuries, available imaging techniques comprise radiographs (X-ray), ultrasound (U/S) images, arthroscopy, magnetic resonance imaging (MRI), computed tomography (CT), scintigraphy and positron emission tomography (PET) scans. Each of these methods offers unique advantages and considerations in the diagnostic process.

Perineural nerve blocks and intra-articular anesthesia aid in the determination of the pain localization area [11].

Radiography stands as a non-invasive and primary diagnostic tool, offering crucial insights into several aspects. It enables the identification of significant information, encompassing the diagnosis of evident bone fractures, defects in articular cartilage, and indications of OA. In radiological OA diagnosis, three primary findings are prominent: the presence of osteophytes, increased subchondral density, and the eventual narrowing of the joint space in its advanced stages [12].

Nevertheless, during the early stages of a lesion, the observation of radiological evidence becomes challenging, potentially leading to inappropriate exclusion or insufficient information regarding the actual extent of the lesions. Moreover, when assessing complex joints, radiography faces limitations as it provides a two-dimensional (2D) representation of three-dimensional (3D) structures. This limitation arises because multiple soft tissues and bony structures are superimposed, making it difficult to evaluate them individually. Consequently, radiography may not be the most favorable method for the assessment of soft tissues [13,14]. Nonetheless, when dealing with cases involving joint or limb distension, radiography is recommended as an initial diagnostic procedure. In instances of acute tendinopathy, desmopathy, or enthesopathy without fragmentation of the insertional surface, radiography may primarily indicate the presence of soft tissue swelling [15].

Ultrasonography has emerged as the preferred technique for diagnosing, assessing, and documenting tendon and ligament lesions, offering valuable insights into therapeutic and healing progresses. The ultrasonographic method for evaluating the pastern region, specifically for palmar/plantar tendon/ligament assessment, is roughly documented in the existing literature [16–18]. A thorough ultrasound assessment of soft tissue injuries is very important to accurately diagnose these lesions [7]. However, in conditions of intra-articular soft tissue lesions, it does not allow for the visualization of structures located deep to the bone [14,19,20]. Since 1990, ultrasonography has been used to complement radiography in cartilage defects diagnosis [21]. Ultrasound has become an essential diagnostic tool as it provides precise information on the synovial membrane and fluid, articular cartilage, subchondral bone, joint margins, ligaments and menisci as well as on the periarticular structures [21]. It requires good ultrasound devices and a strict standardization of the approach technique to every joint. A substantial anatomical knowledge of the equine locomotor system is crucial to warrant the sensitivity and specificity of the diagnostic ultrasonography of joint structures [21,22].

In cases of joint lesions, arthroscopy has proven to be a valuable diagnostic tool with dual functionality for both diagnosis and treatment. This invasive technique enables the intra-articular visualization of the cartilage surface, synovia, and ligaments, offering information on the characteristics of cartilage defects and allowing for grading. Arthroscopy is particularly adept at assessing early, subtle cartilage lesions. However, it should be noted that it has limitations, as it may underestimate the extent of certain lesions and overestimate others. This is attributed to its inability to provide a representative image of the entire articular surface [23,24]. At the same time, it also grants the treatment of some lesions such as the removal of cartilage fragments and joint/synovial debris, osteochondral *curettage* as well as sample collection.

Currently, MRI is the optimal method for the evaluation and monitoring of tendon/ligament and articular cartilage health, providing cross sectional images. While it is a non-invasive assessment technique, it can detect soft tissue changes and assess the cartilage morphology. It also provides volumetric and whole joint assessment without ionizing radiation. This technique is able to non-invasively detect biochemical changes in the tendons, ligaments, and cartilage that precede structural damage and may offer a great advance in the diagnosis and treatment of these debilitating conditions [25–27]. For soft tissue injuries of the palmar aspect of the metacarpal/tarsal regions, MRI is considered more accurate that U/S due to the risk of underdiagnosing injuries [28]. For the diagnosis of distal structure lesions, this may be conducted in a standing manner, with an open U-shaped MRI that is designed to enable imaging up to the level of the carpus and tarsus excluding the need for general anesthesia [29]. However, its high cost prevents its widespread use in initial clinical assessments or during subsequent follow-up examinations to characterize the progression of healing, and the technique requires the availability of professionals trained in interpreting the images obtained.

A CT scan is also a non-invasive procedure that involves the use of radiation, enabling the visualization of bones and soft tissues, producing a 3D image reconstruction [30]. It usually implies general anesthesia, but nowadays, there are also standing CTs. CT arthrography is a very useful diagnostic tool for assessing cartilage defects in metacarpo/tarso-phalangeal joints due to its short acquisition time, its specificity and sensitivity, and it might also be more accurate than MRI. However, MRI allows for a better assessment of the soft tissues and subchondral bone, being a useful technique for joint evaluation [31]. A study was performed comparing CT arthrography (CTA) and MRI sensitivity and specificity in articular cartilage defects in equine metacarpophalangeal joints. The CTA scan sensitivity and specificity were 0.82 and 0.96, respectively, and were significantly higher than those of the MRI (0.41 and 0.93, respectively) in detecting overall cartilage defects (no defect vs. defect). CTA is considered a valuable tool in the diagnosis of articular cartilage defects. CTA specificity is greater than that of MRI, and their sensitivities are very similar, thus concluding that CTA may be preferred, in this case, over MRI due to higher availability and easier image acquisition [26].

Nuclear scintigraphy involves the intravenous administration of a radioisotope that binds to bone structures, reflecting the osteoblast activity and organ function rather than providing detailed anatomical information. This approach enables the diagnosis of skeletal activity and injuries. Nuclear scintigraphy is particularly employed in the evaluation of lameness and poor performance, offering high sensitivity in detecting osseous remodeling associated with stress fractures and stress-related bone injuries [32]. The radioisotope uptake (IRU) by bone might not reflect the pain focus, only bone activity, and can also be associated with other musculoskeletal injuries rather than osseous [33]. Therefore, an in-depth investigation must be conducted, and it is advisable to use this technique together with regional nerve blocks and other diagnostic imaging techniques to better identify the injuries and focus of lameness. Scintigraphy has the potential to provide valuable information, but interpretation is not always straightforward, requiring careful correlation with other clinical and imaging findings [33].

PET scans have recently been added in the diagnostic panorama as a new and valuable tool available for equine musculoskeletal diagnosis, mainly those from the distal limb. Its use is more commonly documented in foot, fetlock, and tarsal injuries [34].

PET scan is a non-invasive nuclear medicine imaging technique, functioning as a cross-sectional modality. This entails that, like scintigraphy, a radiotracer is administered to the patient. Unlike the 2D images obtained in scintigraphy, PET scanning captures images in 3D, enabling the creation of multiplanar reconstructions and volume renderings. The tracer predominantly used in equine PET imaging is the radioactive form known as 18F-sodium fluoride (^{18}F-NaF) [34]. Utilizing radioactive tracers, equine PET scans provide numerous benefits in diagnosing and managing health issues in horses. These advantages include the ability for early detection and comprehensive assessments as they can pinpoint metabolic alterations at the molecular level before structural changes are visible on other imaging techniques, revealing the extent of disease or injury through detailed imaging, thereby offering deep insights into a range of equine health conditions [34,35]. PET scan images can even be integrated with CT and MRI images in order to more accurately diagnose the injury site, and may now be used in a standing manner with the equine under sedation [34].

4. Treatment Options

A diverse array of therapeutic modalities is available to address musculoskeletal injuries, ranging from straightforward pain management and inflammation medications to the use of physical rehabilitation with the shared goal of enhancing the biomechanics and restoring function to affected tissues [36]. A relatively recent entrant into the musculoskeletal therapeutic panorama are regenerative therapies, also known as orthobiologics, which assert the ability to regenerate injured tissues or structures. The knowledge and development in this field are progressing rapidly. Both conservative and regenerative therapies play crucial roles in managing musculoskeletal injuries, offering a spectrum of options for horse owners, trainers, and veterinarians to tailor treatments based on the nature and severity of the condition. The choice between these approaches often depends on factors like the specific injury, the horse's individual characteristics, and the desired outcome for the athlete's performance and well-being. Subsequently, this discussion will delve into the conservative and regenerative therapeutic options available for treating the aforementioned musculoskeletal pathologies (Table 1).

Table 1. Therapeutical options for musculoskeletal injuries. Therapeutic ultrasound (U/S), anti-inflammatories (AIs), hyaluronic acid (HyA), polysulfated glycosaminoglycans (PSGAGs), pentosan polysulfate (PPS), platelet-rich plasma (PRP), autologous conditioned serum (ACS), autologous protein solution (APS), alfa-2-macroglobulin (α2M), autologous chondrocyte implantation (ACI).

Musculoskeletal Injuries Therapeutical Options				
Conservative Treatments		**Surgical Techniques**	**Regenerative Treatments**	
Physiotherapy	**Pharmacological**		**Hemoderivatives**	**Stem-Cell Based Therapies**
• Manual Therapy	• AI's	• Tendon splitting	• PRP	• Stem-cell therapy
• Thermal therapy	• HyA		• ACS	• ACI
• Kynesiotape	• PSGAGs		• APS	• Stem-cell-free therapy
• Therapeutic exercise	• Pentosan PS		• α2M	
• Water exercise	• Polyacrylamide hydrogel			
• Therapeutic U/S	• Biphosphonates			
• Laser				
• Extracorporeal Shockwaves				
• Electromagnetic field				
• Electrostimulation				
• Vibration Plates				

4.1. Conservative Therapies

Conservative therapies have the primary objective of alleviating pain, reducing inflammation, promoting healing, and restoring function. These approaches resort to the use of physical rehabilitation and therapeutic exercises as well as the administration of pharmacological agents. These interventions aim to manage symptoms and promote the gradual recovery of musculoskeletal health without resorting to more invasive procedures [6,15,37]. Conservative therapies include physiotherapeutic, pharmacologic, and surgical methods. Physiotherapeutic techniques include manual therapies, passive stretching, tissue and joint mobilization, thermal therapy, kinesiotape/bandage, and electrotherapy (magnetic field, electrostimulation, therapeutic ultrasound (U/S), laser, extracorporeal shockwave, vibration plates). Pharmacological methods include anti-inflammatories (AIs) (nonsteroidal anti-inflammatories (NSAIDs), corticosteroids (SAIDs)), hyaluronic acid (HyA), polysulfated glycosaminoglycans (PSGAGs), pentosan polysulfate (PPS), polyacrylamide gel, and bisphosphonates to manage pain and inflammation. Surgical techniques include tendon splitting for tendonitis, whose outcome is regenerative.

4.1.1. Physiotherapeutic Modalities

Physical treatment and rehabilitation exercises play a crucial role in promoting the sound musculoskeletal recovery of horses, offering solutions in both preventive and therapeutic scenarios for athletes [36]. In equine sports, physiotherapy primarily targets the soft tissues involved, and is deemed essential to the overall recovery process [36].

Physical and Mechanical Agents

Manual Therapy

Manual therapy consists of applying the therapist's hands to the patient's body for diagnostic or therapeutic purposes. Passive stretching, a modality within manual therapy, is used to improve the range of motion, prevent injury, and decrease pain. It is recommended that each stretch be performed for 3–5 min, once daily, over 3–7 days per week to provide an adequate stimulus [38]. Tissue mobilization implies a deeper mobilization of tissues including massage, myofascial release, tissue mobilization to break down myofascial adhesions such as scar tissue, decrease blood or tissue fluids, and relax muscle tension to optimize fascia, muscle, and tendon function. Joint mobilization implies the active or passive mobilization of joints to increase the range of motion and reduce stiffness and pain. This technique implies small oscillating and gliding movements perpendicular or parallel to the joint axis.

Thermal therapy

Thermal therapy is perhaps the most widely used type of physical therapy and the easiest to apply. It varies from cold to heat therapy; their use is determined by the time elapsed after injury and by the desired objective. The range of thermal modalities is very wide, as demonstrated below [39].

Cold therapy

Cold therapy should be applied in the first 24–48 h after injury. After this, it can be performed in alternance with hot therapy up to 72 h. The main benefits of cold therapy are a decrease in local circulation, tissue swelling, and pain. Reduced blood flow will decrease hemorrhage and the extravasation of inflammatory cells as well as reduce tissue metabolism and enzymatic activity, inhibiting some of the effects of inflammatory mediators, reducing cellular oxygen demands, and decreasing subsequent hypoxic injury. Cold therapy also provides an analgesic effect by decreasing nerve conduction velocity. These benefits are more effective immediately after injury or surgery. After a minimum of 15 min of cold/ice therapy, the tissue temperature decreases 10–15 °C; the average time of cold therapy is 20–30 min [40,41]. Treatments are repeated every 2–4 h during the first 48 h. There are different methods of applying cold therapy—ice water immersion, ice packs, ice bandages,

and cryotherapy. Cryotherapy decreases the skin temperature to 4 °C and is a prohibitive treatment at FEI events in and five days prior to the event due to its analgesic and anti-inflammatory effects. Cold therapy can be administered by directing a cold-water hose onto the specific target site. While this approach is practical, it may not be as effective in reducing tissue temperatures compared to immersion in ice water. However, the physical pressure exerted can still be beneficial in addressing edema and promoting the debridement of wounds [39].

The main advantages of this technique include its simplicity of use and ready availability, relatively low cost (except for spas and baths), effectiveness in acute cases, and multiple effects such as analgesia, restricted blood flow as well as reduced tissue metabolism and activity of inflammatory enzymes.

Heat Therapy

Heat therapy is used from 72 h after injury as it increases the metabolic activity in cells, which leads to induced capillary dilation. This increased blood supply leads to increased supplementation of oxygen and nutrition into the area, and enhances removal of cellular waste products such as prostaglandins, decreasing nerve fiber sensitization and pain.

Heating of dense connective tissues increases extensibility and flexibility due to the effects on collagen molecular bonding. The heating and stretching of tissues around joints over a period of time can increase the range of joint movement. Heat also has effects in muscle spasms as it will relax muscles and decrease spasms. In wounds, it will also increase the healing response and improve edema resorption. An increment of 2–4 °C is required to produce these effects [42]. It is contraindicated in cases with infection or neoplasia, where there is hemorrhage, altered skin sensitivity, burns, circulatory problems, or acute inflammation.

Kynesiotape and Bandage

Kynesiotape is an adhesive tape made of cotton with an elasticity of 130–140% of its neutral state [43]. Kynesiotaping (KT) is a technique consisting of the application of an elastic tape to the skin, capable of acting on its mechanoreceptors to generate analgesic, muscular, and circulatory effects. These effects depend on the way the tape is attached to the skin. The elevation of the tissue triggered by the taping (convolutions) decreases the pressure on the nociceptors and increases blood circulation, providing analgesia [44].

The goal is to enhance the activity of mechanoreceptors and proprioceptive receptors within the skin, fascia, ligaments, and joints. The equine skin boasts a significant presence of sensory nerves and receptors intricately linked to hair follicles, characterized by a thinner epidermis compared to human skin. The application of tape is intended to coordinate the interplay between neural and muscular activity, ultimately achieving the modulation or alteration of locomotion and/or range of motion. There is lack of studies in the literature confirming the efficacy of kynesiotape in horses. Nevertheless, in one study, it did not show any significant effect of kynesiotape in range of motion in extension–flexion or stabilization of the back of the trotting horse [43]. Another study demonstrated that the use of therapeutic bandages resulted in a significant reduction in postoperative swelling of tibio-patellofemoral arthroscopy in horses compared with the control group [45]. In [44], the authors demonstrated that KT led to short-term analgesia.

Therapeutic Exercise

Controlled exercise stands as a fundamental and crucial component of every rehabilitation program, complementing and enhancing the regenerative process. The program typically initiates with complete rest, followed by stall rest, and involves a gradual and systematic escalation in exercise intensity [46].

Regarding soft tissue injuries, hand walking should start very soon after injury to promote optimal fiber alignment and prevent restrictive adhesions. Exercise is started hand walking 5–10 min once or twice a day, depending on lesion gravity. Ultrasound and lameness evaluation should be made periodically, every 4–6 weeks, and the exercise level

may be increased as improvement is achieved [39]. If the ultrasound image deteriorates or lameness grade increases, the exercise plan should be reevaluated. Controlled exercise alone resulted in successful outcomes for 67% to 71% of horses with soft tissue injuries [47].

Maturation of tendon and ligament fibers depends on mechanical loading. After the inflammatory phase of healing, controlled stretching is recommended in order to increase collagen synthesis and improve fiber alignment, resulting in a higher tensile strength [48]. These effects are explained by an increase in the collagen content and extracellular matrix (ECM) produced by tenocytes [22]. The promotion of appropriate orientation and the remodeling of collagen into mature, strong, and optimized tissue is ensured by mechanical stress. Controlled exercise during the chronic remodeling phase provides this conversion and improves the mechanical properties of the healed tendon. The quality of the longitudinal fiber pattern has been linked to prognosis for return to work. Collagen that remains unstressed during the proliferative and remodeling phases remains randomly organized and is weaker than stressed collagen. A prolonged immobilization leads to a tendon with reduced content on water and proteoglycan as well as to weaker and random organized collagen fibers with lower tensile strength and failure at lower strains [49]. It also results in tendon atrophy due to lower vascularization and metabolic rate.

Clinical studies have shown the benefit of early mobilization following tendon repair and the fact that training has improved tensile strength, elastic stiffness, weight, and cross-sectional area of tendons [22,24]. Table 2 presents a suggestion of a controlled exercise program for tendon/ligament injury.

Table 2. Controlled exercise protocol for tendon/ligament injury. The horse is confined to a stall or equivalent size paddock. Adapted from [39].

Weeks after Injury	Exercise	Confinment
0–4	Hand walk, 5–10 min, Twice daily.	Stall rest
5–8	Hand walk, 10–15 min, Three times daily.	Stall rest or small paddock
9–12	Increase time walk 5 min/day Three times daily	Stall rest or small paddock
13–16	If sound and continued improvement in lesion parameters: ride at the walk 20–25 min daily, hand walk 30 min daily.	Stall rest or small paddock
17–20	Ride at the walk 30 min, add 3–5 min trot. On week 18, add 3–5 min additional trot per week.	Stall rest or small paddock
21–recovery	Ride at the walk 30 min, ride at the trot 15 min per session, add 3 min canter. On week 22–24, add 3–5 min canter per session	Small paddock

Concerning articular cartilage, slow progressive physical exercise causes significant adaptive changes, there is enlargement of the cells and nuclei of chondrocytes, and an increase in the proteoglycan content and cartilage thickness. Nevertheless, if the exercise is strenuous or misconducted, it may lead to a cartilage degeneration process. The same happens with bone, as bone tissue adapts to weight-bearing and muscular workout by increasing bone mass and density through osteoblast stimulation. This remodeling cycle is slow, taking several months to occur, and the achieved bone mass also depends on genetic, nutritional, and hormonal factors. Immobilization causes the reverse effect on bone tissue, ultimately leading to osteoporosis [48]. Table 3 presents a suggestion for a controlled exercise program for bone injuries. An ideal program is based on individual patient and lesion specificities and requires periodic controlled veterinary check-ups [46].

Table 3. Controlled exercise protocol for bone injury. In the first month, the horse must be confined to stall rest and then start gradually and increasing exercise. Adapted from [46].

Weeks after Injury	Exercise	Confinment
0–4	-	Stall rest
5–6	Hand walk, 15 min/day.	Stall rest or small paddock
7–8	Hand walk, 30 min/day.	Stall rest or small paddock
9–16	Exercise in small paddock 6 × 6 m.	Stall rest or small paddock
16–recovery	Gradually increase exercise until full work.	Stall rest or small paddock

Water Exercise—Hydrotherapy

The most renowned modalities of exercise in water for horses are swimming pools (complete flotation), and water treadmills (WTs) (semi flotation) [50].

Marked locomotor differences exist between swimming and exercise on a WT. Usually, when swimming, horses stop forelimb movement, presenting only hindlimb movements. From the rehabilitation point of view, it is interesting and important. They use their forelimbs to maintain balance and hindlimbs for propulsion. Extreme range of motion (ROM) though the hip, stifle, and hock joints are observed in horses during swimming. Moreover, horses adopt a lordotic posture with cervical thoracolumbar and pelvic extension, so caution is recommended when using swimming for horses with thoracolumbar, sacroiliac, hip, stifle, or hock injuries [50]. On WTs, as the water depth increases, the buoyancy increases, the impact shock reduces, and hydrostatic pressure on the limbs increases, all of which have potential benefits for the rehabilitation of certain conditions [51]. Additionally, drag increases, which has the potential to limit limb protraction, alter muscle use, and change stride pattern [51]. A WT exercise session is equivalent to a challenging ground schooling session [51].

Water exercise presents a wide range of advantages: increases joint mobility and its ROM, promotes normal motor patterns, prevents muscle atrophy, increases muscle activation and strength, increases in soft tissue flexibility, reduces edema and joint effusion, reduces comorbidities caused secondarily to primary joint pathology as well as stress applied to the limb, increases joint range of motion, and decreases pain and inflammation [52–54]. Limitations of these techniques concern the fact that non-diagnosed injuries may worsen with their overstimulation, and the presence of skin disease or wounds and water mistreatment may lead to cross-infections [55,56]. A good evaluation of each pathology should be carried out before recommending water exercises to understand whether the benefit is real [36]. Regular monitoring of the gait pattern throughout rehabilitation either by a therapist/vet or both is recommended.

Electrotherapy

Therapeutic Ultrasound

Therapeutic ultrasound (U/S), an electric device whose action is based on thermal effects, may be used for superficial and/or the deep heating of tissues. Ultrasound selectively heats tissue with high protein/collagen content. The most intense heating occurs at tissue interfaces such as the skin, tendons, and fluid [31,42]. To achieve therapeutic effects, there must be a temperature increase of at least 2 °C in the tendons [42]. In equine epaxial muscles, the mean temperature rise after 20 min of treatment at 3.3 MHz at 1.5 W/cm^2 was 1.3 °C at a depth of 1.0 cm, 0.7 °C at 4.0 cm, and 0.7 °C at 8 cm. However, temperatures in the tendons were significantly elevated following 10 min of treatment at 3.3 MHz: the mean temperature rise was 3.5 °C in the SDFT and 2.5 °C in the DDFT at the end of the 1.0 W/cm treatment and 5.2 °C in the SDFT and 3.0 °C in the DDFT at the end of the 1.5 W/cm treatment [31]. The other benefit of therapeutic U/S is that sound waves cause a deep massage of tissues known as cavitation. This massage is caused by the expansion and compression of tissues and fluids that enhance tissue healing. For example, fibrous connective tissue scars may be effectively stretched using this technique [42].

This technique is advantageous in articular and tendon disabilities. It addresses joint mobility limitations with the objective of elevating the temperature of connective tissues before engaging in stretching or ROM exercises. Previous observations indicate that warming deep tissues, either before or during stretching, yields a more pronounced impact on tissue length and reduces the risk of injury compared to stretching in isolation. The joint capsule, rich in collagen, frequently contributes to restricting joint motion. Ultrasound energy is efficiently absorbed by collagenous tissue, augmenting its elastic properties [57].

A study found differences among the rates of tissue heating between different tissues. The explanation concerns the thermal and acoustical properties of the tissue through which the continuous sound waves travelled. The difference in the rate of tissue heating among species is likely the result of the distinct acoustic properties of the tissues based on anatomic location and the variation in tissue composition between species [42].

Laser

The term LASER is an acronym for light amplification by stimulated emission of radiation. Laser therapy has been widely used in equine medicine rehabilitation and is gaining much more attention as it is a safe and non-invasive method. It is a device that produces coherent, collimated, and monochromatic light through a process of optical amplification. Laser devices have different classifications—I, II, III, and IV, but only class 3B and IV have therapeutic abilities. Class 3B are therapeutic lasers that have a power output from 5 to 500 mW, and are called low level laser therapy (LLLT). Class IV lasers are therapeutic lasers with a much higher power, above 500 mW, have thermal effects, and are called high intensity laser therapy (HILT) [58]. The use of class IV lasers in FEI competitions is not allowed.

Nowadays, many studies reflect the therapeutic benefits of class LLLT and HILT in tendonitis/desmitis treatment and OA amelioration.

Low-level light therapy generally employs light at the red and near-infrared spectral band (390–1100 nm) to modulate biological activity, without the generation of heat [58,59].

Several studies have presented that LLLT alone can effectively be applied to treat various musculoskeletal disorders [60–64]. LLLT enables tendon healing by promoting angiogenesis under hypoxia, increasing the amount of collagen type III, endorsing the proliferation of fibroblasts, and reducing inflammatory responses. However, it should also be noted that in the final phase of tendon repair, the use of LLLT causes the excessive upregulation of some growth factors, which might lead to tendon fibrosis [62].

LLLT therapeutic efficacy has also been evidenced in joints and articular cartilage, having been demonstrated that beside the anti-inflammatory effects, it promotes a fast recuperation and regeneration of the articular cartilage [65]. In fractures, LLLT increases the bone regeneration process only in the first weeks after the fracture, indicating that LLLT is effective only in the early stages of the process [64]. It is recommended as a physical agent to be used concomitantly with rehabilitation programs [63,66].

Regarding the HILT effects, it presents the same as the LLLT, with additional photothermal effects on soft tissues. The skin temperature rises approximately 3 °C, increasing vascularization and the intensity of metabolic processes in the cells [67,68]. For tendon and ligament injuries, it was demonstrated that it reduced pain, swelling, lameness, and promoted healing, thus reducing the injury percentage, therefore being useful as a supportive therapy for the healing of tendon and ligament injuries in horses [68]. Additionally, HILT seems to be efficient in reducing pain and providing functional improvements in patients with knee OA [69–71].

Before applying this therapy, patient preparation is essential to ensure that the skin is clean and free of any materials that could absorb light [72].

Extracorporeal Shock Wave

Extracorporeal shock wave therapy (ESWT) is a well-investigated and widely used non-invasive treatment modality for many equine musculoskeletal disorders. Acoustic waves are applied to an injury region that trigger a mechano-transduction cascade. Me-

chanical energy causes biological effects that lead to an enzymatic response and to the up-regulation of angiogenic growth factors (GFs) responsible for neovascularization as well as the improvement in blood supply and tissue regeneration, thus improving the healing process [73].

The application of ESWT in chronic tendinopathies stimulates neovascularization, alleviating pain, and initiating the repair of the chronically inflamed tissues [74]. Its therapeutical value in calcified tendonitis is also largely described [75]. Additionally, ESWT has been shown to improve lameness, decrease the time of healing, and improve the ultrasonographic appearance of tendon and ligament injuries. The optimization of collagen synthesis, maturation, and strength progressively increases the tendon tensile strength and hence recovery. These findings account for the gradual and long-term benefits of shock wave therapy in tendinopathy [76].

In knee OA, ESWT has demonstrated clinical benefits for pain and an improvement in physical function. In acute fractures, ESWT treatments enhanced callus formation and induced cortical bone formation. In these cases, the effect of ESWT appeared to be time-dependent [77,78].

Limitations of ESWT are the potential pain and minor hematomas. To overcome these limitations, pretreatment with laser therapy is described, the results being a faster and/or better treatment outcome than ESWT without laser pretreatment. Combining ESWT with laser pretreatment leads to synergistic effects and is thus superior to either treatment modality alone [79].

Electromagnetic Field

Electromagnetic field therapy operates on the principle of the electrical generation of magnetic waves. This therapeutic action can be achieved through high-frequency electromagnetic waves, also known as pulsed diathermy, which induce heat production by increasing the temperature by 3–4 °C or by lower-frequency electromagnetic waves, referred to as pulsed electromagnetic frequency (PEMF) therapy, which produces magnetic fields within the tissues without causing heating. For equines, there are several PEMF devices including blankets and wraps with coils and energy-generating battery units built into them [80]. It is indicated for bone fractures, non-union fractures, and in decreasing pain and muscle tension and spasm [80]. Usually, treatment protocols are established by the device manufacturers and are based on the frequency of the pulses and the treatment time.

Electrostimulation

An electrical current is applied to surface electrodes to produce controlled movement of the skin, muscle, tendon, and associated ligaments. Some of the important advantages of electrotherapy are an improved quality of healing and shortened rehabilitation time. Electrotherapy devices can be placed into two categories: sensory nerve or motor nerve stimulators [81].

Transcutaneous Electrical Nerve Stimulation (TENS)

TENS provides pain relief through electrical stimulation in the low-frequency range (<250 Hz) by using appropriate pulse durations and intensities to activate the desired nerves. It acts primarily via segmental inhibition through pain gating mechanisms [80]. These rely on the activation of larger diameter fibers in peripheral nerves, which in turn help block nociceptive activity in smaller afferent ones. Secondarily, this stimulation of the peripheral nerves can induce a central release of endogenous opiate-like substances, which can have a descending inhibitory effect on pain. Limitations concern skin irritation.

The main indications are pain control in acute and chronic musculoskeletal disorders, edema, and wound healing control [80]. In equines, it is mostly described for superficial flexor tendon injuries in order to decrease pain and edema and epaxial muscle pain [82].

Neural Electrical Muscle Stimulation (NEMS)

Neural electrical muscle devices stimulate motor nerves, producing controlled and visible muscle contractions generated by electrical high intensity impulses that are directed toward the target muscle through a surface electrode [83].

The main indications are muscle stimulation through α-motor nerve activation and stimulation of de-enervated muscles [80]. Effects such as changes in fiber types and physiological factors of equine muscles, muscle strength and hypertrophy, muscle spasm, and hypertonicity have been described [82].

Vibration Plates

The use of vibration platforms in equine rehabilitation is gaining more support. The main indication is to be used prior to exercise to mimic a warm-up effect caused by vibration, reducing injuries during exercise [84]. It is theorized, although not substantiated, that vibration platforms cause longer stride lengths, lower lameness scores, and higher heart rates after treatment [85]. However, it appears to have an acute relaxation effect in stalled, healthy horses [85,86]. Further studies need to be performed.

4.1.2. Pharmacologic Conservative Therapies

Anti-Inflammatories

In musculoskeletal disease, anti-inflammatories—NSAIDs and SAIDs—are the most prescribed and used drugs either administered orally (PO) (both), endovenous (IV) (both), intramuscular (IM) (SAIDs), or injected intra-articularly (IA) (SAIDs). They can relieve pain and reduce inflammation through the inhibition of proinflammatory prostaglandin production by cyclooxygenase enzymes.

Despite this, as soon as tendonitis or OA is triggered, their histopathology clearly reveals that they have a degenerative course instead of an inflammatory one [87], therefore, anti-inflammatories do not alter the course of the disease, they only relieve symptoms inherent to the pathology [37]. Additionally, these drugs can impair healing by the down-regulation of the cycloxigenase-2 (COX-2) pathway for tendon and bone injuries. This entails a profound understanding of the early inflammatory cascade and how it might affect the treatment [88]. Some evidence suggests that NSAIDs may impair the tenogenic differentiation of the mesenchymal stem cells, drawing differentiation toward adipogenic differentiation, and negatively influencing the healing process, thus leading to scar tissue formation and the impairment of functional outcomes [37]. Moreover, the risk of renal, cardiovascular, and gastrointestinal side effects must also be considered.

Corticosteroids and local injections in tendons are not advisable as they may induce tendon fibrosis. Evidence suggests that they are not effective and do not represent any advantage in tendon repair [37,89–91].

Regarding the use of intra-articular (IA) SAIDs with OA, these may be associated with moderate improvement in pain and function but with low duration [92]. The beneficial effects of IA SAIDs are rapid in onset, but may be relatively short lived (approximately one to three weeks) [93].

The use of anti-inflammatories must be very well-balanced because if on the one hand, aberrant cellular activity in the inflammatory phase often results in impaired tissue healing and defective host responses with over-fibrosis and scarring; on the other hand, inflammation is part of the regenerative process and recruits a number of immune cell subtypes that have an impact on tissue healing processes [94]. Thus, in acute stages of inflammation, they might be considered based on a short-term use (3–5 days), but their long-term use is not recommended, as inflammation is critical for normal tissue repair, aiding debris clearance and signaling tissue repair [95].

Hyaluronic Acid (HyA)

HyA is a non-sulfated glycosaminoglycan (GAGs) and is clinically used for the treatment and medical management of equine acute tendonitis and OA [96–100]. There are sev-

eral commercial products licensed for injectable use in equine medicine such as Hyalovet® 20 (Boehringer Ingelheim, Milan, Italy) for IA administration, HY-50® (Dechra, Northwich, UK) for IA or IV administration, Hyonate 10 mg/mL (Boehringer Ingelheim, Amesterdam, The Netherlands) for IA or IV administration, Gel-50® (Equimed, Allentown, PA, USA) for IA or IV administration, and Legend® (Boehringer Ingelheim Animal Health, Duluth, GA, USA Inc.) for IA or IV administration.

HyA is considered a safe and cost-effective therapeutic for helping to lower the side effects of OA and is frequently used in clinical routine. HyA provides lubrication to the joints and chondroprotective effects, secondary to an inhibition of the production of nitric oxide, a mediator that enhances cartilage degeneration and chondrocyte death. It also limits the progression of OA lesions by stabilizing proteoglycan structure, limiting the enzymatic breakdown associated with degenerative arthritis [101]. HyA improves the viscosity of synovial fluid (SF), helping its physiological function by acting as a buffer and stabilizer of lubrication and as an anti-inflammatory and analgesic due to increased joint lubrication, resulting in decreased pain in unstable joints [102]. Treatment can be carried out IA, IV, or PO, with IA being more effective. A rest period (12–24 h) is advised after IA treatment [99,103]. The higher the molecular-weight hyaluronic acid, the more efficacious the treatment of OA [97]. Higher molecular weight HyA may provide superior chondroprotective, proteoglycan/glycosaminoglycan synthesis, anti-inflammatory, mechanical, and analgesic effects [104].

In the past, evidence has demonstrated that the IA injection of HyA and SAIDs improved the performance of race horses with traumatic arthritis, and since then, this association has been widely used [105]. However, there is no scientific evidence that hyaluronic acid combined or not with anti-inflammatory drugs is effective in the long run, and that the association with SAIDs is more effective in reducing lameness than HyA itself [102].

Lately, the development of alternative treatments to the classic HyA and corticoids such as platelet rich plasma (PRP) has created the need to compare the treatment effectiveness of these treatment options. Several studies have demonstrated that PRPs in combination with HyA are more effective reducing pain than PRPs or HyA alone [106–109].

In tendinopathies, HyA also provides analgesia and has been confirmed to be effective on functional improvement as it allows for tendon gliding, reduces adhesions, creates better tendon architectural organization, and limits inflammation [100,110].

Wound healing and immunosuppressive properties have also been reported in in vitro and in vivo studies [96,111]. Its beneficial action to the repair process is stronger after acute tendonitis and should be used soon after injury.

New products that alter the composition of the HyA molecule are continuously being developed as well as combinations with other drugs to enhance their effects. HyA holds significant potential both as a therapeutic agent on its own and as a scaffold when combined with other therapeutic molecules, and it remains the focus of ongoing research. Nowadays, the association of HyA with mesenchymal stem/stromal cells (MSCs) is being largely studied for the treatment of cartilage repair using HyA as scaffolds for MSC implantation [98,112–116].

To sum up, HyA presents analgesic, anti-inflammatory, and lubricative effects, improving organ function [110,111].

PSGAGs

PSGAGs consist of low-molecular-weight polysulfated glycosaminoglycans (GAGs), ranging approximately from 6000 to 10,000 Da, of animal origin, closely resembling the structure of chondroitin sulfate, which is the predominant GAG found in healthy cartilage [117]. In equine medicine, PSGAG is licensed under the name Adequan® (Luitpold Pharmaceuticals, Inc., Shirley, NY, USA) and is administered via IM. PSGAGs have a long history of demonstrated safety and perceived effectiveness in equine OA prevention, being primarily used to prevent, slow down, and reverse the morphological changes in

cartilaginous lesions caused by OA, thus preventing cartilage degeneration [118]. It also presents the ability to reduce inflammation, repair joint cartilage, and promote hyaluronic acid production, thus restoring synovial joint lubrication, alleviating clinical signs, and improving the horse's quality of life and performance. Its application spans early OA indicators to chronic conditions, serving as a standard treatment approach, also being reported for tendon and ligament injuries [119].

PPS

Pentosan polysulfate (PPS) is similar to PSGAG but has a vegetal origin. Its molecular structure and function closely align with those of the naturally occurring glycosaminoglycan substances that play a key role in the maintenance and repair of cartilage and connective tissues. PPS exhibits anti-inflammatory, anti-coagulant, and fibrinolytic properties and promotes the synthesis of hyaluronan, making it effective in endorsing cartilage repair, reducing cartilage fibrillation, improving joint function, and alleviating pain associated with OA [117,120]. Some studies have demonstrated that it presents more benefits than PSGAGs when administered IM. Zycosan® (Dechra, Overland Park, KS, USA) is the licensed PPS for equine medicine and is used for the control of clinical signs associated with OA.

Polyacrylamide Hydrogel

Polyacrylamide hydrogels (PHyds) are licensed in equine medicine under the brands Arthramid® Vet (Polyacrylamide hydrogel 2.5%, Revatis, Aye, Belgium) and Noltrex˜vet™ (Polyacrilamide hydrogel 4%, Bioform®, Moscow, Russia).

PHyds have appeared more recently than HyA, and is a 100% synthetic product, are non-soluble, and essentially act as a substitute for SF, increasing joint lubrication and consequently joint pain/inflammation, thus improving joint function. In an in vivo study with rabbits, it was possible to detect the presence of the hydrogel in the joint cartilage space at day 60 after one single dose treatment [121]. The efficacy of PHyd can be possibly explained because its molecular weight is three times greater than HyA, thus preventing the degenerative process caused by the inflammatory cytokines present in the SF of the joints [122].

The intra-articular administration route is more efficient than IV or PO, is effective at reducing lameness caused by OA in horses, and has a long period of action enabling their physical activities and increasing the welfare of horses [102].

In a recent study, it was demonstrated that intra-articular 2.5% PHyd is highly effective (82.5% free of lameness horses at 2-year follow-up), lasting, and safe for the treatment of equine OA. No other medical treatment has proven such prolonged efficacy. These studies enhanced the belief that the hydrogel exerts its effects through integration in the synovial membrane, increasing joint elasticity and viscosupplementation, protecting articular surfaces, and preventing pro-inflammatory cytokines from exerting their effects, potentiating OA [122,123]. These studies also suggest the absence of intra-articular neurotoxic effects or fibrosis [123].

All of these studies support the application of a polyacrylamide hydrogel in reducing lameness caused by OA in horses due to its long-lasting viscoelastic supplementation. Its association with other therapies such as PRPs or stem cells could be beneficial.

Bisphosphonates: Tiludronate and Clodronate

Bisphosphonates are widely used in both human and equine medicine due to its ability to reduce bone resorption and inhibit osteoclastic activity. In equine medicine, tiludronate was the first bisphosphonate to be approved. It mainly acts as an antiresorptive drug, reducing the ability of the osteoclasts to degrade the bone matrix, although anti-inflammatory and analgesic properties mediated by other mechanisms are also recognized and consensual [124–135]. Tiludronate disodium (Tildren®, Ceva Animal Health LLC, Lenexa, KS, USA) and clodronate disodium (Osphos®, Dechra, Ltd., Staffordshire, UK) are the bisphosphonate drugs that are licensed for use in horses, with its label use on horses

older than 4 years old. These two products are non-nitrogen containing bisphosphonates that reduce osteoclastic bone resorption by causing osteoclast apoptosis [136].

Initially, it was mainly used in navicular syndrome and bone spavin.

In navicular syndrome, horses treated with 1 mg/Kg administered via IV injections daily over 10 days for the treatment of navicular disease showed optimal improvement in lameness and return to normal level of activity 2–6 months post treatment [137]. Several studies have proven bisphosphonate effectivity in improving lameness associated with navicular syndrome [129,138,139].

In bone spavin medical treatment, tiludronate, in association with a controlled exercise program, reduces the lameness score and improves radiological images [140–142].

Nowadays, it is also used for its analgesic action in thoracolumbar spine OA, causing a significant improvement in dorsal flexibility, thus becoming a treatment option for the management of horses with intervertebral lesions and the associated pain [131]. Its use is also valuable to prevent osteopenia in long-term immobilizations [126].

A study conducted in standardbred race horses with fetlock traumatic osteoarticular lesions demonstrated that IV treatment of tiludronate in 500 mL of saline solution decreased the inflammatory process and cartilage degeneration after treatment, meaning that it inhibited the radiographic progression of OA in fetlocks by inhibiting subchondral bone remodeling [128]. The advantage of using tiludronate in young horses to control subchondral bone pain in the initial stages of OA was also highlighted [128].

Despite limited data available on its secondary effects, the current literature suggests a good tolerance of tiludronate, with discomfort or colic [126,143,144] and renal damage [134] being the most frequent side effects.

The perspective of the use of bisphosphonates in horses seems bright and growing, as its advantages in osteoclast activity are consolidated. Nevertheless, its usage must be controlled and properly performed.

4.2. Surgical Techniques

Tendon Splitting

Tendon splitting is a surgical technique performed in acute and chronic tendonitis that has been described for equine tendonitis treatment since the early 70s.

In acute tendonitis, there is collagen fiber damage and an increase in the cross-section area of the tendon due to intratendinous hemorrhage and inflammatory fluid accumulation within the lesion. In this type of lesion, the objective of this technique is to alleviate pressure from the core lesion, as fluid accumulation within the epitendon and paratendon produce "compartment syndrome", increasing pressure in the lesion and therefore reducing the perfusion capacity, causing a slow resolution of inflammation and healing. This decompression of the core lesion allows for the evacuation of accumulated inflammatory fluid and promotes vascular ingrowth within the lesion.

In chronic lesions, the procedure is similar, but the objective is to increase vascularization of the scar lesion to promote healing and increase tissue elasticity through the same technique.

Tendon splitting can be conducted blindly, but guided ultrasound is recommended to avoid any damage to healthy tendon fibers and structures other than the injured ones. Briefly, after trichotomy of the area and aseptic preparation of the limb, patient sedation and a high four-point regional nerve block are performed. The ultrasound probe is covered with a sterile lubricant and a sterile sleeve, and allows for tendon visualization.

The stab incision or splitting begins at the most distal aspect of the core lesion to avoid blood contamination of the next stab incision. A #11 scalpel blade is inserted into the medial or lateral surface of the tendon, perpendicular to the ultrasound probe, being its entry and location observed by U/S. The blade is advanced until reaching the lesion, avoiding normal fibers, and is then rotated in upward and downward movements, parallel to the long axis of the tendon. The blade is removed and subsequent stab incisions are made as needed to split the entire length of the core lesion [26].

Although this technique was more described in the 70s, 80s, and 90s [27], nowadays, it is still used and was referenced in studies describing the treatment of SL branches with stem cells in race horses, where percutaneous splitting of the ligament was performed in lesions with cross-sectional area (CSA) grade III and IV in order to evacuate the inflammatory fluid within the core lesion, reduce edema, and enhance revascularization, as the reduction in intratendinous swelling through the creation of communication between the core lesion and peritendinous/ligament tissue improves circulation, thus reducing the repair size and enhancing tissue repair organization [27,28]. Splitting the ligament in the higher CSA grades—III and IV—was correlated with a beneficial input in treating these lesions, but more studies need to be performed.

The classification of CSA is in percentage estimates lesion area, as follows: Grade 0, 0%; grade 1, lesion is inferior to 25%; grade 2, lesion represents 25 to 50%; grade 3, lesion represents 50 to 75%; grade 4, lesion is superior to 75% of the cross-sectional area [29].

4.3. Regenerative Therapies

The main goal of regenerative medicine is to replace or regenerate cells and tissues, in order to restore the normal structure and function of the injured tissue or organ [145,146].

In contemporary equine orthopedic medicine, there is a growing interest in various regenerative therapeutic approaches, with a notable focus on hemoderivative therapies and stem cell-based therapies. These treatments have gained prominence due to their demonstrated anti-inflammatory effects, immunomodulatory/paracrine properties, regenerative potential, and high tolerability [147].

Hemoderivative therapeutics include PRP [108,148–151], ACS [152–156], APS [157,158], and α2M [159,160].

Mesenchymal stromal/stem cell-based therapies include cell-based and cell-free therapies. Cell-based therapies resort to the use of stem cells themselves; these are multipotent cells that can be harvested from various tissues. MSCs have the potential to differentiate into different cell types and exert immunomodulatory effects, making them valuable for tissue regeneration. Cell-free therapies rely on cell secreted factors such as cytokines, chemokines, GF, extracellular vesicles (EVs), and exosomes, which present many biological activities as well as therapeutic potential in several organ system and disease contexts. Currently, for equine, the only commercially available MSCs cell-based therapies are under the name of Arti-cell® forte (Boehringer Ingelheim Vetmedica GmbH, Ingelheim am Rhein Germany) and Horstem® (Equicord, Madrid, Spain), and Vet-stem is a laboratory that prepares stem cells from adipose tissue and sells the autologous stem cell product.

The interest in these regenerative approaches stems from their ability to address musculoskeletal injuries at a cellular level, providing a more integrated and potentially more effective treatment strategy. As research in equine regenerative medicine continues to advance, these therapies hold promise for enhancing the overall well-being and performance of horses in diverse disciplines.

4.3.1. Hemoderivatives

Hemoderivatives present anti-inflammatory and healing effects, being used in muscle, tendon, ligament, and joint injuries such as strain injuries, tendonitis, desmitis, osteoarthritis, cartilage injury, and synovitis [160]. They also enable the healing and restoration of function in acute and chronic injuries.

In cases of OA treatment, they represent an advantage when compared with traditional intra-articular treatments (HyA + SAIDs), which are only palliative for pain and inflammation control [161] as they improve clinical signs and appear to be chondrogenic and promote chondrocyte homeostasis [161–163]. In cases of tendonitis/desmitis, they also present therapeutic effects, enhancing healing, and leading to the formation of functional tissue without scar formation [164,165].

The common principle across hemoderivatives including PRP, ACS, and APS lies in harnessing the regenerative potential of platelets and their associated bioactive substances

to modulate inflammation, support tissue repair, and facilitate healing processes. Each of these approaches offers a personalized autologous solution, utilizing the horse's own blood components to enhance musculoskeletal health. It is advisable that no NSAID treatments are conducted 1–5 days prior to the preparation of these hemoderivatives [160].

PRP

PRP primarily leverages the therapeutic properties of platelets, which play a crucial role in the natural healing response to injury. When tissue damage occurs, platelets become activated and initiate the clotting process, leading to the release of various bioactive substances. The key components released by α granules of the activated platelets include cytokines, growth factors (GFs), and chemokines such as platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF), and insulin-like growth factor (IGF). These substances are instrumental in modulating the inflammatory response, attracting immune cells to the site of injury. Platelets also contribute to angiogenesis, the formation of new blood vessels, by releasing factors that stimulate the growth and migration of endothelial cells. This process is crucial for supplying oxygen and nutrients to the healing tissue. PRP has garnered significant attention in both equine and human medicine due to its remarkable ability to stimulate the proliferation and migration of fibroblasts, facilitate collagen synthesis, and induce the chemotaxis of macrophages. These cellular processes are crucial for promoting cellular proliferation, tissue healing, and regeneration. PRP has found extensive application in treating musculoskeletal tissue lesions, particularly osteoarthritis (OA) and tendonitis/desmitis, due to its well-established anti-inflammatory and anabolic effects. The proven beneficial effects of PRP underscore its role as a valuable therapeutic tool in promoting tissue repair and regeneration in conditions involving the musculoskeletal system [166,167].

PRP is produced through a centrifugation process of whole blood, during which red blood cells and buffy coat are separated from plasma. Platelets are then aspirated, and a subsequent centrifugation concentrates the platelets in plasma. Platelets release the bioactive factors after degranulation of the α granules in the platelet cytoplasm, which occur upon activation with citrate [168]. Most GFs are released within 1 h of platelet activation and their half-life usually ranges from minutes to hours. This is a simple process that takes approximately 15 min to prepare, being the main device used, a portable centrifuge, which is easy to do in an ambulatory clinic.

PRP can also be obtained through commercial kits for horses: Restigen PRP® (Zoetis, Lincoln, NE, USA), ACP™ (Arthrex GmbH, Munchen, Germany), ACP MAX™ (Arthrex GmbH, Munchen, Germany)), Angel PRP™ (Arthrex GmbH, Munchen, Germany) or through manual procedures. Although it is described in three different manual protocols, in equine practice, the most widely used protocol involves two centrifugations to concentrate the platelets in a small volume of plasma (e.g., 2–5 mL) for injection in the tendons or intra-articular treatment [169]. PRP can be stored for up to 7 days in cooled storage, however, 24 h is the ideal time of storage at 5 °C because it has been demonstrated that the platelet counting and viability did not change under these conditions [170]. When using a commercial kit, PRP can be aseptically and stably prepared with a consistent platelet content, however, the total platelet count is slightly lower than when using double-centrifugation methods [148].

The platelet content of PRP is affected by several factors such as the breed and age of the horse, the administration of AIs, anticoagulants, blood sampling, and the technical skills of the clinician [171,172]. Depending on the PRP preparation protocol, the cellular and cytokine compositions can vary, with such variability being a main clinical concern as it can potentially influence the therapeutic effects of PRP [173,174]. Nevertheless, all of these present higher levels of TGF-β1, VEGF, and PDGF [148,175].

To sum up, PRP provides a growth factor concentrate that enhances the cellular repair of musculoskeletal lesions [167,174]. Other advantages of PRP as a regenerative therapy are its autologous nature, rapid preparation, and non-invasive collection process.

ACS

ACS presents therapeutic effects based on the increase in the interleukin-1 receptor antagonist (IL-1ra) concentration, being therefore known as the interleukin receptor antagonist protein (IRAP). It also presents high concentrations of anti-inflammatory interleukins 4, 10, and 1 (IL-4, IL-10, and IL-1), and growth factors including IGF-1, PDGF, and TGF-β in autologous serum [108,109].

In equine medicine, there are commercial kits for the preparation of ACS: Orthokine® vet IRAP (Dechra, Overland Park, KS, USA) and IRAP Pro EAS® (Arthrex, Naples, FL, USA), which is a natural anti-inflammatory product used for the treatment of OA. They have different preparation protocols, but basically consist of whole blood incubation in a syringe containing borosilicate medical glass beads. The blood is then centrifuged to obtain an IL-1ra enhanced serum product that can then be injected intra-articularly or intralesionally. This product may be applied in joint, muscle, and tendon/ligament injuries.

The role of IRAP is very important in OA control as research in molecular biology has discovered that the major inducer of OA is the general inflammatory cytokine interleukin-1β (IL-1β), which plays a key role in accelerating tissue destruction and the repair mechanisms, being one of the major mediators responsible for the pathogenesis of OA as it activates an inflammatory response, leading to cartilage degradation and bone resorption. The proposed mechanism of ACS action is through the blockade of IL-1 receptors, thus inhibiting IL-1 action and preventing the detrimental effects of IL-1β on articular tissues in OA pathophysiology [176,177].

Recent studies have also referred to the important contribution of other cytokines such as TGF-β, VEGF, and IGF-1 that would positively influence the treatment response as potent anti-inflammatories and cartilage catabolics [152,162]. IGF-1 is responsible for the stimulation of the production of cartilage matrix components—matrix aggrecan and collagen synthesis—with this profile being another major benefit to add to higher levels of IL-1Ra [178,179].

In tendons, it has been demonstrated that ACS treatment causes an early significant reduction in lameness and leads to a temporary improvement in the ultrasonographic parameters of repair tissue as well as a positive effect on histopathological and biomechanical healing [153,180].

APS

APS is an orthobiologic that acts through a combination of cytokines, growth factors, and anti-inflammatory agents, with its main characteristic also being its high concentration of IL-1ra. APS is prepared through an commercially available kit, Prostride® (Zoetis, Lincoln, NE, USA), and the process involves the collection of the horse's own blood, which is processed with the commercial kit and is intended to stimulate white blood cells (WBC) to produce anti-inflammatory cytokines, concentrating its content in a smaller volume of plasma. This product concentrates IL-1ra, 5.8 times more than in plasma, creating a positive ratio of IL1Ra:IL-1β [162,181]. It is reported to include significantly greater concentrations of IL-1RA, IGF-1, TGF-β, IL-10, and growth factors such as vascular endothelial growth factor (VEGF) and PDGF compared with PRP alone [160].

Its preparation takes 20 min, and then the prepared solution is injected directly into the affected joint or tissue. APS can be prepared using portable centrifugation equipment and is a very simple, quick, and non-invasive technique. The intralesional injections can be performed in a single treatment in an ambulatory-based practice [162,181].

It is designed to reduce inflammation, relieve pain, regenerate tissue, and promote angiogenesis and cell proliferation, capitalizing on the horse's own biological resources to enhance the healing processes, making it a personalized and potentially effective treatment.

In horses with naturally occurring OA, APS significantly improved lameness, pain-inflexion, gait analysis, and range of motion up to 14 days after treatment compared with thee baseline and controls. In equine joint fluid, there was a significant decrease in the protein concentration in treated horses compared to the untreated controls [181].

In tendons, it has beneficial effects as an anti-inflammatory and promotes tendon healing [182].

Essentially, the effects of ACS and APS are very similar because they are characterized by higher concentrations of IL-1ra. Nevertheless, the literature has presented some dissimilarities regarding other cytokines, GFs, and anti-inflammatory profiles, attributing some effectiveness variations to these differences [162]. At this point, there is insufficient evidence-based research to support the superiority of APS compared with ACS [162]. However, in the treatment of articular injuries, equine clinicians more widely use IRAP® and Prostride®, although there is no evidence to prove that they are more efficacious than PRP in this type of pathology [183,184].

α2M

α2M is a broad-spectrum proteinase inhibitor, present in a vertebrate's plasma, as it binds to proteinases that induce chronic inflammation, especially those released by granulocytes and other inflammatory cells. It has been demonstrated that it can inhibit many cartilage catabolic factors, attenuating post-traumatic OA degeneration. The upregulation of cartilage catabolic factors seems to be a key mechanism for cartilage damage. Therefore, the inhibition of these molecules will prevent disease progression [185].

α2M is naturally present in high levels in plasma and in low levels in SF. It is produced by the liver—being released to plasma—and by chondrocytes and sinoviocytes—being released in SF. In inflammatory events such as OA, the α2M synovial levels do not significantly increase due to its high molecular weight and it does not pass from the plasma to SF, being unable to inhibit severe intra-articular inflammation. Bearing this in mind, several therapies have been developed to administer α2M intra-articularly. It has been proven that this treatment can inhibit inflammation and delay articular cartilage degeneration and bone resorption mediated by the inhibition of catabolic enzymes [185–187]. It has also been demonstrated that α2M enhanced the cartilage matrix (i.e., collagen type II and aggrecan synthesis). This fact suggests that α2M may have cartilage repair functions or facilitate the synthesis of cartilage matrix [187]. It has also been suggested that the early administration of α2M may provide cartilage protection by reducing the presence of local catabolic enzymes [187]. In chondrocyte culture, a concentrated α2M serum was found to promote chondrocyte proliferation and reduce apoptosis and catabolic gene expression [145].

Nowadays, to create α2M therapeutic levels within the joint, a process was created that isolates and concentrates α2M from a blood sample. This process was developed and is commercialized as a system—Alpha2EQ® (Astaria Global, Houston, TX, USA). Alfa2EQ® isolates α2M from the horse's own blood through α active filtration technology, allowing its use as a potent biological anti-inflammatory molecule—α2M—to address equine lameness, joint inflammation, and soft tissue injury.

To sum up, hemoderivatives represent a new class of regenerative autologous medicinal therapeutics that are evolving rapidly due to their demonstrated efficacy and reduced adverse reactions compared to traditional therapies [188,189]. The production of PRP, ACS, and APS involves the collection of the horse's own blood, followed by centrifugation and serum collection. In the ACS and APS process, an incubation step before centrifugation is also present. They all exert their actions based on bioactive factors released by platelets, with anti-inflammatory, modulation, and regenerative actions and present different concentrations of specific bioactive factors. α2M also involves the use of the horse's own blood and its centrifugation, but isolates the α-2 macroglobulin, a multifunctional protein with diverse roles in inflammation, protease inhibition, and immune modulation.

Since they are autologous, they have a personalized nature and avoidance of compatibility issues, thus minimizing the risks of adverse reactions.

However, they also present some limitations. Although safe, promising, and appealing, its use should always require a good evaluation of the patient and should be conducted in a thoughtful way, considering that this is an autologous product, encompassing a considerable inter-individual variability of cytokine and growth factor content, being

difficult to assure its constancy and homogeneity [152,172,173,190]. The current literature has failed to identify a preparation method where such variability is limited or negligible [155,189,191,192]. With efficacy differences between the various hemoderivatives, this is not possible yet [162,182,189].

4.3.2. Mesenchymal Stromal/Stem Cell-Based Therapies

Stem cells are undifferentiated cells that can self-renew and differentiate into cells and tissues with specialized functions. Therefore, nowadays, they are the focus for the development of regenerative medicinal therapeutics used to overcome the body's inability to regenerate damaged tissues after acute or chronic insults. They are classified by their source as embryonic (ESC), adult, and induced pluripotent stem cells (IPSCs) and by their development and differentiation capacity as totipotent, pluripotent, and multipotent cells. Totipotent stem cells are present only in a very early embryo during the morula stage and can develop into all embryonic and extra-embryonic tissues. During early embryonic development, ESC develops and may give rise to all tissue cells in the body, except for extra-embryonic tissues and germ cells. With further development, they gradually lose their pluripotency and become multipotent, which is characterized by the ability to differentiate into limited types of specific cells, often depending on their germ layer origin [193]. Multipotent stem cells might be hematopoietic stem cells (HSCs) or MSCs depending on their origin. HSCs can differentiate into different cells of the immune system, erythrocytes, and platelets, and MSCs into the cells of bone, cartilage, ligaments, tendons, fat, skin, muscle, neural, and connective tissue. Nowadays, there are proposals to change the acronym MSC to "mesenchymal stromal cell", as these critical advocates suggest that they do not represent true stem cells as there is a lack of some stemness markers [194]. More recently, another nomenclature change was proposed as "medicinal signaling cells", as these cells home into sites of injury or disease due to the profile of secreted cytokines by these tissues, therefore being signaling cells with medicinal intents [195]. However, recent studies have demonstrated that MSCs can release prostaglandin E2 (PGE2). The autocrine effect of PGE2 displays a major role in the self-renewal ability and immunomodulation of MSCs, thus generating a cascade of events on MSC proliferation, a major characteristic of stem cells, demonstrating MSC stemness [196,197].

The International Society for Cellular Therapy proposed a set of standards to define multipotent mesenchymal stromal cells. First, MSCs must be plastic-adherent when maintained in standard culture conditions using tissue culture flasks. Second, $\geq$95% of the MSC population must express the clusters of differentiation (CD)105, CD73, and CD90, as measured by flow cytometry. Additionally, these cells must lack the expression ($\leq$2% positive) of CD45, CD34, CD14 or CD11b, CD79α or CD19, and human leucocyte antigen (HLA) class II. Third, the cells must be able to follow a trilineage differentiation into osteoblasts, adipocytes, and chondroblasts under standard in vitro differentiating conditions [198].

MSCs exert their function through different paths: homing, that is, migration to the site of injury; differentiation into various cell types that can engraft to the damaged tissue for repair; and secretion of bioactive factors [199]. Initially, it was thought that MSCs migrated to injured tissues, became differentiated, and replaced the local cells. It is currently known that the immunomodulatory capacity of MSC is its main characteristic. This ability is due to the paracrine effect of MSC, the secretion of extracellular vesicles, the immunomodulation of apoptosis, and mitochondrial transfer [199].

MSC treatments can be categorized as either autologous or allogeneic, each with its own set of advantages and disadvantages. Opting for autologous treatment offers the advantage of reducing the likelihood of immune reactions, given that the MSCs are derived from the same individual receiving the treatment. However, this approach involves a more time-consuming preparation process including harvesting, processing, and culturing cells from the patient, leading to a delayed treatment onset. Additionally, the individualized production of doses can make autologous treatments more expensive. Furthermore, the patient's specific characteristics such as sex, age, and health may impact the quality and

potency of the MSC treatment. On the other hand, allogeneic treatments, while carrying the risk of possible immune reactions, present the benefit of utilizing cells from a young and healthy donor. This allows for large-scale production and storage in a cell bank, making them readily available for the treatment of acute lesions [200].

No significant differences in efficacy have been established between allogeneic and autologous MSCs for the treatment of musculoskeletal injuries in horses. Therefore, it is suggested that allogeneic MSCs may serve as a safe alternative to autologous MSCs [201]. While autologous MSCs are more commonly used in clinical trials for OA in horses, attributed to their perceived low immunogenicity and lower risk of adverse reactions, recent studies in horses and humans have demonstrated the absence of severe adverse events associated with allogeneic MSCs. This evidence supports the safety of administering allogeneic MSCs [200,202–204].

Mesenchymal Stromal/Stem Cell Therapies

As previously stated, the use of MSC therapy is one of the potential treatments of orthopedic injuries [3,205,206]. Nowadays, there is proof-of-concept that a variety of tissues have been identified as MSC sources for tissue regeneration and engineering. Bone marrow-MSCs (BM-MSCs) [207–210], adipose tissue-MSCs (AT-MSCs) [205,207,211,212], synovial membrane-MSCs (SM-MSCs) [200,202,213–216], amniotic fluid-derived MSCs (AFS-MSCs) [217,218], umbilical cord Wharton jelly's-MSCs (UC-MCS) [219–222], periosteum-MSCs (Po-MSCs) [223,224], dental pulp-MSCs (DP-MSCs) [225,226], and muscle tissue-MSCs (MT-MSCs) [227–229] are some of these.

Currently, BM-MSCs, AT-MSCs, SM-MSCs, and UC-MSCs are four of the most widely used types of MSCs in the treatment of musculoskeletal lesions.

The literature refers to tendon/ligament injuries with MSCs as very efficacious, suggesting that MSCs can contribute to accelerate and improve the quality of tendon healing by improving the tissue strength, providing a more favorable type I collagen composition, indicating a beneficial therapeutic response to these cells [200,202,230–232]. There are several clinical studies using BM-MSCs as the therapeutic option for tendon repair, perhaps because it is the most studied tissue source of MSCs [233,234]. A recent study compared them with UC-MSC, in vitro, and concluded that UC-MSC surpasses other MSCs in its ability to differentiate into tendon-like lineage cells and establish a well-organized tendon-like matrix. In terms of histological properties, UC-MSC promotes a superior regeneration of full-thickness defects when compared to BM- and UCB-MSCs [235]. Notwithstanding, studies with AT-MSCs advocate that this source might be superior regarding their potential to positively influence tendon matrix reorganization and because it is easier to harvest [236,237]. Recently, good results have been achieved by resorting to the use of SM-MSCs, which improved clinical signs and lesion ultrasonographic images, with a return to athletic function and led to no lesion relapse [200,202].

Regarding cartilage defects, BM-MSCs and AT-MSCs have been widely used for the treatment of OA. Each MSC's tissue origin has its own advantages in cartilage regeneration as they have heterogeneous potential concerning their accessibility, invasion during harvest, immunogenicity, proliferative, chondrogenic, and immunomodulatory abilities [238]. However, as synovium and cartilage have the same origin during the development of synovial joints, SM-MSCs are especially suitable for cartilage and have presented a greater proliferation and chondrogenic ability among other MSCs, suggesting superiority in cartilage repair [209,213,239–248].

Comparatively with BM-MSCs, SM-MSCs possess a greater colony-forming potential, have a low-density expansion that allows for the retention of multilineage differentiation capacity, and their gene profile matches the chondrocyte and meniscal cell gene profile closer than BM-MSCs [249]. The implantation of MSCs into cartilage defects have shown great promise in both cartilage and subchondral bone repair and regeneration [203,238,246–248,250–256].

UC-MSCs present higher proliferation potential, differentiation, and immunogenic abilities from the four most widely used tissues, previously referred to in [257]. They can

also release trophic factors that make them an excellent candidate for use in the clinical setting to provide the cell-based restoration of hyaline-like cartilage. Even in allogeneic administrations, these cells stimulate little or no host immune response and can be stored for long periods while maintaining viability [258]. UC-MSCs have also shown the ability for the in vitro induction of the production of glycosaminoglycans and collagen type II [259].

A recent review evidenced significant improvement in pain and function as the main advantages of MSC-based therapy in the treatment of cartilage repair in knees with OA. To sum up, MSCs and the derived exosomes have various functions in the treatment of OA such as an increase in chondrogenesis, proliferation of chondrocytes, reduction in apoptosis, maintenance of autophagy, regulation of synthesis and catabolism of the ECM, regulation of immune response, inhibition of inflammation, and monitoring the mitochondrial dysfunction as MSCs are able to carry out mitochondrial transfer to senescent chondrocytes, thus improving the activity of mitochondrial respiratory chain enzymes and the content of adenosine triphosphates as well as the overall paracrine effect [260].

In skeletal muscle injuries, treatment with AT-MSCs was pointed out as the best choice due to their efficient contribution to myoregeneration. The following characteristics were pointed out as differentiating and advantageous points: their high ex vivo expansion potential and less demanding harvesting than that of BM- or SM-MSCs [261]. Nevertheless, this study refers to autologous treatments.

Overall, the clinical use of MSCs is safe, is an "easy to do" procedure, and the treatment administration is not very invasive [204].

Autologous Chondrocyte Implantation (ACI)

ACI is a novel surgical and regenerative treatment that aims for the regeneration of full-thickness cartilage defects. Chondrocytes are collected from a less loaded area of the joint, digested and expanded, seeded in a scaffold and then injected in the defect region. At the moment, there are several commercial products available such as Cartilife® (Biosolution, Co. Ltd., Seoul, Republic of Korea), MACI® (Vericel Corporation, Sydney, Australia), ChondroCelect® (TiGenix N.V., Leuven, Belgium), Spherox® (CO.DON AG, Teltow, Germany), Chondron™ (CELLONTECH Co. Ltd., Seoul, Republic of Korea), Chondrocytes-T-Ortho-ACI® (Orthocell, Ltd., Murdoch, WA, Australia), and JACC® (Japan Tissue Engineering Co. Ltd., Gamagori, Japan) [262]. Different tissue sources have been used including cartilage, bone marrow, adipose, and umbilical cord tissues to produce chondrocytes. However, those mainly used are autologous bone marrow and cartilage tissues. Recently, there has been a trend shift, with the biggest bet made in allogeneic and adipose tissues [263]. Although these methods can solve the problem of cartilage regeneration to a certain extent, most of the regenerated tissues are fibrous and cartilaginous, which is inferior to hyaline cartilage for the intended purposes of load bearing and joint movement. Therefore, it is difficult to achieve the composition and mechanical properties of natural articular cartilage, and long-term efficacy is not guaranteed [21]. They are relatively successful in relieving pain in patients, but do not result in the regeneration of native tissue [263]. Compared with other techniques such as microfracture or osteochondral autograft/mosaicplasty, ACI seems to be an effective tool for cartilage restoration that may be more efficacious and durable than the other cartilage restoration techniques [264]. Thus, new cell-based and tissue engineering approaches are necessary and continue to be evaluated and optimized with the aim of promoting and inducing cartilage regeneration [263].

Mesenchymal Stromal/Stem Cell-Free Therapies

As previously discussed, the therapeutic efficacy of MSCs primarily arises from their immunomodulatory function. When exposed to inflammatory stimuli, MSCs secrete a variety of bioactive molecules collectively known as the secretome. The secretome is the collective term for the soluble factors produced by stem cells and employed for their intra- and inter-cell communications [265]. These factors are secreted to the extracellular space, which include soluble factors (cytokines, chemokines, and GFs) as well as non-soluble

factors, and extracellular vesicles (EVs) that transport lipids, proteins, ribonucleic acid (RNA), and desoxyribonucleic acid (DNA) subtypes [199,266] (Figure 1).

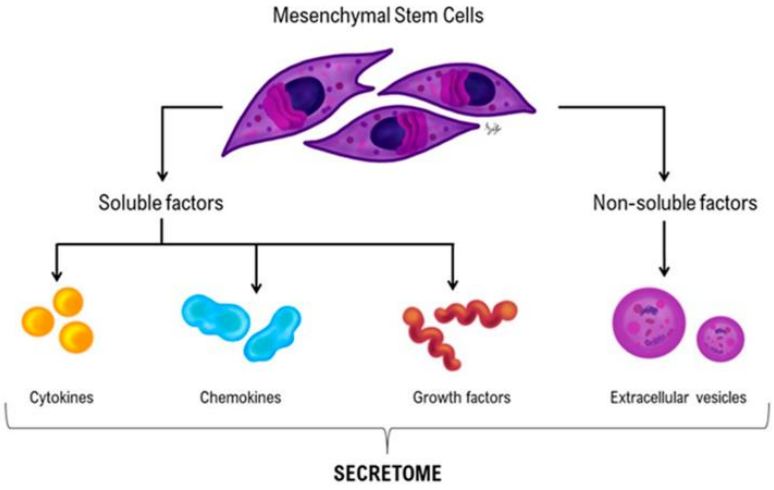

Figure 1. Secretome constitution. The secretome encompasses a varied collection of soluble and non-soluble factors. Soluble factors include cytokines, chemokines, and growth factors. Non-soluble factors include extracellular vesicles (exosomes, apoptotic bodies, and microvesicles) [223].

EVs can be subdivided into apoptotic bodies, microvesicles, and exosomes. This commixture activates the resident stem cells, and hence mediate the endogenous regeneration. However, the secretome of individual cells and tissues is specific, and changes in response to fluctuations in physiological states or pathological conditions [199].

In laboratory settings where MSCs are cultured under specific and adapted conditions, this secretome contributes to the generation of conditioned medium (CM) [267].

Studies have demonstrated that the application of MSC-conditioned medium (MSC-CM) has yielded promising results. This specialized medium, enriched with bioactive factors derived from MSC secretion, has shown efficacy in promoting tissue healing and modulating inflammatory processes due to proangiogenic, antiapoptotic, antifibrotic, anti-inflammatory, and immunomodulatory effects [266]. Previous literature reviews have consistently reported positive outcomes associated with the use of MSC-CM, highlighting its potential as a therapeutic intervention [202,203,267,268]. These findings underscore the importance of exploring MSC-derived secretome and conditioned media as viable treatment options for various inflammatory and tissue injury conditions.

Furthermore, the preservation of the therapeutic action of the parent MSC stands as an additional advantage as each cell type secretes a specific type of bioactive factor [269]. Beyond the biological benefits and addressing the safety concerns associated with the direct application of cells, cell-free therapies offer the potential to avoid immune compatibility, tumorigenicity, and the transmission of infectious diseases potentially related to stem cell therapy as well as several logistical advantages for clinical implementation [265]. These include scalability, ensuring a sufficient supply, and longer shelf-lives [266]. This shift toward cell-free therapies not only enhances the safety considerations, but also streamlines the practical aspects of treatment, making it more accessible, scalable, and feasible for clinical applications.

Certain limitations associated with these treatments pertain to the standardization of MSCs. These include factors such as the age and tissue source of the MSC donor, the duration of MSC preconditioning, the choice of nutritional medium for preculture, the oxygen tension within the culture environment, and the specific preconditioning factors applied [267]. The variability in these factors can influence the characteristics and effec-

tiveness of MSC-derived therapies, emphasizing the need for standardized protocols to enhance consistency and reliability across different treatment approaches. Addressing these considerations will contribute to advancing the field of regenerative medicine and optimizing the therapeutic potential of MSC-based treatments.

Recently, it has been demonstrated that the combination of SM-MSCs and UC-MSC CM could effectively repair a ligament in a reduced time frame, with the achievement of good clinical and imagiological outcomes [202].

A new trend in the regenerative investigation of cell-free therapies is the use of EVs. These have various subtypes and are important mediators in cell-to-cell communication as they carry certain proteins, glycoproteins, lipids, and ribonucleic acids that transmit biological information to support healing in injured tissues [270]. MSC-derived EVs have low-immunogenicity and strong potential for therapeutic applications to treat tissue fibrosis and promote tissue regeneration, and have therefore been proposed as a novel therapeutic agent to mediate immunomodulation and promote regeneration [271].

Recent studies have also enhanced some EV characteristics such as their maintenance in systemic circulation and passage through physiological barriers to ultimately exert their effects on recipient cells. Bearing this in mind, they are being studied for different purposes such as regeneration, drug delivery, activity control strategies for pathological EVs, and targeting technologies [272]. However, it is unclear whether using isolated EVs or exosomes excludes an important component of the associated therapeutic effects of cell-based therapy [273].

Current investigations support the basis for the clinical translation of MSC exosomes as a cell-free therapy for tissue repair. The literature refers to exosomes as joint protectors against OA damage by promoting cartilage repair, attenuating inflammation, balancing cartilage matrix formation, inhibiting synovitis, and mediating subchondral bone remodeling [274,275]. In tendonitis, they also attenuated the inflammatory phase, increased the proliferation and differentiation of tenocytes, had effects of balancing the tendon extracellular matrix, promoting the tenogenesis of tendon stem cells, and improved enthesis [276–278]. In muscular strain and ischemic injuries, exosomes also modulate inflammation, fibrosis, and myogenesis [279,280].

Nevertheless, the use of exosomes is still in its infancy, and approaches for selectively harvesting the exosomes with regenerative potential and screening the regenerative contents have not been achieved yet [276].

Cell-free products 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 MSCs themselves [168]. These cell-free systems also have the advantage of low immunogenicity, non-cytotoxicity, and non-mutagenicity. In this way, they are becoming a center of interest and are being researched as the best candidates to replace cellular systems in the field of regenerative and immunomodulating medicine [272].

5. Prognosis

Conservative treatments for OA typically focus on managing joint inflammation and pain, aiming to provide temporary functional improvement. However, they do not halt the progression of the disease, allowing for a continuous degenerative process to unfold.

In the case of tendon and ligament injuries, conservative treatments include NSAIDs, local cooling, and controlled exercise programs. Unfortunately, these approaches frequently result in prolonged and unsuccessful outcomes. Healing in these cases occurs through fibrosis, restricting the return to function and resulting in a loss of tissue elasticity, making the affected area more susceptible to reinjury.

Muscular injuries are commonly addressed with conservative treatments such as NSAIDs, massage, swimming, and other physiotherapeutic modalities. Despite these efforts, the prognosis is often fair, as fibrosis may develop, leading to mechanical lameness and a potential recurrence of the lesion [281].

The overall prognosis for musculoskeletal injuries treated conservatively is typically moderate to fair, with clinical signs being alleviated, and when healing occurs, it is through fibrosis. In joint injuries, degeneration continues unabated, and as a result, the affected organ fails to fully recover function, preventing a return to the same performance level.

However, recent advancements in medical therapies have introduced regenerative treatments designed to hamper disease progression, reduce inflammation, and promote tissue regeneration. These innovative therapies mark a significant shift in the clinical paradigm of sports medicine, offering a robust and promising contribution. This transformative approach has the potential to improve the prognosis for musculoskeletal injuries, turning it from moderate to fair to a more optimistic outlook [68].

6. Discussion

As emphasized in this work, there is a wide array of therapeutic options for addressing musculoskeletal injuries, with the choice depending on various factors. It is crucial to note that the selection of a specific therapy depends on the type and severity of the musculoskeletal issue as well as the horse's overall health and intended use.

When lesions occur, there are several conservative and regenerative therapeutic options currently available for managing equine injuries. These treatments aim to promote healing, alleviate pain, and restore functionality.

Conservative treatments are considered the first line of intervention and aim to alleviate clinical signs, promote healing, and improve overall well-being without resorting to surgery or other measures. They play a crucial role in managing both acute and chronic pain in horses [282]. The suitability of conservative treatments depends on the specific condition, its severity, and the individual needs of the horse. However, their results are usually unsatisfactory, recovery is slow, and lesion relapses are frequent [37].

It is an undeniable fact that conservative treatments are extensively and commonly used in comparison to regenerative treatments [184]. Conservative approaches including physical therapy, medication, and non-invasive interventions tend to be easier to access and are more cost-effective in contrast to certain biologic therapies. The latter often involve advanced technologies, making them more expensive, however, they are becoming more widely used [184]. Cost considerations may arise regarding the perceived effectiveness of conservative treatments when compared to newer or less-studied biologic therapies and have become particularly significant for horse owners and veterinarians working within budget constraints [282]. Nevertheless, while regenerative treatments may initially appear to be more expensive, they often require fewer treatment sessions compared to conventional methods, demonstrate a lower rate of lesion recurrence, and promote better clinical and functional outcomes, often achieving complete regeneration of the affected tissue [283]. Overall, they are more advantageous, and their perceived outcome is increasing as their use is becoming more common [119].

Conservative treatments often benefit from a long history of use and are supported by a substantial body of empirical evidence. When these treatments prove ineffective in halting clinical signs and pathological traits, regenerative treatments emerge as the most effective means, offering both anti-inflammatory and regenerative effects [169,198–200]. Nowadays, they represent the most promising class of therapeutics and continue to be in constant development. They are biologic and therefore "drug-free", having no concerns with clearance, making it very appealing for use in high-level sports.

Within this class, MSC-based therapies exhibit clinical efficacy, inducing favorable outcomes [193,260,277]. The ability to manufacture or engineer MSCs and their products according to specific pathologies enhances the therapeutic responses based on tissue source, secretome, or cytokine manipulation [199,266,284].

Concerns regarding the immunogenicity and tumorigenicity of stem cell therapies have been mitigated, as no severe adverse reactions have been reported in clinical experiments [197,200,202,203,285]. The use of allogeneic bank cells from healthy donors facilitates prompt treatment in the acute phase, circumventing constraints associated with autologous

treatments such as individual as well as the heterogenic and time-consuming processes of harvest and production that may lead to variations to the product's cell and cytokine composition [286]. All of these factors, which can potentially affect product variability, are a main clinical concern once they can negatively influence the therapeutic effects [287]. Therefore, allogeneic treatments seem to be more advantageous as they skip autologous related limitations.

Among the regenerative therapies, while hemoderivative products have been gaining significant expression in the market with optimistic outcomes, MSC-based products are making strides in that direction. Considerable research has been conducted in this field [288], but there remains a lack of qualitative and quantitative evidence-based data supporting MSC-based regenerative therapies in clinical use, since most of the studies are observational, therefore being dissimilar from each other [4,5,128,138,173,181,203,289–294]. The high heterogeneity of the reviewed studies did not allow for a meta-analysis to compare the results between treatments. However, the results have been unanimous, concluding on the effectiveness and achievement of tissue regeneration, which is the ultimate objective of any musculoskeletal treatment [200,202,284,288,295,296]. In fact, in the last 15 years, there has been a shift in the trend among equine practitioners from OA conservative treatments to regenerative treatments [119].

Significant steps are being taken in the pursuit of standardized protocols for the therapeutic production, storage, and application of regenerative treatments. Establishing accurate therapeutic protocols, identifying optimal hemoderivative or MSC-tissue sources for specific diseases, determining suitable dosages, and establishing the ideal intervals between applications for various pathologies are in the spotlight and warrant further exploration [173,297]. In fact, the expanding market launch of regenerative commercial products is making their utilization more attractive and widespread, with the products exhibiting uniform characteristics, whether through devices that prepare them or as final products available on the market.

Additionally, it is essential to delineate effective physiotherapeutic protocols, employing one or more methods, during rehabilitation period and in routine exercise plans [36,39]. A profound understanding of each physiotherapeutic technique is vital, considering this is a crucial aspect of any musculoskeletal treatment and must be used synergistically with other therapies in order to offer a more favorable return to function and improved clinical outcomes [36].

Recognizing the evolving understanding of a multidisciplinary approach is pivotal for achieving the optimal therapeutic results. Ensuring a well-balanced environment is decisive for the health and performance of the equine. A collective effort of the horse-environment is compelling in preventing and managing injuries effectively, fostering a holistic and informed approach.

7. Conclusions, Challenges, and Future Research Directions

Currently, a diverse array of musculoskeletal treatments is available. While conservative treatments have their merits, they also come with several limitations. This has propelled regenerative treatments into a position of high importance and hope within the field.

The regenerative approaches have demonstrated their value and effectiveness, holding value and promise for addressing musculoskeletal issues in novel and potentially more effective ways. They present anti-inflammatory abilities, enable a return to function and tissue sustainability due to their regenerative competence in a faster time frame, providing a beacon of optimism for both practitioners and the horse community. It is important to bear in mind that the synergistic integration of physiotherapeutic techniques, together with good horsemanship practices and regenerative approaches, holds the promise of delivering superior outcomes.

Promising results in the realm of tissue repair and regeneration are evident in clinical studies with horses. At present, considering MSC-based therapies as the most capable of facing musculoskeletal injuries, the primary hurdles concern the strength of the evidence,

which is currently hampered by the absence of controlled clinical trials, in order to comprehensively understand the advantages and limitations of each therapy. However, this is a field that is rapidly expanding and experiencing significant growth, propelled by the auspicious results that are currently emerging. Consequently, it is anticipated that this will soon cease to be a limitation. The future will dictate the identification of the most effective tissue source, to correspond it to a specific type of lesion, to design orthobiologics that are readily accessible, easier to administer, that carry minimal risk, and are financially feasible. Further research is imperative to validate the efficacy and establish precise guidelines for clinical implementation. Despite the ongoing quest for answers in regenerative therapies, it seems that they have presently emerged as the most effective approach to address musculoskeletal injuries.

The outlook in regenerative medicine is promising, generating high expectations and capturing extensive attention in both equine and human medicine as they accelerate recovery, promote regeneration, and organ functionality, and therefore, a return to peak performance and quality of life.

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Abbreviations

^{18}F-NaF	Fluorine-18-sodium fluoride
2D	Two-dimensional
3D	Three-dimensional
ACI	Autologous chondrocyte implantation
ACS	Autologous conditioned serum
AFS-MSC	Amniotic fluid mesenchymal stromal/stem cell
AI	Anti-inflammatory
APS	Autologous protein serum
AT-MSC	Adipose tissue mesenchymal stromal/stem cell
BM-MSC	Bone marrow mesenchymal stromal/stem cell

CD	Cluster differentiation
CD	Complementary diagnostic exams
cm	Centimeter
CM	Conditioned medium
cm^2	Square centimeter
COX	Cycloxigenase
CSA	Cross sectional area
CT	Computed tomography
CTA	Computed tomography arthrography
DDFT	Deep digital flexor tendon
DNA	Desoxyribonucleic acid
DP-MSC	Dental pulp mesenchymal stromal/stem cell
ECM	Extracellular matrix
ESC	Embryonic stem cell
ESWT	Extracorporeal shock wave therapy
EV	Extracellular vesicles
FEI	Federation Equestre Internationale
FGF	Fibroblast growth factor
FL	Forelimbs
GAG	Glycosaminoglycans
GF	Growth factor
h	Hour
HILT	High intensity laser therapy
HL	Hindlimbs
HLA	Human leucocyte antigen
HSC	Hematopoietic stem cell
HyA	Hyaluronic acid
Hz	Hertz
IA	Intra-articular
IGF	Insulin-like growth factor
IL	Interleukin
IM	Intramuscular
IPSC	Induced pluripotent stem cell
IRAP	Interleukin-1 receptor antagonist protein
IRU	Radioisotope uptake
IV	Endovenous
Kg	Kilogram
KT	Kynesiotaping
LLLT	Low level laser therapy
mg	Milligram
MHz	Megahertz
min	Minute
mL	Milliliter
MRI	Magnetic resonance image
MSC	Mesenchymal stem/stromal cells
MSC-CM	Mesenchymal stem/stromal cells conditioned medium
MT-MSC	Muscle tissue mesenchymal stromal/stem cell
mW	Milliwatts
NEMS	Neural electrical muscle stimulation
nm	Nanometers
NSAID	Non-steroidal anti-inflammatory
OA	Osteoarthritis
°C	Degree Celsius
PDEGF	Platelet derived epidermal growth factor
PDGF	Platelet derived growth factor
PEMF	Pulsed electromagnetic frequency
PET	Positron emission tomography
PGE2	Prostaglandin E2

PHyd	Polyacrylamide hydrogel
Po -MSC	Periosteum mesenchymal stromal/stem cell
PO	Per os
PPS	Pentosan polysulfate
PRP	Platelet-rich plasma
PSAG	Polysulfated glycosaminoglycan
RNA	Ribonucleic acid
ROM	Range of motion
SAIDs	Steroidal anti-inflammatories
SF	Synovial fluid
SL	Suspensory ligament
SM-MSC	Synovial membrane mesenchymal stromal/stem cell
TENS	Transcutaneous electrical nerve stimulation
TGF	Transforming growth factor
U/S	Ultrasound
UC-MSC	Umbilical cord derived Whartons jelly mesenchymal stromal/stem cell
VEGF	Vascular endothelial growth factor
W	Watts
WT	Water treadmill
X-ray	Radiograph
α2M	Alfa-2 macroglobulin

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CHAPTER II

Allogenic synovia-derived mesenchymal stem cells for treatment of equine tendinopathies and desmopathies—Proof of concept

CHAPTER II

- **INTRODUCTION**

- **PAPER II**

Allogenic synovia-derived mesenchymal stem cells for treatment of equine tendinopathies and desmopathies—Proof of concept

I.L. Reis, B. Lopes, P. Sousa, A.C. Sousa, M.V. Branquinho, A.R.Caseiro, S.S. Pedrosa, A. Rêma, C. Oliveira, B. Porto, L.M. Atayde,I. Amorim, R.D. Alvites, J.M. Santos, A.C. Maurício (2023) Animals, 13(8), 1312. doi: 10.3390/ani13081312

- **GRAPHICAL PAPER II**

CHAPTER II INTRODUCTION

In the realm of equine sports medicine, the management of tendon and ligament injuries poses a significant challenge. Typically, the healing process culminates in tissue fibrosis, resulting in compromised functionality of tendons and ligaments. This difficulty underscores a conspicuous deficiency in the available therapeutic modalities demanding redress. Regenerative medicine emerges as a prospective path to address this therapeutical gap. Accordingly, our research team endeavoured to develop a therapeutic intervention tailored to this urgency. Subsequent clinical trials were conducted to assess the efficacy of this therapeutical product in promoting the regeneration of tendon and ligament tissues, with the ultimate goal of reinstating functionality, averting relapses, and facilitating the resumption of athletic activity.

Tendons are fibrous connective tissues, load-bearing structures, transmitting forces from muscles to bones. Tendons such as superficial digital flexor tendon (SDFT) and Achilles tendon also allow energy storage (Shojaee, 2023). This difference concerns with its structure and composition. Tendons intricate collagen architecture is supplemented by a plethora of non-fibrillar proteins crucial for ensuring tendon stability under load (Andarawis-Puri et al., 2015).

To comprehend the cellular dynamics involved in the response to injury, it is crucial to delve into the tendon's anatomy. Cell populations implicated in repair are often categorized into intrinsic and extrinsic origins, yet their specific contributions to the healing process remain ambiguous. Intrinsic cells typically refer to tenocytes which are arranged in alignment between the fibres within fascicles and exhibit a fibroblast-like morphology. In addition to tenocytes, tendons harbour a reservoir of tendon stem/progenitor cells (TSCs), the precise localization of which remains elusive but is speculated to exist within the loose connective tissue surrounding the tendon (epitenon and paratenon), the outer tendon sheath, neighbouring blood vessels (perivascular cells), and inflammatory cells (Walia & Huang, 2019). This extrinsic cell populations are believed to be the initial mesenchymal responders during the inflammatory phase of healing. TSCs are involved in various aspects of tendon physiology and pathology, including tendinopathy. Excessive mechanical loading can trigger the release of inflammatory mediators, potentially driving the differentiation of TSCs into non-tenocyte lineages and contributing to micro-damage within tendons, thereby predisposing them to tendinopathy – (Shojaee, 2023) - **Figure 1.**

It is proposed that intrinsic cell populations participate in the healing process during later stages, contributing to the production of larger and more aligned collagen fibres(Dyment & Galloway, 2015).

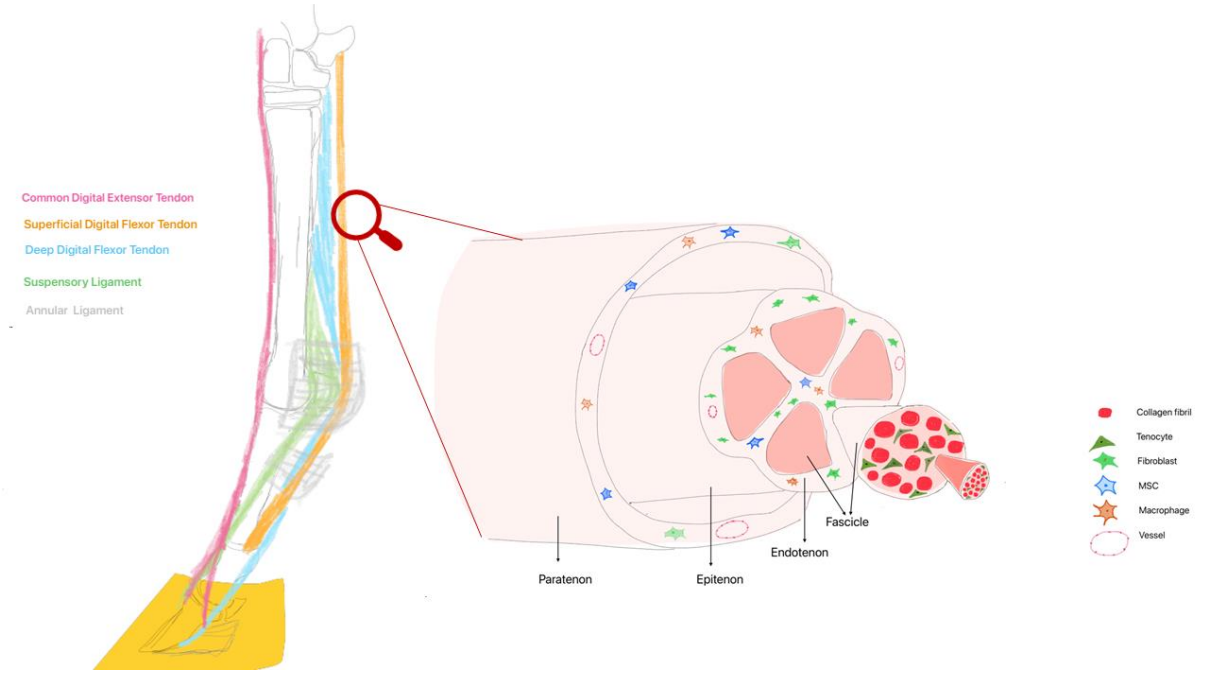

Figure 1. Equine metacarpal tendons and ligaments and tendon structure. In this figure are highlighted the anatomical location of the mentioned structures during this chapter. Superficial digital flexor tendon (SDFT) deep digital flexor tendon (DDFT) and Suspensory ligament (SL). In detail, the tendon structure comprehending the paratenon, epitenon and endotenon and their fascicles. Fascicles are organized structures of collagen fibrils and tenocytes. Fascicles are surrounded by fibroblasts, mesenchymal stromal/stem cells (MSC), the TSCs, white cells such as macrophages and blood vessels.

Tendons undergo structural, compositional, and mechanical alterations in response to mechanical stimuli throughout their development, aging, and in response to injury, a phenomenon termed tissue mechanical adaptation (Zamboulis et al., 2020). These mechanical adaptations are largely mediated by the tenocytes which perceive mechanical loads from the extracellular matrix (ECM) and subsequently influence ECM composition (Walia & Huang, 2019). Thus, mechanical loading plays a pivotal role in maintaining tendon homeostasis, although excessive loading can incite remodelling or degeneration processes. Following tendon degeneration leading to rupture, the ensuing healing process is slow and often fails to fully restore the native tendon structure and often resulting in an inability to attain the complete

range of motion (ROM). It is widely acknowledged that controlled loading of repair tissues is imperative for fostering tendon healing while concurrently minimizing the formation of scar tissue and adhesion within tendons (Andarawis-Puri et al., 2015; Shojaee, 2023). Thus, understanding the biomechanics of tendons is important to design accurate therapeutic protocols.

Tendon injury occurs when the stress imposed is higher than the tissue can withstand and often responds poorly to conventional treatments. To address this deficiency, regenerative medicine has garnered considerable attention as a potential solution, aiming to overcome these challenges through the prospect of tissue regeneration, restoration of normal function and ROM, and the return to athletic lifestyle.

Among the regenerative therapeutical options, mesenchymal stem/stromal cell (MSC)-based therapies seem to be the most promising. Therefore, significant efforts have been dedicated to advancing research in this domain to procure the most efficacious and accessible therapy yielding optimal outcomes.

In pursuit of this objective, a medicinal product has been developed using allogenic equine synovial membrane- MSCs (eSM-MSCs) combined with autologous serum to be injected at tendon/ligament lesion area.

The present study aims to:

1. **Characterize the eSM-MSCs.** Therefore, the cells were collected, isolated, expanded and characterized through trilineage differentiation, karyotype analysis, conditioned medium analysis and immunohistochemistry.
2. **Identify the therapeutical potential** of this combination regarding its **safety**, **easiness to use**, **efficacy** and **clinical and imagological outcome**.

Thus, the study herein presented outlines the characterization of the cells as well as, the preparation, and application of the medicinal product in 20 tendinous and ligamentous structures. Detailed descriptions of the follow-up procedures and outcomes are provided. All patients adhered to a rigorous 12-week physical rehabilitation program, recognizing the critical role of delivering an appropriate biomechanical stimulus in attaining functional success [1].
Regarding clinical outcomes, all treated structures regained functionality and ROM, with all patients resuming their previous lifestyle. Ultrasonographic findings revealed a normal cross-sectional area and optimal fibre alignment and echogenicity. These, coupled with the absence of lameness and positive clinical outcomes, dictated the patients' recovery. Notably, the

athletes returned to sports and achieved their previous performance level, in a shorter timeframe compared to literature findings on identical pathologies treated with other regenerative therapy methods. Also, a proficient diagnosis coupled with access to an allogeneic cell bank enhanced a consistent therapeutic approach on the acute phase.

In a “One-health” perspective, the notable resemblance between the SDFT in horses and the Achilles tendon in humans, as well as between the common digital extensor tendon in horses and the anterior tibialis tendon in humans, is unmistakable(Shojaee, 2023). Therefore, the discovery of a viable and efficient therapy for horses holds promising translational potential for human applications.

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PAPER II

Allogenic synovia-derived mesenchymal stem cells for treatment of equine tendinopathies and desmopathies—Proof of concept.

animals

MDPI

Article

Allogenic Synovia-Derived Mesenchymal Stem Cells for Treatment of Equine Tendinopathies and Desmopathies—Proof of Concept

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Simple Summary: Horses are high-level athletic athletes prone to musculoskeletal injuries. Tendon/ligament injuries are the most frequent types of injuries which that are very difficult to treat. Instead of tissue regeneration, usually, fibrous scar tissue develops which leads to decreased functionality of the injured area and threatens the participation of sport horses. The aim of regenerative medicine is to find a treatment that promotes tissue regeneration and that allows the equine patient to return to the same level of athletic performance in the shortest time period possible. In this study, we developed a solution of equine synovial membrane stem cells and autologous serum, to be injected at the lesion site to promote tissue regeneration. We describe the processes of tissue collection, preparation, isolation of synovial stem cells, expansion, culture, cryopreservation, and posterior preparation with autologous serum. The solution was tested in 16 tendons and ligaments of equines. After treatment, all equine patients underwent a physical rehabilitation program and were monitored with physical and ultrasonographic exams. The results were very promising, and thus, support the use of equine synovial stem cells and autologous serum in the treatment of tendonitis and desmitis.

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Abstract: Tendon and ligament injuries are frequent in sport horses and humans, and such injuries represent a significant therapeutic challenge. Tissue regeneration and function recovery are the paramount goals of tendon and ligament lesion management. Nowadays, several regenerative treatments are being developed, based on the use of stem cell and stem cell-based therapies. In the present study, the preparation of equine synovial membrane mesenchymal stem cells (eSM-MSCs) is described for clinical use, collection, transport, isolation, differentiation, characterization, and application. These cells are fibroblast-like and grow in clusters. They retain osteogenic, chondrogenic, and adipogenic differentiation potential. We present 16 clinical cases of tendonitis and desmitis, treated with allogenic eSM-MSCs and autologous serum, and we also include their evaluation, treatment, and follow-up. The concerns associated with the use of autologous serum as a vehicle are related to a reduced immunogenic response after the administration of this therapeutic combination, as well as the pro-regenerative effects from the growth factors and immunoglobulins that are part of its constitution. Most of the cases (14/16) healed in 30 days and presented good outcomes. Treatment of tendon and ligament lesions with a mixture of eSM-MSCs and autologous serum appears to be a promising clinical option for this category of lesions in equine patients.

Keywords: allogenic; cell-based therapies; clinical trials; ligament; mesenchymal stem cells; sport horses; synovial mesenchymal stem cell; tendon

1. Introduction

Tendonitis and desmitis are defying clinical challenges in equine patients that require long recovery periods, and ineffective tendon repair can affect their sport careers. Tendons operate near their functional limits during maximal exercise, and their ability to adapt to stress and self-repair is limited. A controlled exercise program alone or in combination with a variety of conservative treatments, such as corrective shoeing and nonsteroidal anti-inflammatory drugs (NSAIDs), is still the gold standard therapy for equine tendon disease [1]. Current treatments often do not fully repair or regenerate the injured or affected tendon nor lead to its total functional recovery [1,2].

The aim of tendinopathy treatment is to achieve tissue regeneration and return to complete organ function and performance. Recently, tissue engineering approaches have attracted attention for tissue repair. Among the approaches, the use of mesenchymal stem cell-based therapy has increased, since it is a promising approach for tissue repair and regeneration including tendinopathy and desmitis [1,3–6].

Mesenchymal stem cells (MSCs) can be isolated from several tissue sources such as bone marrow, peripheral blood, dental pulp, umbilical cord, and amniotic fluid [7]. MSC characteristics have been defined by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT), and include plastic adherence when maintained in standard culture conditions; expressing clusters of differentiation (CDs), such as CD44, CD90, and CD105; and no expression of major histocompatibility complex (MHC)-class II markers and of hematopoietic-related markers (CD45 and CD34) [8]. Finally, MSCs must be able to differentiate in vitro into, at least, osteoblasts, adipocytes, and chondroblasts, in the presence of adequate differentiation culture media [8].

Synovial membrane mesenchymal stem cells (SM-MSCs) were initially isolated, in 2001, by De Bari et al. [9], from human knee joints and showed significant proliferative ability in culture, even after Passage 10 (P10), and multilineage differentiation potential in vitro [9]. These cells represent a good source of MSCs and a promising therapeutic tool mostly for musculoskeletal pathologies [10]. Sakagushi et al. compared the properties of different sources of human stem cells, i.e., bone marrow, synovium, periosteum, skeletal muscle, and adipose tissue, and observed the superiority of synovium as a source for MSCs for treatment of musculoskeletal pathologies as they had more ability to chondrogenesis. Pellets of synovium-derived stem cells were larger and expressed more intense staining for chondrogenic differentiation [11].

SM-MSCs have higher chondrogenic capacity than other studied sources of MSCs, such as bone marrow (BM-MSCs) [12,13]. Cartilage pellets from SM-MSCs have been reported to be significantly larger than those from BM-MSCs [12]. SM-MSCs have a higher production of uridine diphosphate glucose dehydrogenase (UDPGD) [13], an enzyme that converts UDP-glucose into UDP-glucuronate, one of the two substrates required by hyaluronan synthase for hyaluronan polymer assembly. In addition, Sox-9, collagen type II (Col-II), and aggrecan, specific markers for chondrogenesis, as well as cartilage-specific molecules such as cartilage oligomeric matrix protein (COMP) have also been found in high amounts in equine synovial fluid-derived MSCs and the extracellular matrix, respectively by reverse transcription polymerase chain reaction (RT-PCR) [13].

In a recent study, using a rabbit model, Bami et al. highlighted the superiority of SM-MSCs in terms of chondrogenesis, osteogenesis, myogenesis, and tenogenesis [14]. A study of xenogenic implantation of SM-MSCs in equine articular defects also confirmed better healing of the cartilage of affected knees as well as a higher expression of collagen type II, indicating the presence of hyaline cartilage in the healed defect [15].

SM-MSCs have been defined as MSCs due to their phenotypic profile and differentiation potential. Even though there are no specific antibody markers to identify these MSCs, there is general agreement that MSCs should be negative to hematopoietic markers CD34 and CD45 and positive to CD44, CD73, CD90, and CD105 [16]. Mochizuki et al. found that SM-MSCs maintained their proliferative ability, regardless of which region they were collected from in the synovium [17].

In 2003, Fickert et al. reported that the markers CD9, CD44, CD54, CD90, and CD166 could be used to identify MSCs isolated from the synovium of human patients with osteoarthritis (OA), and they also confirmed that CD9/CD90/CD166 triple-positive cell subgroups had obvious chondrogenic and osteogenic differentiation abilities [18].

Prado et al. confirmed the mesenchymal nature of equine synovial membrane and fluid-derived stem cells through the expression of significant hematopoietic (CD45, CD34, CD117, and CD133), mesenchymal (CD105, CD90), pluripotency (OCT3/4 and NANOG), embryonic (Tra-1-81), inflammatory, and angiogenesis (vascular endothelial growth factor (VEGF-R1) and LY6a) markers [19]. Although the presence of hematopoietic and inflammatory markers was not expected, variations may occur and must be considered to influence acute or chronic stages of osteochondrosis expression and/or inflammatory events [19,20].

Nevertheless, the immunophenotype characterization of equine MSCs (eMSCs), as well as in other veterinary species, has not yet been completely established [19]. This is a major challenge, since the expression of certain adult stem cell markers may differ between species. For that reason, it is a need to define a set of CD markers which can be uniformly applied for the identification of eMSCs [8,20].

Horses are high performance athletes prone to musculoskeletal diseases, i.e., osteoarticular, as well as tendon/ligament lesions and fractures of various degrees due to sport- and age-related injuries. These pathologies resemble human musculoskeletal conditions, and therefore, horses are a valuable animal model for assessing stem cell and cell-based therapies prior to the translation of results into humans [21]. The use of a therapy that can regenerate these structures and restore their complete functionality instead of ordinary healing is the aim of our study and of equine practitioners throughout the world.

Recent studies have suggested that MSCs can self-renew, migrate to injury sites (homing), perform multilineage differentiation, and secrete bioactive factors, thus, increasing proliferation and migration of tendon stem/progenitor cells via paracrine signaling and increasing the regeneration ability of tissues with poor aptitude [1,3–5,22,23].

In fact, the knowledge of the importance of this paracrine action has opened doors to cell-free therapeutic strategies in regenerative medicine. The soluble factors (cytokines, chemokines, and growth factors) and nonsoluble factors (extracellular vesicles and exosomes) released in the extracellular space by MSCs, commonly known as secretome, have become the focus of novel therapeutic approaches due to their key role in cell-to-cell communication, their active influence on immune modulation, and their pro-regenerative

capacity both in vitro and in vivo [23]. Therefore, in this study, secretome was also analyzed with the prospect of being used therapeutically, in the future, in similar clinical cases.

In the present study, equines used as show jumping and dressage athletes as well as leisure horses with acute and chronic lesions were treated with intralesional administration of the considered combination, i.e., autologous serum and eSM-MSCs. The treatment consisted of two injections, 15 days apart. Pre- and post-treatment evaluations consisted of clinical, orthopedic, and tendon/ligament ultrasound exams. None of the selected equine patients had previously received any other regenerative treatment.

2. Materials and Methods

2.1. Study Design and Horse Selection

This prospective longitudinal study was performed in Portugal between February 2016 and January 2019. Sixteen horses, from 5 to 22 years old with acute and chronic signs of lameness were enrolled in this study (11 males and 5 mares), whose sport activities were distributed over show jumping (14), dressage (1), and leisure (1). The horses were all outpatients from an equine ambulatory clinic. This study included the treatment of 16 tendons, i.e., 14 superficial digital flexor tendons and 2 deep digital flexor tendons, and 4 suspensory ligaments.

Lameness was scored based on the American Association of Equine Practitioners (AAEP) scale (Table 1) and confirmed by using a positive regional nerve block. Flexion and pain to pressure tests were also evaluated [24].

Table 1. Score systems used by the veterinary surgeon to assess lameness, and responses to flexion and pain to pressure tests [25,26].

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

2.2. Inclusion and Exclusion Criteria

In this study, horses with acute or chronic lameness (Table 2), with diagnosed tendonitis and/or desmitis and with no signs of systemic disease were accepted in the inclusion criteria. Injured horses were treated in acute stages of disease, except for two equine patients (Patients 3 and 6). Patient 3 had an injury the year before this treatment and laser therapy had been performed, without a complete recovery. After that, he had a re-injury and, at this time, this treatment was suggested. Patient 6 was referred by another clinician who tried, unsuccessfully, to treat this patient. Patient 6 was sent to the field for one year and then re-evaluated. At this time, and as its trainer wanted to improve its quality of life, this treatment was proposed by its clinician. The lameness grade of each equine patient is specified in Table 2. Considering the established exclusion criteria, the selected equine patients should not have been under any other medical treatment (including nonsteroidal anti-inflammatory drugs, intra-articular corticosteroids, hyaluronan, glycosaminoglycans, platelet-rich plasma (PRP), and other MSC preparations) for at least 2 months before the

allogenic eSM-MSC treatment and did not receive any additional medical treatment (except for that described in the treatment plan) for at least 2 months post the cell-based treatment.

Table 2. Equine patient and lesion characterization. The left column characterizes the equine patients: Sex, male (M) or female (F); age measured in years old (yo); sports modality (SM), show jumping (SJ), dressage (Dre), and Leisure (Lsr); lameness score (AEEP score) pretreatment. The right column characterizes the lesions: Structure affected, superficial digital flexor tendon (SDFT), deep digital flexor tendon (DDFT), and suspensory ligament (SL) left branch (LB); affected limb, right frontlimb (RF), right hindlimb (RH), left frontlimb (LF) and left hindlimb (LH).

Equine Patient					Lesion: Tendonitis/Desmitis			
ID	**Sex**	**Age (yo)**	**SM**	**Lameness Pretreatment**	**Structure**	**Type**	**Limb**	**Evolution**
1	M	22	SJ	3/5	SDFT DDFT	Acute	LF	Favorable in 30 days
2	F	5	SJ	2/5	SDFT	Acute	RH	Favorable in 30 days
3	F	14	SJ	2/5	SDFT	Chronic	RF	Favorable in 30 days
4	M	8	SJ	4/5	SDFT	Acute	RF	Favorable in 30 days
5	M	7	SJ	4/5	LB SL	Acute	LF	Favorable in 30 days
6	M	13	Lsr	4/5	SDFT DDFT SL	Chronic	RH	Tendons: favorable evolution in 30 days; SL in 90 days.
7	M	15	SJ	4/5	SFDT	Acute	RF	Favorable in 90 days
8	M	11	SJ	4/5	SDFT	Acute	RF	Favorable in 30 days
9	F	10	SJ	4/5	SDFT SL	Acute	RF	Favorable in 30 days
10	M	9	SJ	4/5	SDFT	Acute	LF	Favorable in 30 days
11	F	10	SJ	3/5	SDFT	Acute	RF	Favorable in 30 days
12	M	12	Dre	2/5	SDFT	Acute	LF	Favorable in 30 days
13	M	14	SJ	4/5	SL	Acute	LF	Favorable in 30 days
14	M	7	SJ	4/5	SDFT	Acute	RF	Favorable in 30 days
15	M	12	SJ	3/5	SFDT	Acute	LF	Favorable in 30 days
16	F	6	SJ	3/5	SFDT	Acute	RF	Favorable in 30 days

2.3. Ethics and Regulation

This study was carried out in accordance with the 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 equine patient's legal trainer, 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. In addition, no animals were euthanized for this study.

2.4. Donor Selection and SM Collection

The eSM-MSC donor was a young and healthy foal, 7 months old, who died accidentally when running in the arena. The trainer authorized synovial membrane collection from the hocks, knees, and fetlocks. The synovial membrane was evaluated and its appearance was transparent, bright, and smooth; in addition, the presence of viscous and transparent synovial fluid confirmed its soundness. The skin covering the incisional field was surgically cleaned with chlorohexidine and alcohol. The skin and subcutaneous tissue were incised, debrided, the articular capsule was opened, and the synovial membrane was isolated and extracted into a Dulbecco's phosphate buffered saline (DPBS) container. The samples were transported to the laboratory with ice packs for refrigerated temperatures. Figure 1a presents the fresh tissue arrival and Figure 1b shows the preparation at the laboratory. Figure 2 shows a schematic representation of the process from eSM-MSC collection to the

administration of the combination, i.e., eSM-MSCs and autologous serum (1×10^6 cells/mL and 1 mL of autologous serum in a total volume of 2 mL).

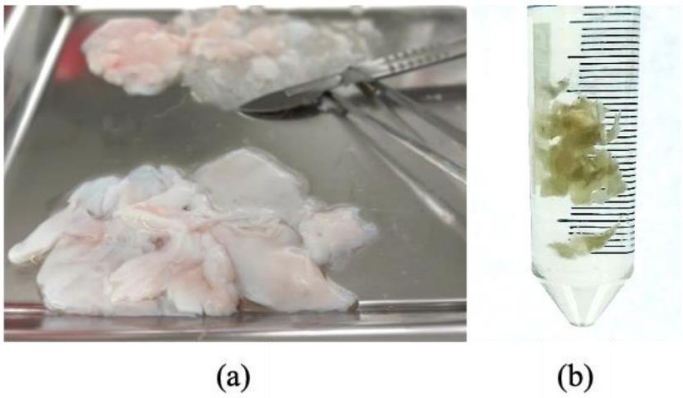

Figure 1. Laboratory arrival and preparation of the fresh tissue to start digestion, isolation, and expansion: (**a**) Tissue collected in the field; (**b**) isolated synovial membrane.

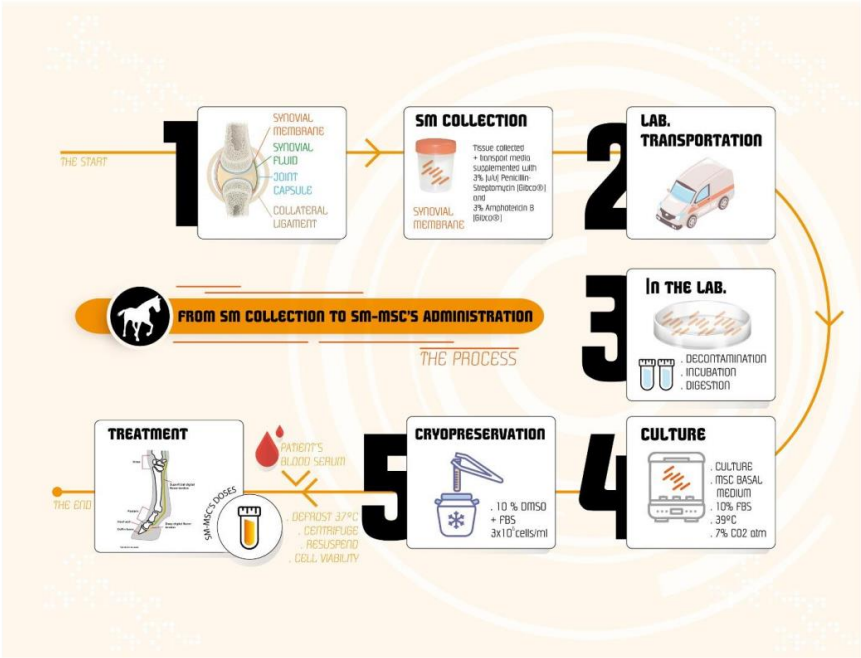

Figure 2. Schematic representation of the event sequence from the collection of synovial membrane to the administration of the therapeutic combination. After the collection, the synovial membrane is transported to the laboratory where it is separated from the whole tissue, decontaminated, incubated, and digested. Then, cells are cultured and expanded and finally cryopreserved in a cell bank. When needed for treatment, cells are prepared with autologous serum, and then applied in the selected equine patient.

2.5. eSM-MSC Isolation

After collection, the equine synovial membrane was prepared at the Laboratory of Veterinary Cell-based Therapies from ICBAS-UP. The isolation protocol for eSM-MSCs was developed by patented proprietary technology Regenera® (PCT/IB2019/052006, WO2019175773, Compositions in use for the treatment of musculoskeletal conditions and methods for producing the same leveraging of the synergistic activity of two different types of mesenchymal stromal/stem cells, Regenera®). Fresh tissue was transported to the laboratory facilities in a hermetically sealed sterile container in transport media (supplemented with 3% (v/v) penicillin-streptomycin (Gibco®, Waltham, MA, USA) and 3% amphotericin B (Gibco®) and processed within a period of up to 48 h. The synovial tissue was digested using collagenase and the isolated cells were incubated in a static monolayer culture using standard MSC basal medium supplemented with 10% fetal bovine serum (FBS) and maintained in standard culture conditions (37 °C, 5% CO_2, and humidified atmosphere) until they reached confluence. Cells from confluent cultures were cryopreserved in 10% dimethylsulphoxide (DMSO) and FBS, at a concentration of 3×10^6 cells/mL, using a control rate temperature freezer (Sy-Lab Cryobiology, SY-LAB Geräte GmbH, Purkersdorf, Austria). For expansion optimization, cells were cryopreserved in passages (P) between P2 and P3 to generate suitable master cell banks (MCBs). Expansion, thereafter, was analyzed during a maximum of 20 cumulative population doublings (cCPDs). The range of cCPDs chosen allowed for enough expansion to maximize the number of cells in the working cell banks (WCB) but kept the cCPDs within the genomic stability range.

2.6. SM-MSC Characterization

2.6.1. Tri-Lineage Differentiation Protocols

For all the differentiation protocols, cells in P4 were used after thawing.

Adipogenic Differentiation and Oil Red O Staining

For the adipogenic differentiation protocol, 1×10^4 cells/cm^2 were seeded in the wells of a 12-well plate (cell culture plates, 12-well, VWR®, Suwanee, Atlanta, GA, USA), with the addition of the standard culture medium. The plate was incubated under standard conditions for 4 days. After this period, the culture medium of 10 wells was replaced by complete adipogenesis differentiation medium (StemPro® Adipogenesis Differentiation Kit, Gibco®, Waltham, MA, USA), 2 wells were used as controls and maintained with the standard culture medium. Following the manufacturer's instructions, the media were replaced every 3–4 days and the cells maintained in differentiation for 14 days. At the end of this period, the oil red O staining protocol was performed using a handmade solution. The culture differentiation medium was removed, and the wells were gently washed with PBS. Cells were fixed with 4% formaldehyde (3.7–4% buffered to pH 7, reference# 252931.1315, Panreac AppliChem®, Darmstadt, Germany) for 10 min at room temperature, and the wells were washed 3 additional times with phosphate-buffered saline (PBS). Oil red O solution was added to each well and the plate incubated for 10–20 min at room temperature. Oil red O was discarded, and any excess dye was removed by several washes with PBS. PBS was added to each well for visualization. The aim of this assay was the identification of rounded cells with intracytoplasmic lipid vacuoles and their red coloration due to the exposure to the oil red O solution.

Chondrogenic Differentiation and Alcian Blue Staining

Thawed eSM-MSCs were automatically counted, and cell viability determined (%). Then, the cells were centrifuged, supernatant removed, and the pellet resuspended in culture medium to generate a cell suspension with 1.6×10^7 viable cells/mL. To generate micro-mass cultures, 5 µL droplets of the cell suspension were placed in the center of 10 wells of a 96-well plate (cell culture plates, 96-well, VWR®, Suwanee, Atlanta, GA, USA) to induce chondrogenic differentiation. The plate was maintained under standard conditions for 2 h. After this time, chondrogenic differentiation medium (StemPro® Chon-

drogenesis Differentiation Kit, Gibco®, Waltham, MA, USA) was added to 8 wells; the other 2 wells were considered to be controls and to these, the standard culture medium was added. Following the manufacturer's instructions, the media were replaced every 3–4 days and cells maintained in differentiation for 14 days. At the end of this period, the Alcian blue staining, pH 2.5, protocol was performed (Alcian Blue 8GX, Sigma-Aldrich®, St. Louis, MO, USA). The culture differentiation medium was removed, and the wells were gently washed with PBS. The cells were fixed with 4% formaldehyde for 20 min at room temperature, and the wells were washed 3 additional times with PBS. Alcian blue solution was added to each well and the plate incubated for 30 min at room temperature. Then, the Alcian blue was discarded, and the wells were rinsed 3 times with 3% acetic acid (v/v). For neutralization of acidity and for visualization by inverted phase contrast microscopy, distilled water was added to all wells. The aim of this assay was the identification of chondrogenic aggregates and their coloration in blue due to the exposure to Alcian blue solution.

Osteogenic Differentiation and Alizarin Red Staining

For osteogenic differentiation, 8×10^3 cells/cm^2 were seeded into the wells of a 12-well plate. The plate was maintained under standard conditions for 4 days. After this period, the culture medium of 10 wells was replaced by complete osteogenic differentiation medium (StemPro® Osteogenic Differentiation Kit, Gibco®, Waltham, MA, USA), and 2 wells were used as controls and maintained with the standard culture medium. Following the manufacturer's instructions, the media were replaced every 3–4 days and the cells maintained in differentiation for 21 days. At the end of this period, the alizarin red s staining protocol was performed using a commercial solution (alizarin red staining solution, Milllipore®, Burlington, MA, USA). The culture differentiation medium was removed, and the wells were gently washed with PBS. The cells were fixed with 4% formaldehyde for 30 min at room temperature, and the wells were washed twice with distilled water. One ml of 40 mM of alizarin red solution was added to each well and the plate incubated for 30 min. Then, the alizarin red solution was discarded, and the wells were rinsed 3 times with distilled water until the supernatant became clear. For visualization by inverted phase contrast microscopy, PBS was added to all the wells. The aim of this assay was to identify calcium-containing osteocytes stained in red after exposure to alizarin red solution.

2.6.2. Karyotype Analysis

The eSM-MSCs in two different passages (P4 and P7) were submitted to cytogenetic analysis to determine the genetic stability in terms of chromosome number and occurrence of neoplastic changes. For both passages, 70–80% confluence was reached. Then, the culture medium was changed and supplemented with 10 µg/mL colcemid solution (KaryoMAX® Colcemid™ Solution, Gibco®, Waltham, MA, USA). After 4 h, the eSM-MSCs were collected and resuspended in 8 mL of 0.075 M KCl solution, followed by incubation under standard conditions for 15 min. After centrifugation (1700 rpm), 8 mL of ice-cold fixative comprising methanol and glacial acetic at a proportion of 3:1 was added and mixed. Afterwards, the cells were centrifuged again. Three fixation rounds were carried out. After the last centrifugation, the suspension of eSM-MSCs was spread over glass slides. A karyotype analysis was performed by one scorer on Giemsa-stained cells. For the different passages, a specific number of cells in metaphase were evaluated depending on the number of cells with a normal karyotype identified, guaranteeing a better representation of the population under study.

2.6.3. Secretome Cell Conditioned Medium (CM) Analysis

The eSM-MSCs were harvested from equine synovial membrane and maintained in culture, as previously described. The cells in P4 were subjected to an analysis of their conditioned medium (CM) 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.

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. After collection, it was centrifuged for 10 min at 1600 rpm, and 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 to complete drying. Finally, the upper compartment of the concentrator tubes was filled with plain CM (1 × concentration) and subjected to new centrifugation cycles, 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). The list of searched biomarkers includes 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 (GRO/KC). All samples were analyzed in duplicate.

2.6.4. Immunohistochemistry

Early passages of eSM-MSCs-P0 and -P3 were maintained in culture until a confluence of 70–80% was reached, and then enzymatic detachment was performed with 0.25% trypsin-EDTA solution. A cytoblock was performed fixing the cells with Sure Thin® (Statlab®, Gerwig Ln Columbia, Columbia, MD, USA). Consecutive sections were cut at 2 µm, deparaffinized, hydrated, and submitted to immunohistochemical analysis using the Novolink™ Polymer Detection Systems (Leica Biosystems®, Vista, CA, USA) kit, according to the manufacturer's instructions. Information regarding the primary antibodies and antigen retrieval recovery methods used in this study is summarized in Table 3.

The antibodies were selected to confirm the pluripotent and mesenchymal origin of the eSM-MSCs' octamer-binding transcription factor 4 (OCT4), homeobox protein (NANOG), proto-oncogene receptor tyrosine kinase or stem cell factor receptor (c-kit), synovial origin (lysozyme), and non-epithelial origin histogenesis (vimentin). Additionally, pan-cytokeratin (AE1 and AE3), synaptophysin, CD31, and glial fibrillary acidic protein (GFAP) were used to confirm there were no vascular, epithelial, neuronal, and neuroendocrine origins of cells, respectively. For each antibody, appropriate negative and positive controls were included, and all primary antibodies were incubated overnight.

The final step consisted of microscopic cell observation, evaluation, and photograph using the microscope Eclipse E600 (Nikon®, Tokyo, Japan) and the software Imaging Software NIS-Elements F Ver4.30.01 (Laboratory Imaging®, prague, mmun republic). A semi-quantitative score was used for mmunoexpression evaluation, consisting of the percentage of labeled cells (<5%, 5–80%, and >80%) and labeling intensity (0, negative; +, weak; ++, moderate; and +++, strong). Immunoreactivity was considered positive when distinct nuclear and cytoplasmic staining was recognized in at least 5% of the cells.

Table 3. List of antibodies investigated, dilutions, and antigen retrieval methods applied in the immunohistochemical analysis. Oct 4—Octamer-binding transcription factor 4; NANOG—Homeobox protein NANOG; GFAP—Anti-Glial Fibrillary Acidic Protein; CD31—Platelet endothelial cell adhesion molecule.

Marker	Type/Clone	Supplier	Dilution/ Incubation Period	Antigen Unmasking	Positive Control	Cells of Interest	Reference
OCT4	Polyclonal	Abcam	1/100 ON	RS/WB	Canine mast cell tumor	Stem cells	Ab18976
NANOG	Clone Mab	ABGENT	1/10 ON	RS/WB	Canine testicular carcinoma	Stem cells	AM1486b
c-Kit (CD117)	Polyclonal	Dako Denmark	1/450 ON	RS/WB	Canine mast cell tumor	Stem cells	A4502
Lysozyme	Polyclonal	Dako Denmark	1/400 ON	RS/WB	Canine synovial membrane	Synovial cells	A0099
Vimentin	Clone V9	Dako Denmark	1/500 ON	RS/WB	Canine mammary gland	Non-epithelial cells	M0725
Pan-cytokeratin	Cocktail AE1/AE3	Thermo Scientific	1/300 ON	RS/WB	Canine mammary gland	Epithelial cells	M3-343-P1
GFAP	Polyclonal	Merck Millipore	1/2000 ON	RS/WB	Mouse brain tissue	Neuronal cells	AB5804
Sinaptophysine	Clone SP11	Thermo Scientific	1/150 ON	RS/WB	Mouse brain tissue	Neuronal cells	RM-9111-S
CD31	Clone JC70A	Dako Denmark	1/50 ON	Pepsine	Canine spleen	Platelet endothelial cells	M0823

2.7. eSM-MSC Solution Preparation

The eSM-MSC solution for local clinical application in the 16 equine patients, was a combination of allogenic eSM-MSCs suspended in autologous serum. Prior to preparation of the final therapeutic combination, autologous serum was isolated from whole blood. Then, 10 mL samples of whole blood were collected into dry blood collection tubes, and after clotting, they were centrifuged at 2300 rpm for 10 min and their supernatant (serum) collected and transferred to a 15 mL falcon. Then, the serum was inactivated through a water bath at 56 °C for 20 min followed by cooling on ice. Finally, the serum was centrifuged and filtered using a 0.22 µm syringe filter and stored at −20 °C until further use. Cryopreserved P3 eSM-MSC batches were thawed in a 37 °C water bath, and the content was transferred to a 10 mL tube with autologous serum and slowly diluted, followed by the addition of sterile DPBS until reaching 10 mL. Then, the mixture was centrifuged at 1600 rpm for 10 min. The supernatant was discarded, and the cell pellet was re-suspended in a mixture of autologous serum at a ratio of 0.8:1. Cell counting and viability were determined by using the trypan blue exclusion dye assay (Invitrogen ™, Waltham, MA, USA) using an automatic counter (Countess II FL Automated Cell Counter, Thermo Fisher Scientific®, Waltham, MA, USA). Then, the cell number was adjusted to 5×10^6 cells/mL, and then 2 mL of the solution of eSM-MSCs suspended in autologous serum was transferred to a perforable capped vial and preserved on ice until the time of administration.

2.8. Treatment Protocol

Twenty structures, tendons and ligaments, were treated with a mixture of allogenic eSM-MSCs and autologous serum. The same treatment protocol was used in every case. All equine patients were submitted to 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 s followed by trot). Lameness was evaluated at a walk and a trot on hard surface and scored on a scale from 0 to 5, according to the AAEP parameters. Complementary diagnostic exams included regional nerve blocks (to identify the pain area), radiographs, and ultrasound image as reported in other studies [21,24,25,27–32].

Following the assumptions of the exclusion criteria, the horses did not receive any treatment before or after the administration of the therapy protocol. In the case of adverse events occurring, such as inflammatory/anaphylactic reactions or infections, the horses should be immediately evaluated and treated with anti-inflammatories or antibiotics, in accordance with their clinical status. The equine patients were monitored in the 48 h after treatment and any occurrences were registered. Following the treatment, the equine patients were assessed periodically to control the equine patient's healing evolution and to provide valid comparative data among equine patients within the same study group. Table 4 presents the lesion type casuistic.

Table 4. Lesion type casuistic.

Lesion Type	No. Clinical Cases	Total Number (2019)
Tendonitis	16	20
Desmitis	4	

2.8.1. Intralesional eSM-MSC Injection

Selected horses were sedated with detomidine (0.02 mg/kg), trichotomized, a regional nerve block was performed with lidocaine 2% (20 mg/mL, 2 mL/point), and the surgical skin was disinfected with chlorohexidine and alcohol. The therapeutic combination was aspired to a 2 mL syringe and homogenized, ultrasound was used to identify the lesion site, and an ultrasound guided injection was performed at the lesion over three different points. Finally, a bandage was applied to the limb. All equine patients were injected with phenylbutazone (2.2 mg/kg, IV, SID) at the end of the treatment. The established protocol included a second eSM-MSC administration 15 days after the first treatment using the same protocol.

2.8.2. Clinical Evaluation—Serial Evaluations

Tissue regeneration was estimated through a lameness evaluation, pain to pressure test, limb inflammation, sensitivity, and ultrasound image (reduction of hypoechoic area and fiber alignment). Lesion ultrasonographic evaluations were performed using a 7.5 MHz linear transductor probe (Sonoscape A5®, Shenzhen New Industries Biomedical Engineering Co Ltd., Shenzhen, China). For each assessment, a complete examination of the structure was conducted by means of longitudinal and transverse scans. The obtained images were evaluated at each examination for two parameters: lesion echogenicity and lesion longitudinal fiber alignment (FA). The contralateral healthy limb was used as comparison. The evaluation was performed on the treatment day (Day 1) as well as on Days 15, 30, and 45 post-treatments, as presented in Figure 3. According to the classification proposed by Guest et al., this is a short term period study [33].

The rehabilitation program consisted of an exercise-controlled program with stall confinement and increasing the amount of time for exercise. Early mobilization included weight-bearing activities, strengthening, and flexibility, and stall rest alone was used as infrequently as possible, as presented on Table 5 [34–38]. Regular ultrasound evaluations were also performed.

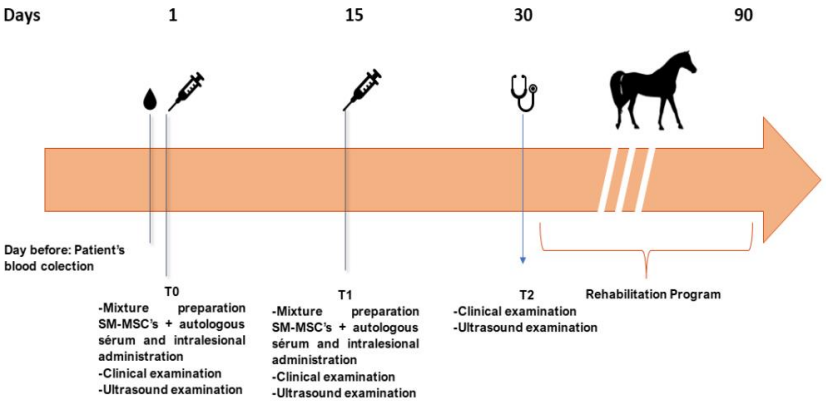

Figure 3. Timeline of the eSM-MSC treatment protocol and rehabilitation program. The day before the first treatment (T0), blood from the equine patient was collected to prepare autologous serum. At T0, the mixture of autologous serum and eSM-MSCs was injected intralesionally after a clinical and ultrasound examination. After 15 days, the same procedure was repeated. At day 30 (T2), a clinical and ultrasound examination was performed and if a favorable outcome was considered, the horse progressed to a physical rehabilitation program. During the physical rehabilitation program, the equine patient was also re-evaluated at days 60 and 90.

Table 5. Physical rehabilitation program. After eSM-MSC treatment, all horses were submitted to a rehabilitation program consisting of two days of box rest followed by 13 days of 10 min of hand walking. The bandage applied on treatment day was removed 24 h after treatment. At Day 15, the second treatment was performed followed by another 15 days of rehabilitation, until Day 30. Between Days 30 and 45, the work consisted of 20 min hand walking; between Days 45 and 60, the work was 30 min of hand walking; between Days 60 and 75, the work was 30 min of hand walking plus 5 min trotting; and finally, between Days 75 and 90, each horse was submitted to 30 min of hand walking plus 10 min of trotting. After this, the horses could return to full work.

Week	Exercise
0–2	Stall confinement, 2 days Hand walking, 10 min Day 15, new treatment
3–4	Stall confinement, 2 days Hand walking, 10 min VET-CHECK on Day 30
5	Hand walking, 15 min
6	Hand walking, 20 min VET-CHECK on Day 45
7	Hand walking, 25 min
8	Hand walking, 30 min VET-CHECK on Day 60
9–10	Hand walking, 30 min + 5 min trotting
11–12	Hand walking, 30 min + 10 min trotting VET-CHECK on Day 90

3. Results

3.1. eSM-MSC Isolation

eSM-MSCs were successfully isolated from equine synovial membrane samples and the average total number of cells isolated from the samples was 1.2×10^5 and 5.6×10^5 at Days 6 and 11, respectively, and expanded from the donor. Cells were observed radiating from the explants and those identified in culture showed clear plastic adherence and mostly fibroblast-like morphology, an essential feature to characterize cells as MSCs (Figure 4a,b).

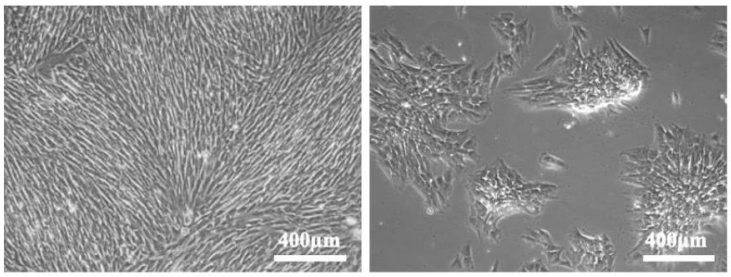

Figure 4. eSM-MSCs in culture, isolated through enzymatic digestion: (**a**) Passage 0 (P0); (**b**) Passage 1 (P1). Plastic adherence, monolayer, and fibroblast-like shape of eSM-MSCs may be observed. Magnification, 100×.

3.2. eSM-MSC Characterization

3.2.1. Tri-lineage Differentiation

Tri-lineage differentiation was confirmed (Figure 5).

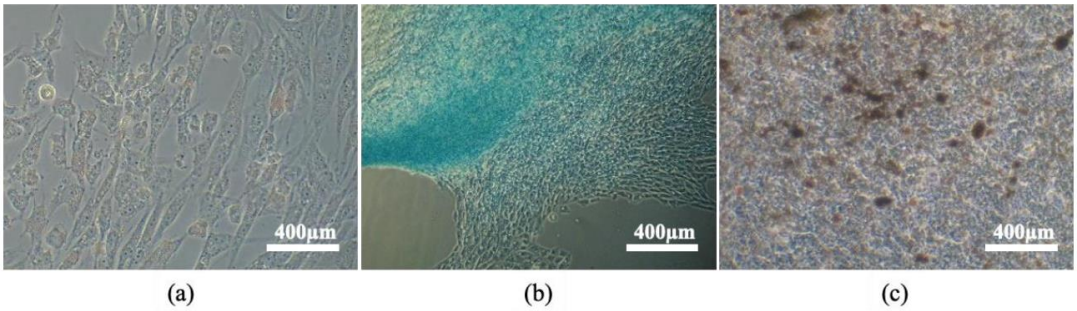

Figure 5. Tri-lineage differentiation: (**a**) eSM-MSC's adipogenic differentiation, cytoplasmatic lipid vacuoles stained in red (Oil red staining); (**b**) eSM-MSC's chondrogenic differentiation, proteoglycans in extracellular matrix stained in blue (Alcian blue staining); (**c**) eSM-MSC's osteogenic differentiation, extracellular calcium deposits stained in red (alizarin red staining). Magnification 100×.

Adipogenic Differentiation—Oil Red O Staining

Adipogenic differentiation was confirmed by the presence of large red stained lipid vacuoles in the cytoplasm due to exposure of oil red O staining.

Chondrogenic Differentiation—Alcian Blue Staining

Chondrogenic differentiation was confirmed by the presence of proteoglycans' marked deposition in the extracellular matrix which stained blue, confirming the presence of chondrogenic aggregates.

Osteogenic Differentiation—Alizarin Red Staining

Osteogenic differentiation was demonstrated by the presence of extracellular calcium deposits stained red by alizarin red solution, which dyes chelate complexes with calcium.

3.2.2. Karyotype Analysis

The cytogenetic analysis revealed the presence of 36% normal cells in P4 and 32% normal cells in P7. Tetraploidy was present in 4% of P4 cells and 8% of P7 cells. Aneuploidy represented 60% of the cells in both passages, hypoploidy being the most representative (56%), as shown at Table 6 and Figure 6.

Table 6. Cytogenetic analysis in Passages 4 and 7 (P4 and P7). Percentage of normal cells, tetraploid cells, and aneuploid cells.

P4	Cytogenetic Analysis	P7
36%	Normal cells 64, XY	32%
4%	Tetraploid cells 128 XXYY	8%
60%	Aneuploid cells:	60%
56%	Hipoploidy 54–63	56%
4%	Hiperploidy 71	4%

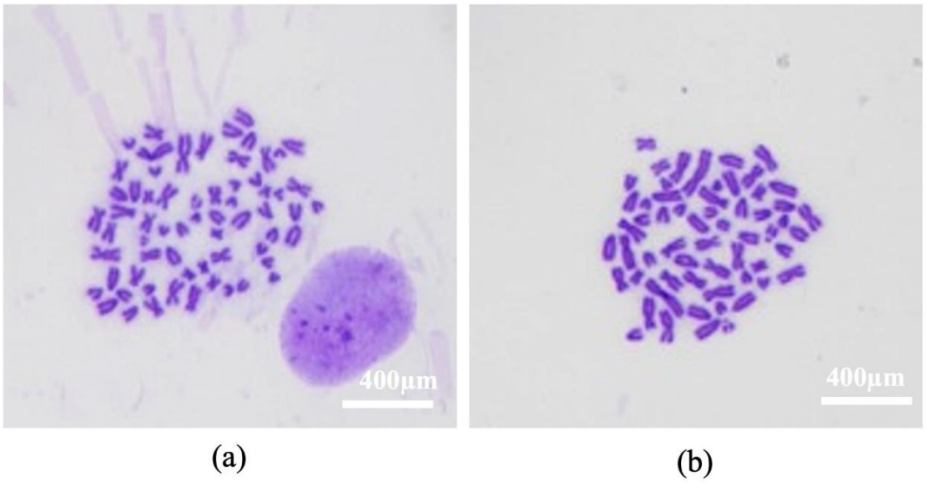

Figure 6. Karyotype. Images of eSM-MSC cytogenetic analysis Passage 7 (P7): (**a**) Normal karyotype, 64 chromosomes, XY; (**b**) hypoploid cell, 59 chromosomes, 3 acro and 2 submeta. Magnification 100×.

3.2.3. Secretome Analysis

The analysis of CM revealed the production and secretion of several factors with immunomodulatory functions, capable of intervening beneficially in tissue regeneration. The results of the eSM-MSC CM analysis are shown in Figure 7. Seven biomarkers were identified: keratinocyte chemoattractant/growth regulated oncogene (KC/GRO), monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6), fibroblast growth factor (FGF-2), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), and interleukin-8 (IL-8). The most expressive were KC/GRO and MCP-1.

3.2.4. Immunohistochemistry

The eSM-MSCs showed strong expressions of OCT4/NANOG, vimentin, and lysozyme which confirmed marked stem cells, non-epithelial cells, and synovial cells, respectively; weak expression of GFAP; and no expression of CD31, synaptophysin, and pan-cytokeratin, as seen in Figure 8, which confirmed no vascular, neuronal, and epithelial origins of cells. Except for GFAP, in which a smaller number of cells exhibited weaker cytoplasmic immunolabeling in P3 as compared with in passage P0, there was preservation of immunoexpression of all the antibodies between passages P0 and P3. The combination of the positive and negative expressions of these different markers confirmed the expected mesenchymal origin of the cells. Figure 8 presents the immunolabeling of the eSM-MSCs.

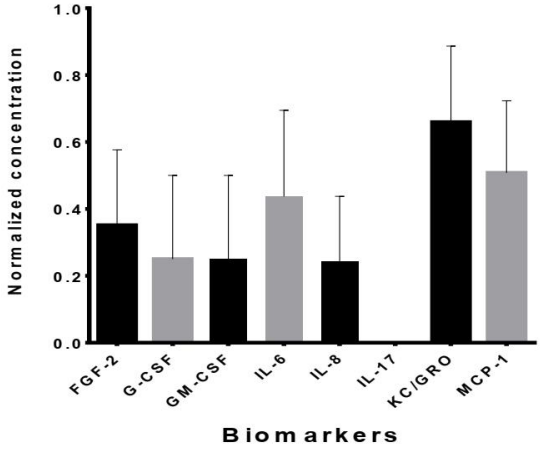

Figure 7. Biomarkers, normalized concentration of each biomarker in the conditioned medium of eSM-MSCs P4 (mean ± SEM).

3.3. Treatment Results

No horse had any adverse event that required study cessation, unplanned procedures, or additional treatments. All intra-tendinous injections and follow-up procedures had no adverse reactions (inflammation, infection, deterioration of the lesion, increased lameness), as shown by Godwin et al. (2012) [39]. No horse had abnormalities identified on the weeks following the injection.

Tendon/ligament regeneration occurred in a time frame of less than 30 days in 80% of the cases and between 30–90 days in 20% of the cases. In this study, eight horses had a lesion on the right front limb, six horses had a lesion on the left front limb, and two horses had a lesion on the right hind limb. There were 14 acute cases and two chronic cases. Chronic cases were diagnosed 6 months before our approach.

After Day 90, meaning they had completed the proposed rehabilitation physical program, the horses started cantering and started to return to their usual work plan. By Day 120 post the first treatment, 87.5% of the horses were back to full work, with the exception of the 12.5% who needed another 30 days to return to full work.

All horses returned to the same level of sport activity they had before injury. Tables 2 and 7 summarize the recovery progress, with the respective ultrasound images in Figures 9 and 10. At Day 30, the group that fully recovered demonstrated both a fulfilled ultrasound cross-sectional area and good fiber alignment. There was also no evidence of pain and lameness. Below, transversal and longitudinal ultrasound images of four cases on Day 1 and on Day 30 are presented. After the eSM-MSC treatment, all horses were submitted to a rehabilitation program, as explained in Table 4.

Radiograph exams were performed to rule out the presence of other associated pathologies and regional nerve blocks were performed to better localize the injured region originating the pain.

Ultrasound images at Day 1 and at Day 30 clearly illustrate the evolution of tendon regeneration. Changes in echogenicity, fiber alignment, and cross-sectional area are evident, as seen in Figure 10.

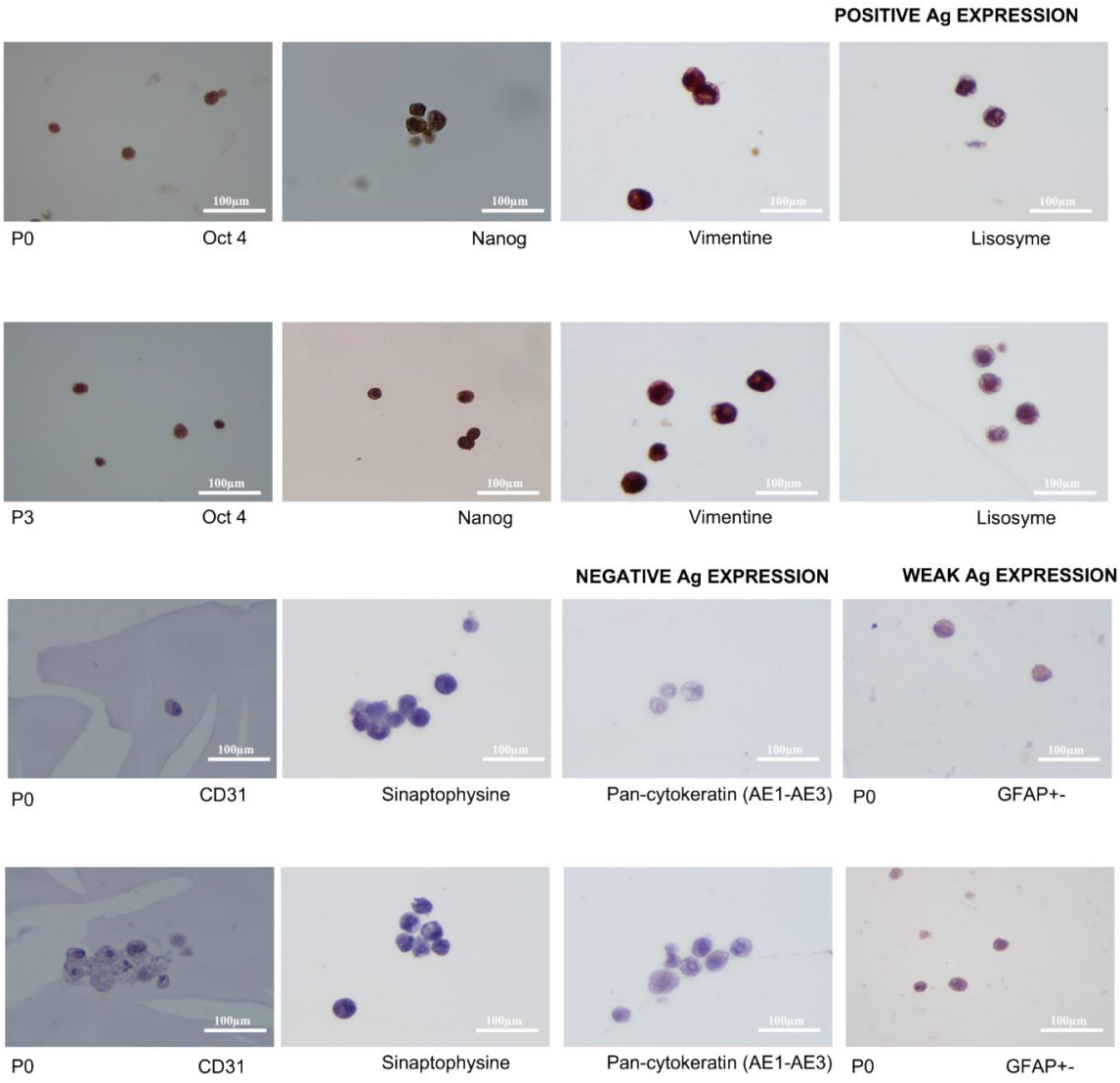

Figure 8. Immunolabeling of eSM-MSCs P0 and P3. Magnification 600×. Images present positive Ag expression of OCT4 and NANOG confirming stem cells, positive expression of vimentin confirming non-epithelial cells, and positive expression of lysozyme confirming synovial cells. Positive expression was revealed by cytoplasmatic staining of the cells. CD31, synaptophysin and pan-cytokeratin had negative expressions, did not stain, and confirmed no vascular, neuronal, or epithelial origins of cells. GFAP represents a neuronal origin of cells and had a weak expression in P0, which reduced in P3. Magnification 600×.

Table 7. Ultrasonographic lesion characterization at Days 1, 15, and 30. Equine patient identification, structure affected, lesion ultrasonographic location, cross-sectional area, and longitudinal fiber pattern are characterized. Assessment outcome is also evaluated. Affected structure superficial digital flexor tendon (SDFT), deep digital flexor tendon (DDFT), suspensory ligament (SL), and left branch (LB). Lesion ultrasonographic location (zones 1A-1B, 2A-2B, 3A-3B) [27], cross-sectional area % (0, 0%; 1, <25%; 2, >25–50%; 3, >50–75%; 4, >75%), longitudinal fiber pattern (0, 0%; 1, <25%; 2, >25–50%; 3, >50–75%; 4, >75%) [27], and assessment outcome (full function, acceptable function, and unacceptable function) [33].

Patient ID	Day	Structure	Location	Cross-Sectional Area	Longitudinal Fiber Pattern (%)	Assessment Outcome
1	1	SDFT	1A-1B	1	1	
		DDFT	1A-1B	1	1	
	15	SDFT	1A-1B	1	1	
		DDFT	1A-1B	1	1	
	30	SDFT	1A-1B	0	0	
		DDFT	1A-1B	0	0	Full function
2	1	SDFT	1A-1B	1	1	
	15	SDFT	1A-1B	1	1	
	30	SDFT	1A-1B	0	0	Full function
3	1	SDFT	1A-1B	1	1	
	15	SDFT	1A-1B	1	1	
	30	SDFT	1A-1B	0	0	Full function
4	1	SDFT	1A-1B	2	2	
	15	SDFT	1A-1B	2	2	
	30	SDFT	1A-1B	0	0	Full function
5	1	LB SL	3A-3B	2	2	
	15	LB SL	3A-3B	2	2	
	30	LB SL	3A-3B	0	0	Full function
6	1	SDFT	2A-2B	2	2	
		DDFT	2A-2B	2	2	
		SL	2A-2B	2	2	
	15	SDFT	2A-2B	1	1	
		DDFT	2A-2B	1	1	
		SL	2A-2B	2	2	
	30	SDFT	2A-2B	1	1	Unacceptable function. Only at day 90.
		DDFT	2A-2B	1	1	
		LS	2A-2B	1	1	
7	1	SDFT	2A-2B	3	3	
	15	SDFT	2A-2B	3	3	
	30	SDFT	2A-2B	2	2	Unacceptable function.
8	1	SDFT	2A-2B	2	3	
	15	SDFT	2A-2B	2	2	
	30	SDFT	2A-2B	0	0	Full function
9	1	SDFT	2A-2B	2	1	
	15	SDFT	2A-2B	1	1	
	30	SDFT	2A-2B	0	0	Full function
10	1	SDFT	1A-1B	3	3	
	15	SDFT	1A-1B	2	2	
	30	SDFT	1A-1B	0	0	Full Function
11	1	SDFT	1A-1B	2	2	
	15	SDFT	1A-1B	2	2	
	30	SDFT	1A-1B	0	0	Full function
12	1	SDFT	2A-2B	1	1	
	15	SDFT	2A-2B	1	1	
	30	SDFT	2A-2B	0	0	Full function
13	1	SL	1A-1B	2	2	
	15	SL	1A-1B	1	1	
	30	SL	1A-1B	0	0	Full function
14	1	SDFT	2A-2B	1	1	
	15	SDFT	2A-2B	1	1	
	30	SDFT	2A-2B	0	0	Full function
15	1	SDFT	2A-2B	2	2	
	15	SDFT	2A-2B	2	2	
	30	SDFT	2A-2B	0	0	Full function
16	1	SDFT	2A-2B	2	2	
	15	SDFT	2A-2B	2	2	
	30	SDFT	2A-2B	0	0	Full function

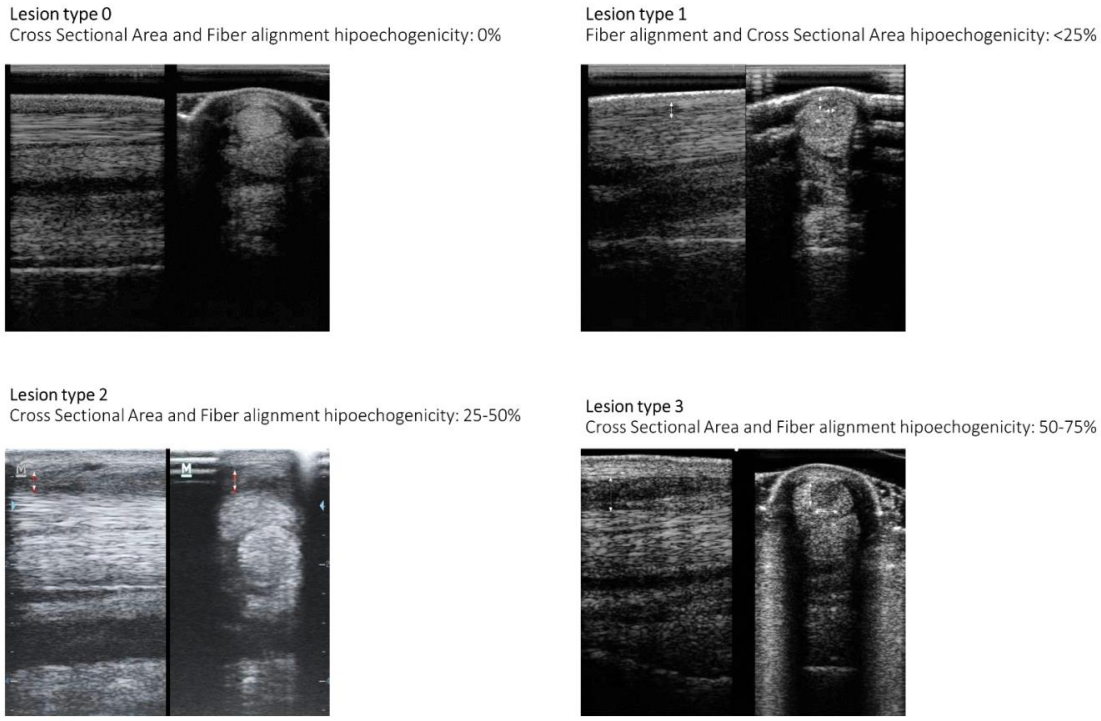

Figure 9. Illustration of ultrasonographic lesion characterization summarized in Table 7. Longitudinal fiber alignment and cross-sectional area echogenicity loss is presented [27].

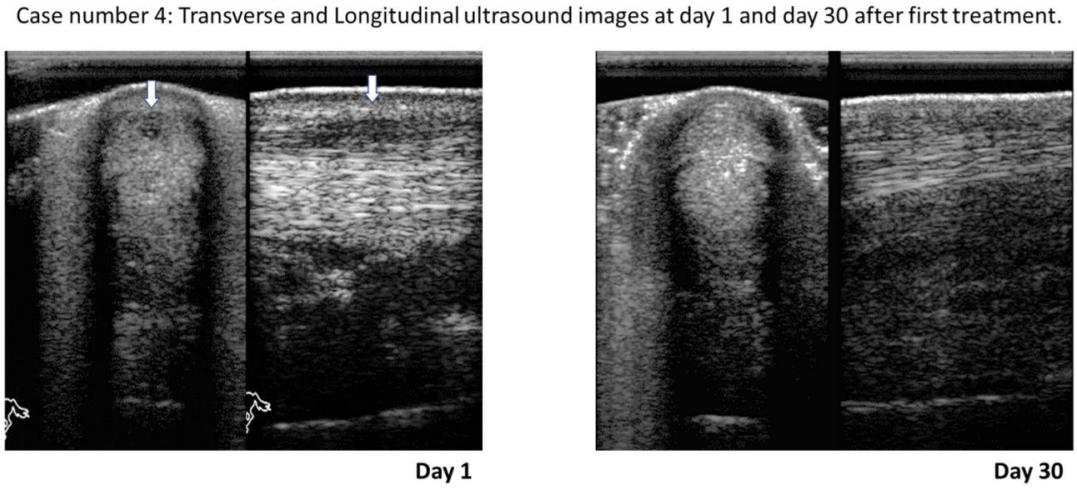

Figure 10. *Cont.*

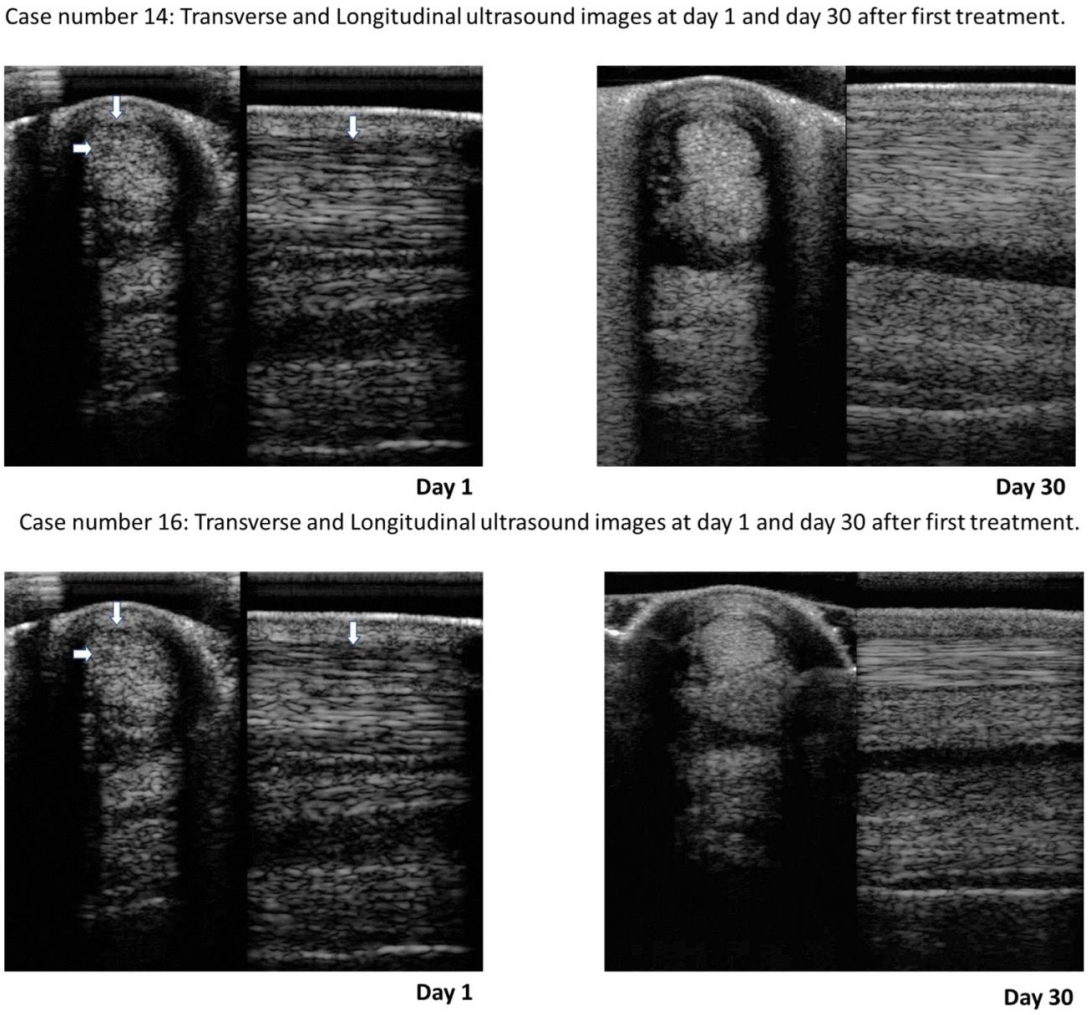

Figure 10. Ultrasound images of Case numbers 4, 14, and 16 that represent the clinical cases concerning superficial digital flexor tendon (SDFT). Transversal and longitudinal images at Day 1 and at Day 30 after first treatment with eSM-MSCs. These ultrasounds are representative of the cases and very illustrative of good fiber alignment and cross-sectional area reduction, evidencing tissue regeneration.

4. Discussion

Recently, eSM-MSCs have become 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, tendons, and ligaments. Regarding the collection, isolation, expansion, freezing, and thawing protocols used in this clinical trial, it was possible to use these cells in equine tendon regenerative treatments. The full characterization of eSM-MSCs presents a significant challenge since eSM-MSCs are not as well studied as MSCs from other species, namely human MSCs. However, in this study, their stemness and origins were confirmed through different processes: trilineage differentiation, karyotype, secretome, and immunohistochemistry. All the SM-MSC cultures presented monolayer culture, plastic adherence capacity, and fibroblast-like shape [40–43], accomplishing some of the minimal criteria defined by ISCT. Successful osteogenic, chondrogenic, and adipogenic differentiation

was also demonstrated. De Bari et al. [9] were the first group of researchers to isolate MSCs from synovial tissues.

The karyotype presented some genomic variations when the number of passages was increased. That was consistent with some studies regarding genomic variations along cell passages [44–48]. DNA replication is a critical event for timely genome duplication. Errors in replication lead to genomic instability across evolution [49]. Prieto Gonzalez et al. considered that genomic instability, incurred during the process of stem cell isolation, culture expansion, and reprogramming, might be the most critical point of a stem cell-based therapeutic approach as a viable option from the clinical perspective [50]. Peterson et al. highlighted that there was very little evidence linking genomic abnormalities, for example, in human pluripotent stem cells (hPSCs) with tumorigeneses [44]. The frequency and effects of variations have increased with the development of even more sensitive methods for detecting genomic variation [45].

As reported by Simona Neri, the interpretation of genetic instability and senescence of cultured MSCs is controversial, but the increasing incidence of genetic alterations at advanced culture times clearly indicates that few culture passages correspond to a reduced chance to harbor dangerous alterations. Therefore, prudent behavior is desirable with a reduction in culture times as much as possible to avoid safety concerns [51]. More studies must be performed in this area.

During the last decade, it has been shown that the therapeutic effectiveness of MSCs is due mainly to the release of paracrine factors, namely CM, composed of soluble (cytokines, chemokines, and growth factors) and nonsoluble factors (extracellular vesicles) that are primarily secreted in the extracellular space by stem cells [52]. CM's paracrine signaling can be considered to be the primary mechanism by which MSCs contribute to the healing process, and therefore, their study has become an interesting subject [53,54].

In our study, eSM-MSCs revealed a CM with a high level of KC/GRO, MCP-1, Il-6, FGF-2, G-CSF, GM-CSF, and IL-8. This highlights the intense activity of fibroblasts, producing KC/GRO that is chemotaxic for neutrophils during inflammation. MCP-1 is essential for reperfusion and the successful completion of musculoskeletal tissue after an ischemic injury [55]. Macrophages are tissue resident cells involved in tissue regeneration along with their inflammatory and infection responses [56]. IL-6 is a proinflammatory and angiogenic interleukin capable of increasing the expression of growth factors; reactivating, for example, intrinsic growth programs of neurons; promoting axonal regrowth; and creating a link between inflammation and tissue regeneration [57,58]. FGF-2 is a recognized GF responsible for proliferation of tenogenic stem cells. FGF-2 signaling has been reported to produce a tendon progenitor population that expressed scleraxis during somite development [59]. FGF-2 plays a crucial role in cell proliferation and collagen production, becoming a useful GF for tissue regeneration by promoting stem cell proliferation [60]. G-CSF is a cytokine that mobilizes bone marrow-derived cells (BM-DCs) to peripheral blood. A study suggested that injection of G-CSF to promote BM-DC release in the target area, i.e., rotator cuff, effectively enhanced rotator cuff healing by promoting tenocyte and cartilage matrix production [61]. Wright et al. presented a study that confirmed skeletal muscle damage, including muscle damage following strenuous exercise, induced an elevation in plasma G-CSF, implicating it as a potential mediator of skeletal muscle repair [62]. Recent human trials have shown the benefits of G-CSF administration as a treatment for neuromuscular diseases, considering that G-CSF affects skeletal muscle, leading to functional improvements [63–68]. GM-CSF is an hematopoietic growth factor with proinflammatory functions [69]. Major sources of GM-CSF are T and B cells, monocyte/macrophage endothelial cells, and fibroblasts. Neutrophils, eosinophils, epithelial cells, mesothelial cells, Paneth cells, chondrocytes, and tumor cells can also produce GM-CSF [70]. Paredes et al. evidenced that elevated levels of proinflammatory factors such as those found at these cells CM (GM-CSF, G-CSF, Il-6, IL-8 and IL-17), were implicated in the activation of resident tendon cells for effective healing, stimulating tendon cell proliferation [71,72]. IL-8 is one of the major mediators of

inflammatory response and is a potent angiogenic factor. This is similar to IL-6, but IL-8 has a longer half-life [73].

A recent study highlighted that hematopoietic factor promoted tendon healing in aged mouse tendons. Histochemical results demonstrated that vascularization of the injury site was significantly elevated. It was concluded that vascular endothelial growth factor (VEGF) played an important role in decreasing adipocyte accumulation and also improved vascularization of the tendon during aged tendon healing. Active regulation of VEGF may improve the treatment of age-related tendon diseases and tendon injuries [74].

Studies with human BM-MSCs using a human-specific proteome profiler array with different angiogenic factors such as VEGF-A, IL-6, IL-8, platelet-derived growth factor A (PDGF-A), endothelin-1 (ET1), and urokinase plasminogen activator (uPA), which had not been previously reported in the CM of human MSCs, were also identified in an equine array, confirming what we found in this study [75]. This factor has been proposed as a modulator of the different neovascularization stages, through the enhancement of VEGF gene promotor activity [75,76]. Schokry et al. [77] reported that BM-MSC therapies have recovery times of 3–6 months and conservative therapeutic methods allow recovery in 12–18 months without regeneration but with formation of fibrous scar tissue. Retrospectively, no re-injuries of tendons have occurred in horses treated with this new approach, during the study frame time. In the literature [78], Smith et al. referred to a low percentage re-injury rate of 27% for SFD tendonitis treated with bone marrow stem cells. Horses returned to "full function" as defined by Cook et al. and modified by Guest et al. [33,79].

A study using a murine osteoarthritis (OA) model demonstrated that an injection of MSCs CM, similarly to injection of MSCs, resulted in early pain reduction and had a protective effect on the development of cartilage damage in a murine OA model, by using the regenerative capacities of the MSCs-secreted factors [80].

Interestingly, the results accumulated so far have provided evidence that veterinary patients affected by naturally occurring diseases should provide more reliable outcomes of cell therapy than laboratory animals, thus, allowing translating potential therapies to the human field. More recently, a cell-free therapy based on MSCs CM has been proposed. Even though there are very few clinical reports to refer to in veterinary medicine, recent acquisitions suggest that MSC-derived products may have major advantages compared to the related cells, for example, they are considered safer and less immunogenic [52]. As evidenced before, eSM-MSC CM factors are able to promote tendon healing by reducing inflammation and fatty infiltration, stimulating cell proliferation and tenogenic differentiation [81].

In this study we used a cell-based therapy instead of CM itself, but we were aware of its effect and potential on cell-based therapies; its advantages and therapeutic effects were the reason why this study was performed.

To better characterize the cells under study, we performed immunohistochemistry assays. The choice of markers was based on a previous work [8] and included several of the criteria used for humans, as determined by the ISCT. Results of our study demonstrated the presence of the embryonic stem cell markers OCT4 and NANOG. Detection of these markers has been previously described by Beltrami et al., in multipotent adult stem cells (HMASC) from human bone marrow [82], as well as, by Riekstina et al., who also demonstrated the presence of these markers in HMASC derived from bone marrow, adipose tissue, heart, and dermis [83]. Greco et al. also evidenced elevated expression of OCT4 in P3 MSCs and hypothesized OCT4 expression could be an indicator of MSC differentiation potential in clinical diagnostics [84]. In equine characterization of synovial fluid and membrane-derived MSCs, Prado et al. also evidenced the presence of NANOG and OCT4 markers [19]. In contrast, Fulber et al. had no positive results for these two markers in equine mesenchymal stem cells of synovial tissues [43]. Vimentin, a mesenchymal stem cell marker, was also detected, suggesting the mesenchymal origin of cells. The presence of lysozyme confirmed the synovial origin of cells, as stated by Fulber et al. [43].

The immunohistochemistry analysis showed the absence of CD31, sinaptophysine, and pan-cytokeratin expressions, confirming no vascular, neuronal and epithelial origins of cells. GFAP was weakly expressed, being less expressed in P3 than in P0 cells. CD31 was performed to investigate the presence of hematopoietic cells in eSM-MSCs. The expression of VEGF was not found, these results being similar to those from Fulber et al., and to other authors that evidenced the absence of hematopoietic markers [43,85]. The absence of neuronal and dermal markers was also consistent with other studies [19,43].

In our clinical trial, we treated mainly early acute lesions; 87.5% of the cases were acute lesions of tendons or ligaments. Therefore, we created a master cell bank of allogenic eSM-MSCs suitable for treatments in early acute phases versus treatments with autologous cells where time of tissue collection, preparation, and cell culture need to be considered. Furthermore, cell harvesting for autologous treatment is an invasive procedure which is unnecessary with this new eSM-MSC solution. The possibility of having a master cell bank enables faster healing of the organ and a quicker return to sport life. Horses spend less time in recovery time and have a regenerated tissue instead of a fibrotic tissue. These are some advantages of the eSM-MSC solution. Another concern is that in the early stages of the lesion there is an inflammatory phase; however, the paracrine factors released by eSM-MSCs also have anti-inflammatory action, reducing inflammation.

Chronic cases represented 12.5% of the cases, involving four structures. Three of the horses recovered in 30 days and one of the horses had a delayed recovery time.

The delayed recovery time in 20% of the structures, meaning 12.5% of the horses, was due to, in Case 6, an increased number of involved structures (more than one tendon or ligament) and a foot conformation abnormality, as the horse had a fetlock hyperextension that was impairing correct tendon healing. This was corrected with special shoeing. Inappropriate rehabilitation program (Case 7) was another cause of delayed recovery time. As soon as the corrective shoeing was performed, ligament regeneration started.

We could also conclude that lameness grade was not directly correlated with lesion cross-sectional area. Horses with ultrasonographic cross-sectional grade 1, 2, and 3 lesions presented lameness grade 4/5, which was observed in 9 of 16 patients. Lameness grade 3/5 was presents in 4 of 16 of equine patients with ultrasonographic cross-sectional grade 1 and 2 lesions. Lameness grade 2/5 was present in 3 of 16 equine patients with ultrasonographic cross-sectional grade 1 lesions.

Kamm et al. (2021) concluded that based on the evidence to date, tendons appear to have improved healing when treated with allogeneic MSCs, and the use of these treatments in equine tendon and ligament lesions is warranted [86]. Colbath et al. (2020) claimed that some of the advantages of using allogenic stem cells include the ability to bank cells and to also reduce the treatment time, to collect MSCs from younger donor animals, and the ability to manipulate banked cells prior to administration [87]. Some of the disadvantages focused on the risk of immunological reactions. However, currently, there are several studies in horses accumulating evidence that allogeneic MSCs may be a safe alternative to autologous MSCs [87]. Nevertheless, the donor's health must always be taken into consideration as well as the donor's age [88].

5. Conclusions

To sum up, this study accomplishes the criteria for reporting veterinary and animal medicine research for MSCs in orthopedic applications [33] and the ISCT perspective on immune assays for MSC's criteria for advanced phase clinical trials [89], confirmed by plastic adherence, tri-lineage differentiation, synovial membrane origin, spindle-shaped cells, as well as proliferative and immune modulatory capacity proven by immunohistochemistry and CM.

From a clinical point of view, the idea of having an allogenic eSM-MSC cell bank is very interesting. Therefore, the possibility of having a universal donor who can provide a large amount of eSM-MSCs, to culture and preserve non-immunogenic cells whose

availability is immediate, allowing a quick and effective therapeutic answer in acute stages of musculoskeletal lesion is the paramount goal of orthopedic medicine.

From a "one-health" perspective, equines play an important role as a model for human musculoskeletal disorders; the high-level analogy between human and equine structures may have a great translational value for both species for future clinical aspects [28,90]. There are significant resemblances between equine SDFT and human Achilles tendon with respect to the size of anatomical structure and load, function (energy store), pathophysiology of tendon injury, and the healing response under activity or traumatic rupture compared to other species [90]. Moreover, considering the result of tendinopathy in equine species which reflects the conditions encountered in humans, the horse is accepted as an appropriate model in this area by the research community and by other authorities such as the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA).

Based on the clinical, ultrasonographic, and performance outcomes identified in the present study, the use of eSM-MSCs together with autologous serum solution has proven its efficiency for tendon and ligament repair and contributes to reduce the recovery period and subsequent rapid return to athletic activity. The therapy was demonstrated to be safe and had no adverse findings. The clinical results and athletic outcomes of the horses were very positive. Comparing our study with others, using for example BM-MSCs, it seems that our new approach has shorter recovery times and fewer re-injuries [39,77]. These results encourage the use of eSM-MSCs and autologous serum for the treatment of tendonitis and desmitis, since they can regenerate tendon and ligament tissue and regain organ function, enhancing the return to competition in excellent time frames.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

AAEP	American Association of Equine Practitioners
AE1/AE3	Pan-cytokeratin
BM-MSC	Bone marrow mesenchymal stem cell
CD	Cluster differentiation
c-Kit	Proto-oncogene receptor tyrosine kinase or stem cell factor receptor
CM	Conditioned medium
cm^2	Square centimeter
Coll-II	Collagen type II
COMP	Cartilage oligomeric matrix protein
CPD	Cumulative population doublings
DMSO	Dimethylsulphoxide
DPBS	Dulbecco's phosphate buffered saline
eSM-MSC	Equine synovial membrane mesenchymal stem cell
FBS	Fetal bovine serum
FGF-2	Basic fibroblast growth factor
G-CSF	Granulocyte colony stimulating factor
GFAP	Glial fibrillary acidic protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
GRO/KC	Human growth-regulated oncogene/keratinocyte chemoattractant
ICBAS-UP	Instituto de Ciências Biomédicas Abel Salazar–Universidade do Porto
IL	Interleukins
ISCT	International Society for Cellular Therapy
IV	Endovenous
kg	Kilogram
MCB	Master cell banks
MCP-1	Monocyte chemoattractant protein-1
mg	milligram
MHC-II	Major histocompatibility complex
MHz	Megahertz
min	Minutes
mL	Milliliter
MSCs	Mesenchymal stem cell
NSAIDs	Nonsteroidal anti-inflamatory drugs
OA	Osteoarthritis
OCT4	Octamer-binding transcription factor 4
ORBEA	Organismo Responsável pelo Bem-estar Animal
P	Passage
PBS	Phosphate-buffered saline
PRP	Platelet-rich plasma
RT-PCR	Reverse transcription polymerase chain reaction
SID	Once a day
SM-MSC	Synovial membrane mesenchymal stem cell
UDP	Uridine diphosphate
UDPGD	Uridine diphosphate glucose dehydrogenase
VEGF-R1	Vascular endothelial growth factor
WCB	Working cell banks

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GRAPHICAL PAPER II

2 Allogenic SM-MSCs for the treatment of equine tendinopathies and desmopathies

A eSM-MSCs + Autologous serum

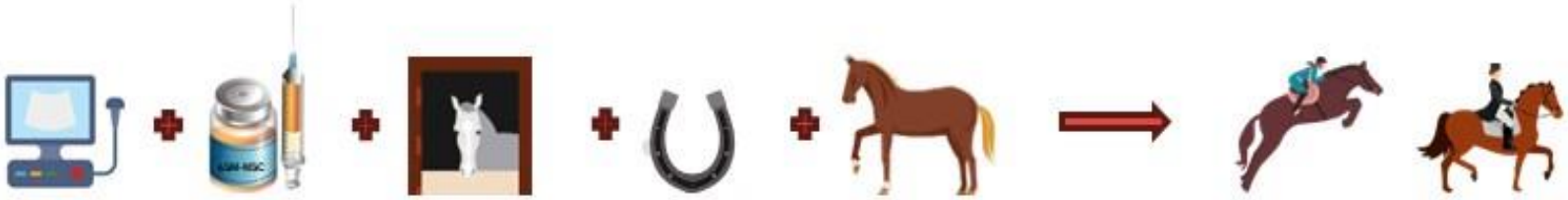
16 Tendons

4 Ligaments treated

- Structures: SDFT, DDFT, SL, LB SL
- Acute and chronic lesions
- Discipline: Show jumping, Dressage and Leisure horses

Treatment protocol

- 2 doses of $1x10^7$ eSM-MSCs, 2 ml, intralesional,15 days apart
- 90 days of physical rehabilitation program.
- Corrective shoeing
- Clinical and imaging assessments



- Impressive clinical and imaging recovery in a short time frame.
- Early return to work with no relapses.
- Easy to do procedure
- Treatment in acute lesion phase

80% recovered in 30 days

20% recovered in 90 days

CHAPTER III

Treatment of Equine Tarsus Long Medial Collateral Ligament Desmitis with Allogenic Synovial Membrane Mesenchymal Stem/Stromal Cells Enhanced by Umbilical Cord Mesenchymal Stem/Stromal Cell-Derived Conditioned Medium: Proof of Concept

CHAPTER III

- **INTRODUCTION**

- **PAPER III**

Treatment of Equine Tarsus Long Medial Collateral Ligament Desmitis with Allogenic Synovial Membrane Mesenchymal Stem/Stromal Cells Enhanced by Umbilical Cord Mesenchymal Stem/Stromal Cell-Derived Conditioned Medium: Proof of Concept

I. L. Reis, B. Lopes, P. Sousa, A.C. Sousa, M.V. Branquinho, A.R.Caseiro, A. Rêma, I. Briote, C. Mendonça, J.M. Santos, L.M. Atayde, R.D. Alvites, A.C. Maurício, (2024) Animals 14(3), 370, ***doi.org/10.3390/ani14030370***

- **GRAPHICAL PAPER III**

CHAPTER III INTRODUCTION

Equine tarsus pathologies present a challenging puzzle to solve due to the intricate relationship among various structures: synovial structures, bones, bursae, ligaments, tendons, and tendon sheaths. This complexity contributes to a notable incidence of pathology (Clegg, 2003). Tarsocrural desmitis, in particular, stands as a significant cause of severe hindlimb lameness in horses. However, it is frequently underdiagnosed clinically because it is relatively uncommon and carries a guarded prognosis for athletic soundness (Stashak, 2008).

An overview of kinematic and a good knowledge of anatomy are essential for a better understanding of tarsus pathology (Kümmerle & Kummer, 2013) .

Tarsus movement is linked with stifle movement, they work in coordination due to reciprocal apparatus (Nagy, 2016). This reciprocal mechanism is responsible for the long-standing position of equines, keeping their hindlimbs straight without muscular effort (Schuurman et al., 2003). 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.

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 (Schuurman et al., 2003).

When the joint is flexed, the distal limb is flexed and is pulled slightly to one side due to the outward slant of the trochlear ridges. Among the joints in the equine hindlimb, the tibiotarsal joint exhibits the highest range of motion, contributing to approximately 90% of the total motion range, while the three lower joints below account for the remaining 10% (Lohse & Trout, 1984; Schuurman et al., 2003). To limit movement in the distal tarsal bones, a system of collateral ligaments ensures minimal mobility.

There are four collateral ligaments in the tarsus. Long lateral collateral ligament: 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: 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: 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: 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 collateral ligaments exhibit tension during extension and loose during flexion of the tarsocrural joint. Conversely, the short collateral ligaments display a more intricate behaviour throughout the tarsocrural joint's range of motion, with the majority being taut during flexion and lax during extension – **Figure 1** (Kümmerle & Kummer, 2013).

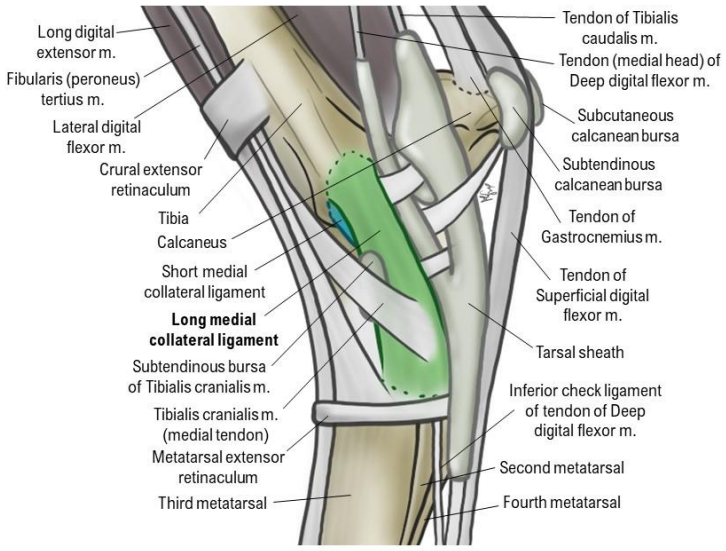


Figure 1. Anatomy of medial aspect of right tarsocrural joint. This image evidences the complexity of the tarsocrural joint structures. Long medial collateral ligament is highlighted in soft green. (Image by Mário Ribeiro)

Intra-articular soft tissue injuries of the tarsus are inadequately documented due to their nonspecific clinical signs, which may manifest as oedema over the affected collateral ligament, effusion within the tarsocrural joint, and mild to non-weight bearing lameness with positive joint flexion (Desmaizières & Cauvin, 2005; Lamb et al., 2012). Collateral ligament injuries of the tarsocrural joint can lead to chronic pain and secondary osteoarthritis due to joint instability, resulting in cartilage damage in affected horses. Tarsal pain accounts for 80% of chronic, low-grade hind limb lameness in horses, with common diagnoses including bone spavin, osteochondrosis, tendon and ligament injuries, and synovial disease (Blaik et al., 2000).

Hemarthrosis, a major source of pain, may also develop and has been documented in both equine and human studies (Barker et al., 2013; Blaik et al., 2000; Clegg, 2003; DYSON, 1986; Garrett, 2022; Judy & Galuppo, 2005; Kawamura et al., 1994; Tokateloff et al., 2011).

Thus, being a difficult lesion to diagnose, when tarsal desmitis occurs, they usually heal through fibrosis, being a recurrent cause of chronic lameness and/or tarsal distension (Blaik et al., 2000).

However, with a comprehensive clinical and imagological examinations, a diagnosis is possible to obtain.

The primary therapeutic goal for horses afflicted with tendonitis and/or desmitis is the restoration of the horse to its previous athletic capacity while minimizing the risk of reinjury. Rehabilitation has traditionally been the basis of therapy following tendon and ligament injuries. However, in recent years, regenerative medicine has emerged as a promising approach to enhance self-healing through different ways: delivery of exogenous cells and bioactive factors and recruitment of endogenous cells (Ortved, 2018). The main goal of these therapies is to promote healing of the tissues thorough their regeneration, resembling native tissues, thereby restoring normal biomechanical function and structure. Ultimately, achieving these objectives would enable horses to perform at their previous athletic levels with a reduced likelihood of experiencing reinjury (Ortved, 2018).

Assuming this paradigm, a product combining equine synovial mesenchymal stromal/stem cells (eSM-MSCs) with umbilical cord mesenchymal stromal/stem cells derived conditioned medium (UC-MSC CM) was created to promote regeneration of musculoskeletal structures, namely a ligamentar structure. This new formulation was developed in order to enhance the previous one with the benefits of UC-MSC CM namely their immune modulatory abilities enabling the universal donor/allogeneic approach. UC-MSC are the responsible for the maternal avoidance of the foetal tissues. Also, they present a high tenogenic, anti-inflammatory and multipotency abilities, being, therefore, very appealing for tendon and ligament regeneration (Bami et al., 2020; Fan et al., 2009; Murata et al., 2014; Nagamura-Inoue & He, 2014).

The main goals of this work were to:

1. **Formulate the therapeutic blend comprising eSM-MSCs with UC-MSC CM** and investigate the UC-MSC secretome concerning interleukins -6 and -8 expression.

2. Identify the **therapeutical potential** of this combination regarding its safety, easiness to use, efficacy and clinical and imagological outcome in ligamentar lesions.
3. Assess the **time** required for **clinical and imaging-based recovery**.

To accomplish our objectives, a long medial collateral ligament desmitis of a young 5-year-old show jumping equine athlete, was treated. The patient clinically and imagological recovered within 60 days and resumed athletic activities at the conclusion of the rehabilitation program. Two years after injury, the horse is jumping and progressing toward a higher athletic level compared to before the injury occurred.

The treatment demonstrated safety, eliciting no adverse reactions, and proved to be easy and simple to administer, as it only requires intra-lesional administration of the therapeutic combination, yielding effective results as evidenced by the patient's rapid recovery. Consequently, this therapeutic combination seems to emerge as a safe and efficacious approach for managing ligamentous injuries in equine patients.

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PAPER III

Treatment of Equine Tarsus Long Medial Collateral Ligament Desmitis with Allogenic Synovial Membrane Mesenchymal Stem/Stromal Cells Enhanced by Umbilical Cord Mesenchymal Stem/Stromal Cell-Derived Conditioned Medium: Proof of Concept.

animals

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Treatment of Equine Tarsus Long Medial Collateral Ligament Desmitis with Allogenic Synovial Membrane Mesenchymal Stem/Stromal Cells Enhanced by Umbilical Cord Mesenchymal Stem/Stromal Cell-Derived Conditioned Medium: Proof of Concept

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Simple Summary: Musculoskeletal injuries in sport horses are relatively common and quite worrisome. Tendon and ligament injuries in sport horses usually result in a long period of time out of competition. Their healing usually results in tissue fibrosis and concomitant loss of elasticity, which, depending on the severity, might prevent the horse's recovery to the same performance levels or even to athletic levels. The continuous development of regenerative medicine offers therapeutical promise. Synovial membrane mesenchymal stem/stromal cells (SM-MSCs) and umbilical cord mesenchymal stem/stromal cells (UC-MSC), as well as their growth factors, have been described as having optimal characteristics for tendon and ligament regeneration. Therefore, a therapeutical combination of SM-MSC and a conditioned medium of UC-MSC was developed, produced, and administered on a tarsal long medial collateral ligament desmitis of a show-jumping horse. The production and application of the orthobiologic therapeutical combination as well as the clinical outcome are presented herein.

Abstract: Horses are high-performance athletes prone to sportive injuries such as tendonitis and desmitis. The formation of fibrous tissue in tendon repair remains a challenge to overcome. This impels regenerative medicine to develop innovative therapies that enhance regeneration, retrieving original tissue properties. Multipotent Mesenchymal Stem/Stromal Cells (MSCs) have been successfully used to develop therapeutic products, as they secrete a variety of bioactive molecules that play a pivotal role in tissue regeneration. These factors are released in culture media for producing a conditioned medium (CM). The aforementioned assumptions led to the formulation of equine synovial

membrane MSCs (eSM-MSCs)—the cellular pool that naturally regenerates joint tissue—combined with a medium enriched in immunomodulatory factors (among other bioactive factors) produced by umbilical cord stroma-derived MSCs (eUC-MSCs) that naturally contribute to suppressing the immune rejection in the maternal–fetal barrier. A description of an equine sport horse diagnosed with acute tarsocrural desmitis and treated with this formulation is presented. Ultrasonographic ligament recovery occurred in a reduced time frame, reducing stoppage time and allowing for the horse's return to unrestricted competition after the completion of a physical rehabilitation program. This study focused on the description of the therapeutic formulation and potential in an equine desmitis treatment using the cells themselves and their secretomes.

Keywords: allogenic; conditioned medium; equine; ligament; mesenchymal stem/stromal cells; MSC-based therapies; orthobiologic; secretome; synovial membrane; umbilical cord

1. Introduction

Tendon and ligament injuries account for a large proportion of a sport horse's wastage and early retirement. Most injuries are overstrained injuries, as these structures 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 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 a decrease in tissue functionality and, therefore, in athletic performance, as well as recurrent reinjuries and lameness. Tarsocrural desmitis is an inflammatory event, causing moderate to severe hindlimb lameness in horses, and it is often clinically underdiagnosed due to unspecific clinical signs such as synovial effusion and minimal radiographic changes [3]. Ultrasonographic examination allows for desmitis diagnosis characterized by increased ligament size, decreased echogenicity, and abnormal fiber pattern. This pathology has a guarded prognosis for athletic soundness [4].

Medial tarsal collateral desmitis has been rarely described in the veterinary literature; however, it is one of the most common soft tissue injuries of the tarsus. Conventional treatments are often referred to in the literature, but the outcome is not very advantageous, since there is a guarded prognosis to return to the same performance level, and osteoarthritis (OA) is usually secondarily associated with these approaches [4–9].

In an attempt to overcome these obstacles, regenerative therapies are gaining increased interest, and the use of multipotent Mesenchymal Stem/Stromal Cells (MSCs) therapy holds immense promise [4–6]. The MSCs are adult multipotent progenitor cells found in many organs and tissues, able to self-renew, to migrate to injury sites (homing), to differentiate into multiple lineages, and to secrete bioactive factors, providing immunomodulation, increasing proliferation and migration of tendon stem/progenitor cells via paracrine signaling, and increasing the regeneration ability of tissues with a poor aptitude [10–13].

Interestingly, in light of the many findings regarding their identity and function, the adequate nomenclature of the MSCs acronym has become itself a field of debate. Classically, the "Mesenchymal Stem Cells" designation, as proposed by the International Society for Cellular Therapy (ISCT) in defining the minimal criteria, has been the most widely accepted [14]. However, other scientific voices currently advocate that these do not represent "true" stem cells, since there is still a lack of indisputable evidence of some critical stemness features (such as the asymmetric cell division and complete lineage renewal). Thus, the term "Mesenchymal Stromal Cell" is proposed to better describe the conventionally isolated populations [15]. Recently, it was proposed to rename it to "Medicinal Signaling Cells" to more accurately reflect the fact that these cells home in on sites of injury or disease and secrete bioactive factors that are immunomodulatory and trophic (regenerative), meaning that these cells make therapeutic drugs in situ, which are medicinal [16]. Therefore, the aim of regenerative stem cell medicine is to regenerate cells

and tissues and to restore their normal structure and function [1,3]. These therapies consist in the administration of MSCs themselves or MSC free products [1,17,18].

The latest studies highlight the importance of the paracrine action of MSCs through the release of soluble and non-soluble factors, primarily secreted in the extracellular space by MSCs, known as the secretome [19] whose paracrine signaling is considered the primary mechanism by which MSCs contribute to healing processes [20,21], avoiding the need for living-cell implantation [22,23]. This spurs the design of MSC-based therapies that do not require cell administration being immediately available for the treatment of acute conditions, with the possibility of being massively produced from commercially available cell lines, avoiding invasive cell collection procedures [11,19,24,25]. Stem-cell-free products have demonstrated preclinical efficacy and safety, as they appear to have non-cytotoxicity, non-mutagenicity, and low immunogenicity. They also overcome the challenge of cell viability maintenance and potency throughout the manufacture, storage, and delivery, maintaining the advantages of therapeutic ability [26]. Several cell-free preparations have shown encouraging outcomes in early-stage clinical trials [27].

Equine synovial membrane and umbilical cord mesenchymal stem cells (SM-MSCs and UC-MSCs), the object of our research, are an interesting alternative cellular and cell-based therapy due to their promising articular, tendon, and ligamentous high regenerative capacity. Additionally, the evaluation of these cells' secretome is important to understand their biological potential and their synergistic action, as MSCs isolated display similar appearances but different biological functions and markers depending on the their origins [27]. Equine SM-MSCs' tenogenic superiority presents them as good candidates for tendon and ligament regeneration [28–30]. Also, it has been proven that UC-MSCs present a superior ability in differentiating into tendon-like lineages and forming a tendon-like matrix, improving the regeneration of these structures [31]. Additionally, the UC-MSC secretome is characterized by the presence of angiogenic factors, reduced levels of metalloproteinases (MMPs), and elevated synthesis of transforming growth factor β 1 (TGF-β1), chemokines, and anti-inflammatory cytokines, with interleukyn-6 (IL-6) being one of the most secreted. These characteristics suggest that the UC-MSC secretome presents the ability to control inflammatory responses [32,33].

The aim of the present research is to evaluate the beneficial effect of a newly developed stem-cell orthobiologic therapeutic combination, consisting of eSM-MSCs and a UC-MSC conditioned medium (CM), in the treatment of equine tarsal ligament desmitis. Herein, a diagnosis and innovative treatment approach of a seven-year-old show-jumping horse, who sustained an injury of the long medial collateral ligament (LMCL) of the right tarsus, is thoroughly described. Additionally, the orthobiologic product preparation and application, as well as the determination of the eSM-MSC and UC-MSC therapeutic potential, using the cells themselves and the cell-derived CM, are demonstrated.

2. Materials and Methods

2.1. Ethics and Regulation

This research was carried out in accordance with "Organismo Responsável pelo Bem Estar Animal" (ORBEA) from ICBAS-UP recommendations and authorization (reference P289/ORBEA/2018). Treatments were performed with permission and signature of informed consent from the patient's legal tutor after a thorough explanation of the procedure itself and possible associated risks, in accordance with the national legislation from the competent authorities.

2.2. Patient Identification and Clinical Evaluation

A seven-year-old showjumper stallion was examined for a complaint of a swollen right tarsus. Upon examination, signs compatible with acute lesion of the right tarsus, such as oedema and significant effusion of the tarsocrural joint, were observed (Figure 1).

Figure 1. Horse's clinical inspection. Evidence of increased volume of the right tarsocrural joint. (**a**) Frontal view and (**b**) medial view.

The animal was subjected to a complete physical and orthopedic examination including dynamic examination, where the evaluation of lameness was performed while walking, trotting, and galloping in a straight line and in circles, on both rough and soft surfaces. Flexion tests of the main joints, followed by trotting in a straight line on a rough surface, were also performed, and lameness was scored on a scale proposed by the American Association of Equine Practitioners (AAEP) (Table 1).

Table 1. Score systems used to assess lameness and response to flexion test [34].

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

On the first assessment day, complementary diagnostic examinations included radiographs and ultrasound images, as reported in other studies [5–7]. The follow-up evaluations of the patient included a physical examination, as well as lameness and ultrasonographic evaluations.

2.3. Complementary Diagnostic Exams

2.3.1. Radiological Examination

Radiological examination (X-ray) of the right tarsocrural joint was performed with a digital system—CareRay Cw series® (CareRay, Suzhou, China), radiological constants: 72 Kv, 0.8 mA. The distance between the X-ray generator (Orange 1060 HF, EcoRay, Seoul, Republic of Korea) and the flat panel was approximately 66 cm. Four standard views—lateromedial, dorsoplantar, dorsolateral-plantaromedial and dorsomedial-plantarolateral—were obtained at patient's first examination.

2.3.2. Ultrasound Examination

Ultrasound examination (U/S) of the right and left tarsocrural joint was performed in longitudinal and transversal scans using a 7.5-MHz linear probe, digital portable ultrasound system—Sonoscape A6® (SonoScape, Shenzhen, China).

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.

2.4. Donor Selection and SM Collection

The equine SM-MSCs' donor was a young and healthy foal (six months' old) who suffered an accident and died. Briefly, the tutor authorized synovial membrane collection from hocks, knees, and fetlocks. Before the procedure, the incisional field was subjected to surgical asepsis with chlorohexidine and alcohol. Sequentially, the skin was incised, the subcutaneous tissue was debrided, the joint capsule was incised and opened, and the synovial membrane was isolated and extracted. The collected tissues were immersed in Dulbecco's phosphate-buffered saline (DPBS—14190-144, Gibco®, Waltham, MA, USA) and transported to the laboratory facility, wrapped in ice packs to maintain refrigeration. These procedures have been previously described [28].

2.5. Equine SM-MSCs' Isolation, Culture, and Characterization

The collected synovial tissue was processed at the Laboratory of Veterinary Cell-based Therapies—ICBAS-UP, following the patented eSM-MSCs' isolation protocol (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®, Barcelona, Spain) previously described [28].

Before cryopreservation of the cells' batch, the culture medium was subjected to a bacteriological control using the BACT/ALERT® (BioMérieux Portugal®, Linda-a-Velha, Portugal) medium to rule out the presence of any bacterial or fungal contamination.

2.6. Equine UC-MSCs' Isolation, Culture, and Characterization

Equine UC-MSCs were isolated from the equine umbilical cord matrix (Wharton's jelly), a birth residue, collected during a full-term parturition of a different foal from which the synovial tissues were obtained.

Briefly, tissue samples were collected and placed in transport media supplemented with penicillin (300 U/mL)–streptomycin (300 µg/mL) (15140-122—Gibco®) and amphotericin B (5 µg/mL) (15290-026—Gibco®). Upon arrival, umbilical cord tissues were decontaminated and dissected for the isolation of the stromal tissue, which was digested using Collagenase I (17100-017—Gibco®) and Dispase II (17105-041—Gibco®). Single-cell suspension of the digested tissues was obtained through a 70 µm cell strainer (CLS431751—Corning® Falcon®, Corning, NY, USA) and cultured in DMEM-HG (11965092—Gibco®), 20% (*v*/*v*) MSC qualified FBS (04-400-1A, Biological Industries Israel Beit-Haemek Ltd., Migdal HaEmek, Israel), penicillin (150 U/mL)–streptomycin (150 µg/mL), and amphotericin B (3.75 µg/mL) for the first 24 h. Non-adherent cells were discarded after 24 h, and the remaining cells were further expanded in DMEM-LG (11885-084—Gibco®), 10% (*v*/*v*) FBS, penicillin (100 U/mL)–streptomycin (100 µg/mL) (Gibco®), and amphotericin B (2.5 µg/mL) (Gibco®) to form a culture of adherent cells with fibroblastic morphology. This process was performed and is patented proprietary technology (PCT/IB2019/052006, WO2019175773—Regenera®).

Before cryopreservation of the cells' batch, the culture medium was subjected to a bacteriological control using the BACT/ALERT® (BioMérieux Portugal®) medium to rule out the presence of any bacterial or fungal contamination.

As previously described, isolated populations' immunophenotypes were validated in Passage 5 [28]. The antibodies were selected to confirm the mesenchymal/stromal histogenesis and discard epithelial and endothelial histogenesis of eUC-MSCs. For each antibody, appropriate negative and positive controls were included, and all were incubated overnight. Detailed immunocytochemistry information is provided in Table S1 of Supplementary Material.

Trilineage differentiation was also validated in Passage 5: adipogenic, chondrogenic, and osteogenic differentiation (StemPro® Differentiation kits: A1007201—Osteogenesis; A1007001—Adipogenesis; A1007101—Chondrogenesis, Gibco®) were assessed using Oil Red-O, Alcian Blue, and Von Kossa stainings, respectively. All protocols were performed as described [28], except for Von Kossa stain employed to detect mineral extracellular deposition, in which cells were fixated and dehydrated with increasing ethanol concentrations and then rehydrated and incubated in 2% Silver Nitrate (7761-88-8, Sigma-Aldirch, St. Louis, MO, USA) solution under UV light and sodium thiosulfate (1091471000, Merk™, Rahway, NJ, USA) 5% for 3 min. Wells were rinsed, and microphotographic records were obtained.

2.7. Secretome—Conditioned Medium Preparation and Analysis

The conditioned medium of eSM-MSCs and eUC-MSCs in Passages 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. Then, the culture flasks were further washed two to three times with the basal culture medium of each cell type without any supplementation. The conditioning procedure was based on maintaining the culture flasks in standard culture conditions after the addition of non-supplemented DMEM/F12 GlutaMAX™ (31331-093—Gibco®). The culture flasks were kept under these conditions for 48 h, stimulating the cells to secrete paracrine factors (CM). After this period, the CM was collected and subjected to centrifugation at 3000× *g* for 10 min. The sediment was eliminated by collecting the supernatant, which was filtered with a 0.2 µm syringe filter (Filtropur S, PES, Sarstedt®, Nümbrecht, Germany). Then, the CM was concentrated five times (5×) using a Pierce™ Protein Concentrator, 3k MWCO, 5–20 mL tubes (88525, Thermo Scientific®, Waltham, MA, USA). The concentrators were sterilized before the procedure. Initially, the upper compartment of each concentrator tube was filled with 70% ethanol (*v*/*v*) and centrifuged for 10 min at 3000× *g*. Then, 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 a new centrifugation cycle, under the conditions described above, for the number of cycles necessary to obtain the desired CM concentration (5×).

Equine SM-MSCs secretome has been previously characterized [28]. Similarly, eUC-MSCs CM was stored at −20 °C, concentrated 5×, and assessed for the production and secretion of the selected equine biomarkers, IL-6 and IL-8, through Multiplexing LASER Bead analysis (Eve Technologies, Calgary, AB, Canada). The average concentration for each interleukin in eUC-MSC was evaluated in triplicate.

2.8. Equine SM-MSCs + eUC-MSC CM Solution Preparation

The eSM-MSCs solution for intra-ligamental clinical application was a combination of allogeneic eSM-MSCs suspended in eUC-MSCs CM.

Cryopreserved P3 eSM-MSCs batches were thawed and suspended in the recipient animal's heat-inactivated autologous serum. For this purpose, 10 mL of whole blood was collected into two serum tubes (367820—BD Vacutainer®, Franklin Lakes, NJ, USA). The

tubes were then centrifuged at 6000× *g* for 10 min, and the supernatant (autologous serum) was collected into a 15 mL tube. The serum sample was heat-inactivated for 20 min at 56 °C (water bath), quickly cooled down in an ice bath, and sterile-filtered with a syringe filter with 0.22 µm into a new 15 mL tube. For one 9×10^6 eSM-MSCs dose, 3 × 2 mL eSM-MSCs vials containing around 3×10^6 cells each were thawed in a 37 °C water bath, and the cell suspensions of the 3 vials were mixed into one 15 mL tube. Then, 2–3 mL of autologous serum was slowly added to the tube (drop-wise), and the suspension was gently mixed. A total of 5 mL of PBS was slowly added into the tube, and the suspension was gently mixed and centrifuged at 3000× *g* for 10 min. After eliminating the supernatant, the cell pellet was resuspended in autologous serum, maintaining a ratio of 0.8:1. The cells were then counted, and their viability was determined in an automatic counter (Countess II FL Automated Cell Counter, Thermo Fisher Scientific®, Waltham, MA, USA) using the Trypan Blue exclusion dye assay (T10282—Invitrogen™, Waltham, MA, USA). Around 8.58×10^6 cells were counted with an average viability of 98% (triplicate count) before the first administration, and 9.42×10^6 cells were counted with an average viability of 100% (triplicate count) before the second one. The cell number was then adjusted to 1×10^7 cells/mL. At this point, the conditioned medium from eUC-MSCs was thawed and added to the suspension to a final 1:1 concentration. Then, 2 mL of the solution of eSM-MSCs suspended in eUC-MSCs CM was transferred to a perforable capped vial and preserved on ice until the moment of administration.

2.9. Treatment Protocol

The injured structure—LMCL—was treated with the mixture of allogenic eSM-MSCs and eUC-MSCs CM. Patient received a single endovenous administration of phenylbutazone (Phenylarthrite®, 2.2 mg/kg, IV, Vetoquinol, France) at the end of the treatment. No other medical treatments (including nonsteroidal anti-inflammatory drugs, intra-articular corticosteroids, hyaluronan, glycosaminoglycans, hemoderivative treatments, and other MSCs preparations) were administered, except for those described in the treatment protocol, before and after allogenic eSM-MSCs + eUC-MSC CM treatment.

Patient was monitored for 48 h after treatment, and no complication 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".

Intralesional eSM-MSCs + eUC-MSCs CM Administration

Patient was sedated with detomidine (Domosedan®, 0.02 mg/kg, IV, Orion Corporation, Finland), the right tarsus was trichotomized, and the skin was surgically disinfected with chlorohexidine and alcohol. The prepared therapeutic combination was aspired with a 18G needle to a 2 mL syringe and gently homogenized. Ultrasound-guided injection with a 20G needle was performed at the lesion site. The stablished protocol included a second eSM-MSCs + eUC-MSCs CM administration 15 days after the first treatment using the same protocol.

2.10. Post-Treatment Monitoring—Clinical Evaluations

Tissue regeneration was indirectly estimated through lameness evaluation, pain to pressure, limb inflammation, limb sensitivity, and ultrasound imaging. In each assessment, the ultrasonographic examination was performed in transverse and longitudinal scans, and three parameters were evaluated: 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 days 15 (T1—second administration), 30 (T2), 45 (T3), 60 (T4), and 90 (T5) post treatment (Figure 2).

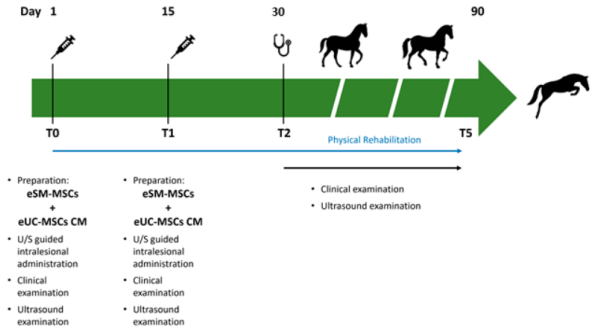

Figure 2. Timeline of eSM-MSCs treatment protocol and rehabilitation program. T0 is the day of the first treatment with the administration of eSM-MSCs + eUC-MSCs CM combination. Besides the intralesional application of the therapeutic combination, clinical and ultrasound examinations were also performed. T1 refers to the second application of the composition 15 days after T0, when the same procedure was repeated. On the other assessment days—T2, T3, T4, and T5—clinical and ultrasound examinations were performed. At the same time, a physical rehabilitation plan was carried out.

The rehabilitation program consisted of an exercise-controlled program including stall confinement and regular and increasing-time exercises, as presented in Table 2 [1,35–38]. Exercise was initiated early, on the second day after treatment, with low-level movements—hand walking. Early movements should include weight-bearing, strengthening, and flexibility activities, whereas stall rest alone should be used as infrequently as possible [36].

Table 2. Physical rehabilitation program.

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

After eSM-MSCs + eUC-MSCs CM treatment, the patient underwent a rehabilitation program consisting of two days of box rest followed by 13 days of 10 min hand-walk. Bandage applied on the treatment day was removed 24 h after treatment. On day 15, the second treatment was performed, followed by the same 15-day 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 min of hand-walking; between day 60 and day 75, the work consisted of 30 min of hand walking plus 5 min of trotting; and, finally, between day 75 and day 90, the patient underwent 30 min of hand-walking plus 10 min of trotting.

Veterinary assessment on day 90 (T5) determined if the horse could return to regular work based on clinical recovery (limb sensitivity and lameness evaluation) and ultrasonographic lesion improvement, evidenced by normal echogenicity, good fiber alignment, and normal cross-sectional area of the ligament when compared with contralateral limb.

3. Results

3.1. Clinical Evaluation

On the first assessment day, there was no pain at palpation and manipulation, and no swelling in the distal 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 pain to pressure were identified.

3.2. Complementary Diagnostic Examinations

3.2.1. Radiological Examination

A radiological examination was performed. The horse did not present significant articular abnormalities within the tarsocrural joint. There was tarsocrural joint distension, soft tissue distension, and slight evidence of tissue thickening at the injured long medial collateral ligament. Radiographs are presented in Figure 3. X-ray examination was not carried out again during the follow-up period.

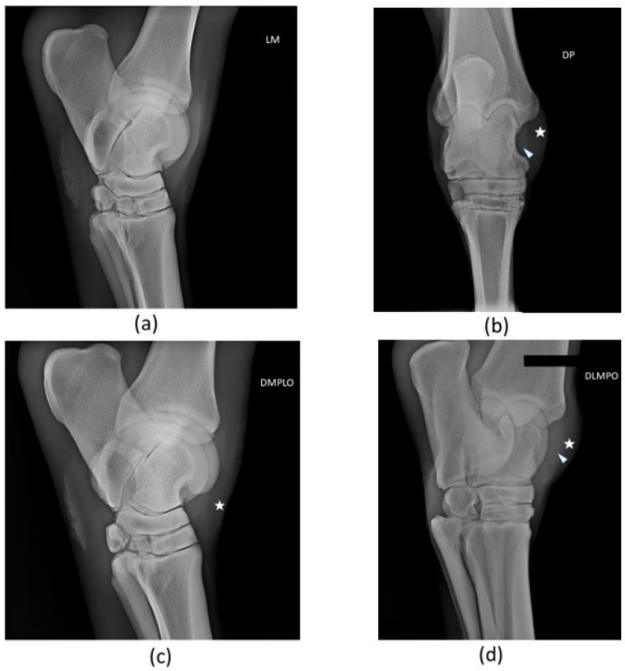

Figure 3. Radiographic views of patient's right tarsus: (**a**) Lateromedial (LM), (**b**) Dorsoplantar (DP), (**c**) Oblique dorsomedial-plantarolateral (DMPLO), (**d**) Oblique dorsolateral-plantaromedial (DLPMO). The white head of the arrow (△) points to increased radiopacity of the long medial collateral ligament, and the star (*) signals soft tissue swelling and joint distension. There are no significant radiological alterations of articular surfaces.

3.2.2. Ultrasound Examination

At the first veterinary assessment, ultrasonographic examination evidenced an increased amount of hypoechoic fluid containing areas of increased swirling (heterogeneous echogenicity) (Figure 4a), suggestive of an organized hematoma and/or fibrin within the

joint—hemarthrosis. Also evidenced was a moderate fiber pattern disruption of LMCL (hypoechoic region) at medial malleolus insertion as well as an increased cross-sectional area (Figure 4b). Cartilage surface was normal.

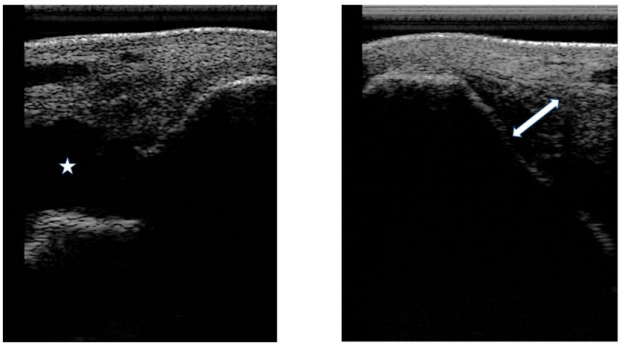

Figure 4. Images of the first ultrasonographic examination. Desmitis of LMCL insertion at the medial malleolus: (**a**) increased amount of hypoechoic fluid within the joint, signaled with the star (*); (**b**) disruption of the fibers at the insertion, signaled with the double arrow (↔).

3.3. MSCs Isolation and Characterization

Equine SM-MSCs were fully characterized, with the goal of confirming the presence of the minimum classification criteria necessary to classify their identity as MSCs, as previously described [4–6]. Briefly, the plastic-adherence of the cells when in culture as well as their fibroblast-like morphology were confirmed. The tri-differentiation capacity was confirmed by exposing eSM-MSCs to specific media to induce adipogenic, chondrogenic, and osteogenic differentiation. In addition to the morphological changes, the differentiations were confirmed qualitatively through staining with Oil Red O, Alcian Blue, and Alizarin Red, respectively. These cells were previously characterized by immunocytochemically, and positive staining was identified for markers indicating stemness (Octamer-binding transcription factor 4 (OCT4) and Homeobox protein NANOG (NANOG)), mesenchymal/stromal histogenesis (vimentin), and synovial histogenesis (lysozyme). Karyotype evaluation revealed chromosomal structural normality, normal number of chromosomes (64, XY), and absence of neoplastic alterations. The bacteriological control confirmed the absence of bacterial growth in both aerobiosis and anaerobiosis, and also the absence of fungal contamination after five days of incubation.

Equine UC-MSCs have been successfully isolated from UC tissue (Figure 5a). Cells in culture presented clear plastic-adhesion and fibroblast-like morphology (Figure 5b).

Figure 5. Isolation of MSC from equine umbilical cord tissue. (**a**) Umbilical cord tissue. (**b**) Isolated population of eUC-MSCs at P3—plastic adhesion, monolayer, and fibroblast-like shape of eUC-MSCs may be observed.

Equine UC-MSC trilineage differentiation was confirmed: adipogenic differentiation was observed by the presence of large red-stained lipid vacuoles in the cytoplasm due to exposure of Oil Red O staining; chondrogenic differentiation was observed by the presence

of proteoglycans-marked deposition in the extracellular matrix, which was stained blue, confirming the presence of chondrogenic aggregates; and osteogenic differentiation was demonstrated by the presence of extracellular phosphate deposits brown-stained by Von Kossa solution (Figure 6).

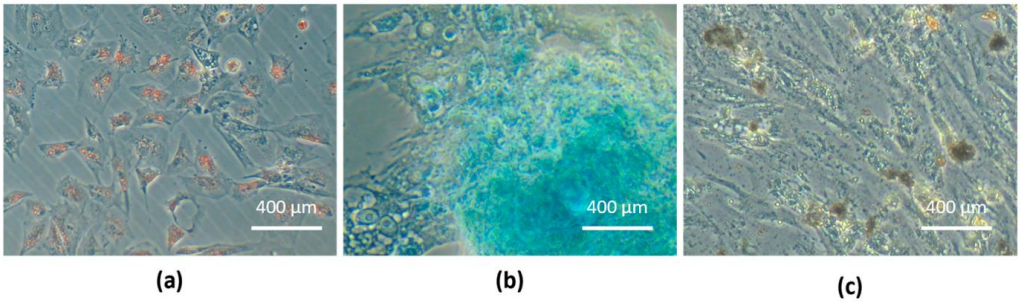

Figure 6. Tri-lineage differentiation: (**a**) eUC-MSCs adipogenic differentiation, cytoplasmatic lipid vacuoles stained in red (Oil Red O stain); (**b**) eUC-MSCs chondrogenic differentiation, proteoglycans in extracellular matrix stained in blue (Alcian Blue staining); (**c**) eUC-MSCs osteogenic differentiation, extracellular phosphate deposits stained in brown (Von Kossa staining).

The eUC-MSCs showed strong expression in vimentin, confirming their mesenchymal/stromal and non-epithelial origin and no expression of pan-cytokeratin (AE1 AE3); and a platelet endothelial cell adhesion molecule (CD31) which confirms the non- epithelial and non-vascular histogenesis of the cells, respectively. Altogether, these results confirm the expected mesenchymal/stromal origin of the cells (Figure 7).

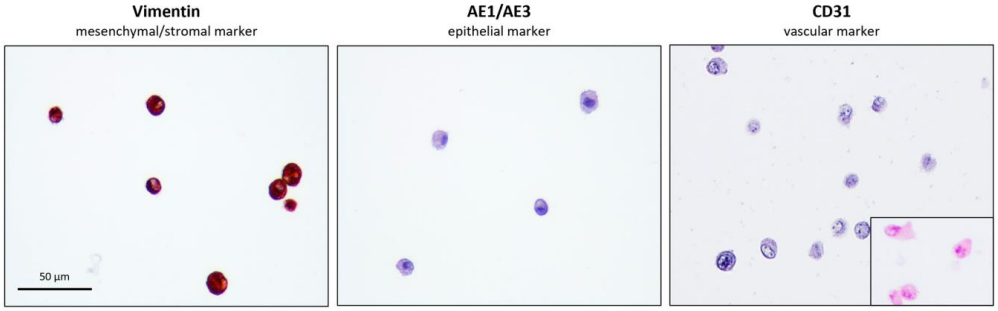

Figure 7. Immunocytochemistry of eUC-MSC. Positive staining for mesenchymal/stromal marker vimentin (**left)** and negative for the epithelial marker AE1/AE3 (**middle**) and vascular marker CD31 (**right**), H-E staining (lower right insert).

3.4. Secretome: Conditioned Medium Analysis

The results indicated that IL-6 and IL-8 were produced in high levels ranging from IL-6: 29.66 ± 2.64 pg/mL to IL-8: 14.05 ± 0.96 pg/mL. The average concentration quantified for each interleukin is depicted in Table S1 of Supplementary Materials.

3.5. Treatment Results

The patient did not present any adverse event that required treatment cessation, unplanned procedures, or additional treatments. The two intra-ligamentous administrations and follow-up procedures did not display adverse reactions (inflammation, infection, deterioration of the lesion, increased lameness), neither at treatment time (T0 and T1) nor during the following weeks.

On day 30 (T2), there was no evidence of pain and lameness (grade 0/5). Ultrasonographic evaluation evidenced increased echogenicity of the lesion as well as a reduction

in the cross-sectional area with good fiber alignment. The tarsocrural joint swelling and oedema were reduced. Nevertheless, compared with the contralateral limb, the right tarsocrural joint diameter was still larger than the left.

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

To sum up, the assessment of the patient's clinical recovery was performed by the presence of no pain in the tarsus and no lameness achieved by day 30. Lesion ultrasonographic improvement and indicators of regeneration were evidenced by a progressive increase in echogenicity and fiber alignment, and decrease in ligament cross-sectional area and synovial fluid accumulation within the joint space, observed through ultrasound examination during the follow-up on days 1 (T0), 15 (T1), 30 (T2), 45 (T3), and 60 (T4) (Figure 8).

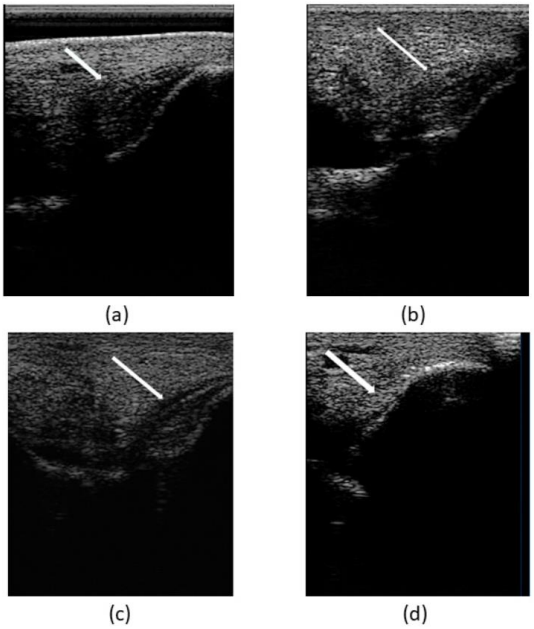

Figure 8. Images of ultrasonographic follow-up. (**a**) Day 1 (T0), (**b**) day 15 (T1), (**c**) day 30 (T2), and (**d**) day 60 (T3). Indicators of ligamentous regeneration: increased echogenicity and fiber alignment, as well as decrease in cross-sectional area and synovial fluid accumulation within the joint space.

On day 90 (T5), the patient returned to regular work with no lesion relapse reported up to 18 months after injury. Additional information reports that patient is already participating in competitions at a higher level than before the injury.

4. Discussion

This research discloses the application of a treatment strategy that focuses on the follow-up and healing features of an equine injured ligament, as well as on the patient rehabilitation and return to competition. Herein, we also highlight the effect of combining eSM-MSCs and eUC-MSCs populations in the treatment, using either the cells themselves or the cell-derived secretome.

In the current study, the use of the therapeutic combination of eSM-MSC + eUC-MSC CM in the treatment of LMCL desmitis was considered successful as clinical and ligament ultrasonographic parameters returned to normal.

On day 30 (T2), no pain and lameness (Score 0/0, AAEP Lameness Score) were registered. On day 45 (T3), there was an impressive ultrasonographic recovery, and on day 60 (T4), a complete ultrasonographic ligament recovery with the achievement of good fiber alignment, normal cross-sectional area, and good echogenicity was observed. It must be highlighted that, usually, with conventional treatments, only scar tissue repair occurs, and regeneration is never accomplished. In addition, full ultrasonographic recovery time was achieved in 50% of that described in the literature, being considered successful [5,6].

Clinically, on day 90, the patient was able to resume full work. This period of time also corresponds to half of that reported in literature when different therapeutic protocols are used in the same type of lesion. On the other hand, in the present case, there was a significative reduction in the rest period. In 2 days, this horse started the rehabilitation program versus 30–180 days of rest, and it returned to full work after 90 days versus 180 days presented in other studies, which accounts for almost 50% of the reduction in the total recovery time [6,9,18]. A study referred to the treatment of this type of lesion with platelet-rich plasma (PRPs), achieving a return to the same level of work in 180 days in 81% of the horses versus 90 days with this combination product [18]. The recovery times obtained in the current case remain a great attainment compared with those described by others concerning equine clinical trials of desmitis of collateral ligament of tarsus and equine tendonitis [5–9].

Remarkably, after the rehabilitation program was completed, the patient returned to the same physical work conditions and even exceeded the previous performance levels, exceeding the usual jumping level from 1.0 m to 1.30 m.

It is important to highlight that no side effects were registered during the treatment and rehabilitation periods, and 18 months after injury, no lesion relapse was noteworthy. Nevertheless, a slight distension of the right tarsus grossly remains currently perceptible when compared with the contralateral limb, and, ultrasonographically, the affected joint also presents sparse oedema.

To the best of our knowledge, there are no references to LCML healing with recovering of the normal ligament ultrasonographic features and absence of clinical signs in such a short period of time. 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 [39].

Importantly, besides the effective result, there are other advantages concerning this treatment protocol: its allogenic origin and easy application and access. The allogeneic source is a key factor in this therapeutic product with no observed adverse or rejection reactions supporting its potential as an alternative to autologous therapies, promoting its ready-to-use application. Furthermore, the existence of a cell and secretome bank that enables the production and validation of both MSCs and secretome, and offers well-characterized orthobiological products with recognized benefits in musculoskeletal tissue regeneration which might be stored and readily available for administration, becomes clinically very appealing and advantageous. This allows for early medical intervention in acute cases based on prompt and easy procedures such as an injection, contributing to a better functional outcome and a rapid and sustainable return to the sportive career.

As previously reported, SM-MSCs and UC-MSCs present a high tenogenic, anti-inflammatory, multipotency, and low-immunogenicity abilities, being, therefore, very appealing for tendon and ligament regeneration [29,40–42]. Equine SM-MSCs secrete high levels of human growth-regulated oncogene/keratinocyte chemoattractant (KC/GRO), monocyte chemoattractant protein-1 (MCP-1), IL-6, basic fibroblast growth factor (FGF-2), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), and IL-8 [28]. This profile supports their reported benefits in fibroblast-intense activity and lesion reperfusion, proliferation of tenogenic stem cells, enhancing cell

proliferation and collagen production [43]. Other factors such as G-CSF and GM-CSF also depict potential as skeletal muscle repair mediators, including those with pro-inflammatory functions [44,45]. Pro-inflammatory factors such as those found in these cells secretomes (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 [46,47].

The current study also demonstrated the presence of high levels of IL-6 and IL-8 in eUC-MSC CM. However, the exact meaning of this finding remains to be clarified.

Interleukin-6 bares pro-inflammatory and angiogenic functions, capable of increasing the expression of other growth factors (GF). Immunosuppressive properties are also associated with IL-6, which may be prime motors for the success of allogenic MSC implantation [48,49]. This pro-inflammatory nature is associated with the induction of acute-phase proteins, inducing a potent regeneration of various tissues and supporting their potential as a therapeutic approach for regenerative medicine [50,51]. Previous studies have, likewise, demonstrated that IL-6 is a potent anti-inflammatory cytokine significantly up-regulated in injured human tendons [52]. This cytokine has been demonstrated to have an important role in regulating tendon-derived stem cell (TDSC) activity and differentiation, however inhibiting their tenogenic differentiation in vitro [53], while in an in vivo model (IL-6 −/− mice), it has been demonstrated to be involved in the complex mechanisms that contribute to mechanical and organizational properties of injured tendons [54]. Another in vivo study demonstrated that a human Achilles tendon presented high levels of various growth factors after exercise. Among these, IL-6 was largely present, suggesting a responsibility in transforming collagen under biomechanical stimulation. After an experimental infusion of IL-6 in the peritendinous tissue, followed by exercise, collagen synthesis stimulation was observed, corroborating the hypothesis that IL-6 is an important growth factor for connective tissue in healthy human tendons [55].

Interleukin-8 is also a recognized pro-inflammatory mediator and a potent angiogenic factor associated with the increase in vascular endothelial growth factor (VEGF) concentration. Interleukin-8 was directly related to VEGF stimulation, helping in the revascularization and ligamentization of a grafted tendon [56]. Interleukin-8 has a similar effect to IL-6 but has a longer half-life [57].

A study on human Achilles tendon showed that IL-6, IL-8, and IL-10 were upregulated in a tendon healing phase with the absence of inflammation, indicating that these cytokines may be associated with anti-inflammatory and regenerative activity in the tendon healing process [52].

Herein, the high in vitro production of IL-6 and IL-8 by the MSCs populations under study suggests a putative involvement and contribution of these bioactive molecules in diverse biological functions related to immunomodulative and regenerative processes, magnifying the potential benefits of this therapeutic combination [58].

Hypothetically, eSM-MSCs and eUC-MSCs secretome factors are able to promote tendon/ligament healing by stimulating fibroblastic and angiogenic proliferation, reactivating growth programs, reducing inflammation, and stimulating cell proliferation, collagen production, and tenogenic differentiation, accomplishing not only lesion repair with regenerated tissue but also strengthening of the entire ligament, reducing the risk of lesion recurrence [39]. Nevertheless, further research is needed to more accurately understand the real influencing role of IL-6 and IL-8 in vivo. Having this potential in mind, one might believe that the combined action of eSM-MSCs and UC-MSC CM was a therapeutical advantage in this study case.

As typical of other studies, the current also has limitations, and the most important is related to the fact that only one animal case is presented herein, impairing conclusions about the real benefits of the therapeutic product tested. Also, this work may contain some bias as it refers to a specific patient treated with a certain batch of cells and a CM preparation that might present some variations in its composition in other cases. In this way, further

investigations involving a larger number of patients in more controlled and standardized clinical conditions should be carried out to prove the effectiveness of this treatment.

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

5. Conclusions

The outcome of the use of the therapeutic combination of eSM-MSCs and eUC-MSCs CM in the treatment of the presented clinical case of desmitis of LCML was considered successful. The clinical re-evaluations showed a fast, efficient, and safe clinical recovery with very positive outcomes: no lameness, and ultrasonographic images were compatible with regeneration (lesion fulfillment, parallel fibers, and normal cross-sectional area). The patient returned to a sportive career, reaching higher jump performance levels. After 18 months, there were no evidences of lesion relapse.

Clinical injuries of LMCL are very difficult to treat, in part due to their frequent misdiagnosis as well as their long-term recovery, meaning outcomes have frequently poor prognosis in terms of competition return. The fact of having a complete clinical recovery and ultrasound images compatible with LMCL regeneration in 60 days remains a very encouraging and promising accomplishment. Nevertheless, whether this result is due to the eSM-MSCs or to the eUC-MSCs CM or their patented combination cannot be fully determined since this study reports results of only one patient. Therefore, further research into the specific mechanisms of therapeutic action and clinical trials are required to further validate the approach and confirm the real benefits of this combination.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani14030370/s1, Table S1. Antibody reagents used in eUC-MSC immunocytochemistry: marker, type, supplier, dilution, incubation period, positive control, and cells of interest. Table S2. Average concentration of IL-6 and IL-8 in eUC-MSC CM. Concentrations obtained through Multiplexing LASER Bead analysis (Eve Technologies, Calgary, AB, Canada). The average concentration for each interleukin in eUC-MSC was evaluated in triplicate. (nd—not determined).

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Abbreviations

2D	Bidimensional
3D	Tridimensional
AAEP	American Association of Equine Practitioners
BM	MSC bone marrow multipotent mesenchymal stromal cells
CL	Collateral ligament
CM	Conditioned medium
cm^2	Square centimeter
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
eSM	MSCs equine synovial membrane-derived multipotent mesenchymal stromal 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	Milliamperes
MCB	Master cell banks
MCP-1	Monocyte chemoattractant Protein-1
mg	Milligram
MHz	Megahertz
min	Minutes
mL	Milliliter
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

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GRAPHICAL PAPER III

3 Treatment of equine tarsus long medial collateral ligament (LMCL) desmitis with allogenic SM-MSC + UC-MSC CM: Proof of Concept

B eSM-MSCs + eUC-MSC CM

- Stallion, 7yo, show jumper
- Acute lesion of the right tarsus

1 Clinical + orthopedic examination + Xray + U/S

- RH tarsus effusion,
- Lameness grade 2/5
- Hemarthrosis
- **Desmitis of LMCL**

2 Treatment protocol

- 2 doses of $1x10^7$ eSM-MSCs + e UC-MSC CM, 2 ml, intralesional,15 days apart.
- 90 days of physical rehabilitation program + corrective shoeing
- Clinical and U/S assessments

3 Clinical and U/S recovery at day 60

- Rehabilitation program until the end
- 20 months later the patient is competing at high level

CHAPTER IV

Case report: equine metacarpophalangeal joint partial and full thickness defects treated with allogenic equine synovial membrane mesenchymal stem/stromal cell combined with umbilical cord mesenchymal stem/stromal cell conditioned medium

CHAPTER IV

- **INTRODUCTION**

- **PAPER IV**

Case report: equine metacarpophalangeal joint partial and full thickness defects treated with allogenic equine synovial membrane mesenchymal stem/stromal cell combined with umbilical cord mesenchymal stem/stromal cell conditioned medium

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- **GRAPHICAL PAPER IV**

CHAPTER IV INTRODUCTION

Articular cartilage presents as a significant healing challenge, being injuries to this tissue a major concern in the fields of trauma and orthopaedic surgery. Such lesions are particularly impactful in equine athletes, leading to reduced athletic performance, decrease of quality and life span and, consequently, substantial economic losses.

Equine articular cartilage is susceptible to long-term damage if subjected to excessive loading forces. This is further exacerbated by its relatively thin nature, with thickness generally varying from 1.5 to 2mm, and its limited intrinsic healing ability, largely due to its avascular nature. These characteristics highlight the critical importance of developing effective management and therapeutic approaches to maintain the health and function of equine joints (T. P. Liu et al., 2022).

Conventional therapeutic approaches manage pain and reduce inflammation, alleviating symptoms but neither enable disease progression nor repair the damaged tissue. Therefore, there's an urgent demand for innovative treatments capable of regenerating tissue and restoring functionality, allowing equine athletes to resume their physical activities and achieve their performance goals (Crawford et al., 2013; Deuse et al., 2011; Linardi et al., 2019; S. Liu et al., 2022). Intensive efforts in regenerative medicine have resulted in the creation of innovative treatments for bone and cartilage disorders. These new approaches are aimed at controlling and slowing down the progression of the disease while also reassuring the regeneration of tissues, restoring them to a state that closely resembles their original anatomical and physiological conditions. The ultimate objective is to shift the environment within the joint from a catabolic state, that is inflammatory and breaks down tissue, to one that is anabolic and conducive to growth, thereby permitting the regeneration of cartilage.

In order to develop and refine regenerative treatments targeting joint health, a deep comprehension of both anatomy and biomechanics is essential.

Articular cartilage, a specialized form of connective tissue, is noted for its unique viscoelastic properties. Its primary roles include providing a smooth, lubricated surface to minimize joint friction, ensuring friction remains low during joint surfaces movement and to minimize shear stresses. This cartilage specific composition enables it to withstand significant contact pressures and distribute these forces evenly across the subchondral bone, reducing the impact (Nam et al., 2018).

Articular cartilage is composed of two tissue phases: a fluid and a solid phase. Fluid phase is composed of water, the main component (80%), and inorganic ions such as sodium, calcium, chloride, and potassium (Prockop et al., 2003). The solid phase is characterized by the extracellular matrix (ECM), which is porous and permeable, and its production is directly related with chondrocyte volume or function. ECM is composed of three major types of macromolecules: fibres (collagen and elastin), proteoglycans and glycoproteins, which are synthetized and maintained by chondrocytes. Collagen, the principal fibre within the ECM, exists in various molecular types: collagen II, the predominant collagen type in hyaline cartilage (>90%), collagen III (<10%), collagen IX (≈1%), collagen XI (≈3%) and collagen VI (<1%, exclusively in the pericellular matrix surrounding chondrocytes). Collagen type X appears in hypertrophic cartilage, in the calcified portion (Prockop et al., 2003). Proteoglycans are hydrophilic proteins that have one or more glycosaminoglycan (GAG) chains - keratin sulphate and chondroitin sulphate - covalently attached to a protein core. The relationship between proteoglycan aggregates and interstitial fluid provides compressive resilience to articular cartilage (Segawa et al., 2009) – **Figure 1**.

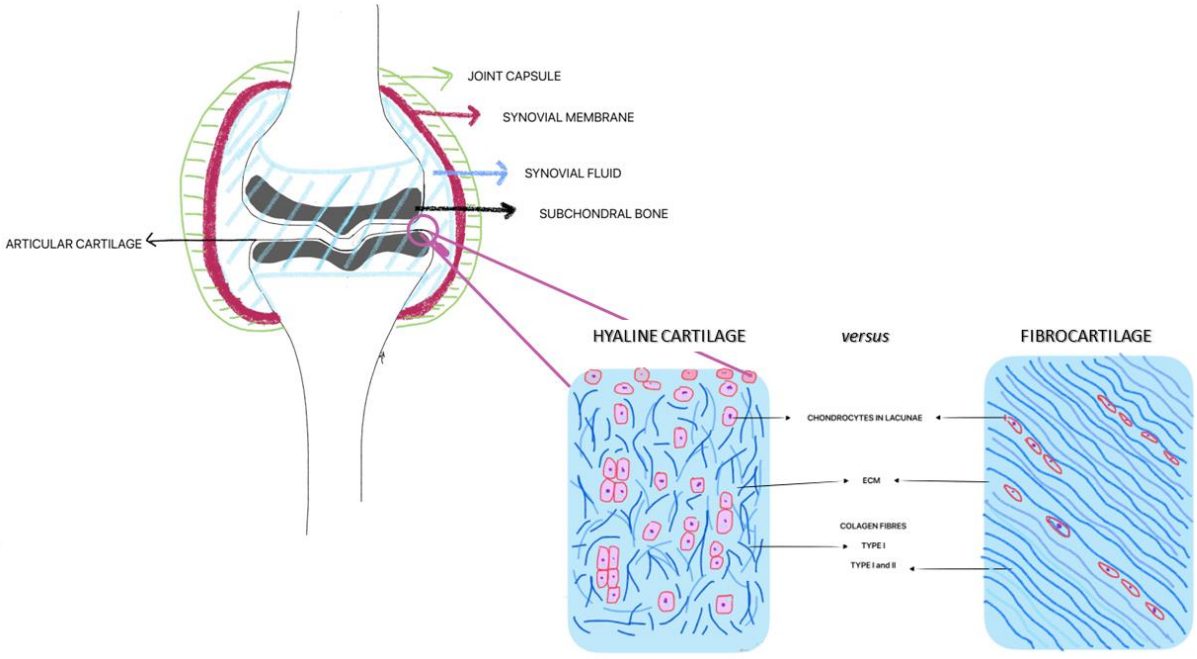

Figure 1. Synovial joint structure: joint capsule, synovial membrane, synovial fluid, cartilage and subchondral bone. In evidence hyaline cartilage structure: articular hyaline cartilage does not present perichondrum, ECM with mainly collagen type II fibres, homogeneous cartilaginous matrix, chondrocytes with lacunae (an ovoid space within the matrix). Fibrocartilage is composed of collagen

type I and II, cartilaginous matrix is composed of thick collagen fibres located between parallel rows of chondrocytes that are smaller than those of hyaline cartilage.

Synovial fluid (SF) plays an important role in the biomechanical behaviour, lubrication, and nutrition of the articular cartilage, being its major source of nutrients. SF is a plasma ultrafiltrate, sharing many protein similarities with blood plasma, thus being considered a dynamic reservoir of proteins, with the superimposed effect of those derived from cartilage and synovial tissue due to its anatomical proximity. Lubricant ability of SF, which contributes to the low friction and wear of the articular surface, is achieved by the synergistic action of the three lubricating agents: hyaluronic acid, lubricin and phospholipids at physiologic concentrations. Cytokines and growth factors present in SF are important regulatory factors for the cell populations lining and within the synovial joint space, including chondrocytes and synovial cells. Proteolytic enzymes are also present in normal SF, matrix-degrading enzymes, such as matrix metalloproteinases -1 and -3, and become elevated in joint injury and disease. Normal SF is relatively acellular when compared to whole blood. Each of the SF components can serve as biomarkers used in the monitoring of synovial joints heath, through the use of advanced molecular markers methods (Hui et al., 2012; Popov et al., 2021). Analysing the variations in their concentrations can reveal the health status and pathological conditions of the joint.

In recent times, the role of synovial inflammation in the onset and progression of diseases has gained widespread acknowledgment, with SF offering a precise snapshot of the joint's local microenvironment (de Grauw, 2011).

As stated before, articular cartilage injuries possess a constrained ability to regeneration and self-repair, primarily due to the limited growth potential of mature chondrocytes, their insufficient capability to produce a significant amount of ECM, and due to its avascular nature. This leads to the inability to form blood clots, an essential step in the healing process, and prevents progenitor cells from the blood and bone marrow, as well as resident chondrocytes, from migrating to the areas of damaged cartilage. Such constraints contribute to the onset of osteoarthritis (OA) following cartilage injuries (Mochizuki et al., 2006). Even so, various cell types can be activated post-injury to synthesize new matrix material. However, this newly formed matrix is typically both morphologically and mechanically inferior to the original articular cartilage (Jin et al., 2013).

Articular cartilage injuries are characterized into two types: partial-thickness defects, affecting only cartilage, lacking self-repair ability, and full-thickness defects, which extend into

the subchondral bone and possess a limited capacity for self-repair, influenced by the defect's size and location. This repair potential is attributed to the engagement of the subchondral bone, triggering a local influx of blood, mesenchymal stromal/stem cells (MSCs), and the formation of a hematoma that includes a fibrin clot along with platelets, red and white blood cells. Studies have shown that full-thickness cartilage defects can spontaneously regenerate into fibrocartilage (which is more abundant in collagen type I than type II), rather than reverting to native hyaline cartilage (Koga et al., 2008). Mechanical damage affects the ECM directly or indirectly through chondrocytes, which are stimulated to express matrix-degrading enzymes. Compression injuries can lead to chondrocyte death via necrosis and apoptosis, while the surviving cells diminish their biosynthetic function. Over time, the concentration of proteoglycans in the injured cartilage decreases, impairing its ability to react to physiological levels of mechanical stimulation (Čamernik et al., 2019). Alterations in the mechanical environment, a crucial factor in maintaining cartilage health, significantly influence the production of proinflammatory mediators within the articular cartilage. Joint injuries can substantially elevate levels of pro-inflammatory cytokines, contributing to OA and the progressive deterioration of articular cartilage (Koga et al., 2008; Mochizuki et al., 2006). The hallmark features of OA include degradation of articular cartilage, thickening of the subchondral bone, formation of osteophytes, synovial inflammation, ligament degeneration, and capsule hypertrophy, leading to symptoms such as pain, deformity, instability, and a decrease in mobility and function (Chen et al., 2018) – **Figure 2**.

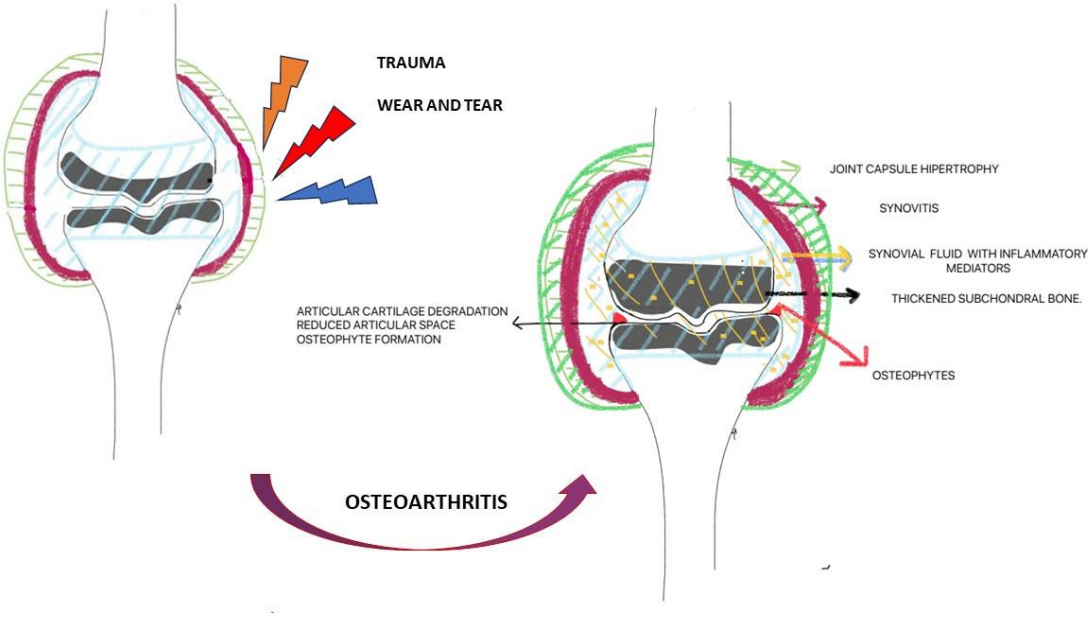

Figure 2. Osteoarthritis hallmark features. After repeated trauma and wear and tear lesions on joints, several pathologic features develop within the joint.

Regenerative treatments aim to halt and reverse these processes, maintaining joint integrity and fostering the regeneration of tissue in both anatomical and functional terms.

The use of MSCs for the treatment of cartilage and underlying bone defects is presenting significant potential, with these cells migrating to the damaged areas, releasing bioactive factors, aiding in both repair and regeneration. The primary sources of MSCs used in the field of cartilage tissue engineering include those from bone marrow (BM-MSCs), adipose tissue (AT-MSCs), synovial membrane (SM-MSCs), and umbilical cord stroma (UC-MSCs). Each type offers unique benefits in fostering cartilage regeneration and varies in terms of ease of collection, potential immune response, growth capabilities, capacity for developing into cartilage (chondrogenic potential), and ability to modulate the immune system. Notably, SM-MSCs present advantages in cartilage defects regeneration as they have the ability to produce hyaline cartilage repair tissue with an abundance of type II collagen and sulphated GAGs and variable cell morphology in accordance to local microenvironment (Goh et al., 2023; Koga et al., 2008; Ouzin & Kogler, 2023; Santos, 2023; Sekiya et al., 2015). Also, UC-MSCs present themselves as an excellent candidate for use in the clinical setting to provide cell-based restoration of hyaline-like cartilage. Although allogeneic, these cells stimulate little or no host immune response and can be stored for long periods while maintaining viability (Sadlik et al., 2018). Their aptitude to secrete growth factors positions them as an interesting option for cell-based and cell-free regenerative treatments (Sriramulu et al., 2018). Recent research endorses the application of MSCs conditioned medium (CM) as an innovative approach for the repair of articular cartilage. CM is created by altering the culture environment of MSCs, enabling the selective production and secretion of bioactive components, known as the secretome, in a targeted manner. These factors with both anti-inflammatory and pro-inflammatory effects related to chondrocyte catabolic and anabolic processes interplay allowing remodelling to occur and ultimately improving cartilage's structure and biomechanical properties (Rosochowicz et al., 2023; Solursh & Meier, 1973). The simple modification of the local inflammatory environment is partially the key to the deceleration of OA progression by attenuating activated macrophages and sinoviocytes secreting the massive amount of metabolites, harmful for articular cartilage ECM and chondrocyte (Rosochowicz et al., 2023). It has been highlighted that CM has a remarkable articular-protective effect, maintaining subchondral bone structure, producing a significantly more abundant cartilage matrix and

inhibiting chondrocyte apoptosis with enhanced autophagy, when compared with PBS in in vivo(Chen et al., 2018).

Considering this context, a gap in osteoarticular regenerative therapies spectrum was detected. The distinctive properties of SM-MSCs and UC-MSCs laid the groundwork for the creation of an innovative therapeutic product. Theoretically, this new approach holds considerable potential as an alternative solution for repairing cartilage damage.

Therefore, the main objective of this research is to:

1. **Formulate** the therapeutic blend comprising eSM-MSCs with UC-MSC CM.

2. **Identify** the therapeutical **potential** of this combination regarding its safety, easiness to use, efficacy and clinical and imagological outcome when applied in partial and full thickness cartilage defects of an equine patient.

To evaluate the efficacy of the therapeutic approach, a young stallion with both partial and full-thickness defects in the metacarpophalangeal joint was administered the combination of eSM-MSCs and UC-MCS CM treatment. Throughout the rehabilitation process, the patient underwent regular clinical examinations and imaging assessments. A follow-up period extending to 20 months post-treatment provided evidence of the safety of this therapeutic approach, as no adverse events were recorded. Furthermore, both clinical evaluations and imaging studies confirmed the recovery of the joint, with the stallion successfully returning to competitive activities.

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PAPER IV

Case report: equine metacarpophalangeal joint partial and full thickness defects treated with allogenic equine synovial membrane mesenchymal stem/stromal cell combined with umbilical cord mesenchymal stem/stromal cell conditioned medium

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Case report: Equine metacarpophalangeal joint partial and full thickness defects treated with allogenic equine synovial membrane mesenchymal stem/stromal cell combined with umbilical cord mesenchymal stem/stromal cell conditioned medium

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Here, we describe a case of a 5-year-old show-jumping stallion presented with severe lameness, swelling, and pain on palpation of the left metacarpophalangeal joint (MCj). Diagnostic imaging revealed full and partial-thickness articular defects over the lateral condyle of the third metacarpus (MC3) and the dorsolateral aspect of the first phalanx (P1). After the lesion's arthroscopic curettage, the patient was subjected to an innovative regenerative treatment consisting of two intra-articular injections of equine synovial membrane mesenchymal stem/stromal cells (eSM-MSCs) combined with umbilical cord mesenchymal stem/stromal cells conditioned medium (UC-MSC CM), 15 days apart. A 12-week rehabilitation program was accomplished, and lameness, pain, and joint effusion were remarkably reduced; however, magnetic resonance imaging (MRI) and computed tomography (CT) scan presented incomplete healing of the MC3's lesion, prompting a second round of treatment. Subsequently, the horse achieved clinical soundness and returned to a higher level of athletic performance, and imaging exams revealed the absence of lesions at P1, fulfillment of the osteochondral lesion, and cartilage-like tissue formation at MC3's lesion site. The positive outcomes suggest the effectiveness of this combination for treating full and partial cartilage defects in horses. Multipotent mesenchymal stem/stromal

cells (MSCs) and their bioactive factors compose a novel therapeutic approach for tissue regeneration and organ function restoration with anti-inflammatory and pro-regenerative impact through paracrine mechanisms.

KEYWORDS

case report, equine, osteochondral defect, synovial membrane mesenchymal stromal/stem cell, umbilical cord conditioned medium, cell-based medicinal product

1 Introduction

Human and animal athletes are prone to articular lesions due to trauma or stress-induced pathologies. The high-impact nature of equine sports leaves their articular cartilage particularly susceptible to damage (1). Articular defects might be partial-thickness, affecting only articular cartilage, or full-thickness, also reaching subchondral bone. Full-thickness defects may occur in young and mature sport horses, secondary to stress-related trauma when cartilage is exposed to excessive loading forces (2).

Cartilage tissue has low regenerative efficiency due to its avascular nature, among other factors (3). This poor intrinsic ability to heal favors the development of osteoarthritis (OA), a common chronic joint disease characterized by pain, deformity, instability, and reduction of motion and function (4). OA's pathologic findings might include articular cartilage degradation associated or not with subchondral bone thickening, osteophyte formation, synovial inflammation, ligament degeneration, and capsule hypertrophy (5). Traditional treatments predominantly focus on pain and inflammation control, relieving symptoms but having limited ability to correct the underlying pathology, to delay disease progress and to, ultimately, regenerate damaged tissues (6). When conservative treatment fails, surgical approaches, such as autologous chondrocyte implantation (ACI), might be considered but present limitations: they do not alter disease progression, do not regenerate cartilage, and do not enhance organ function (7). They usually result in inferior quality cartilage (fibrocartilage) formation, donor site morbidity, loss of phenotype from differentiation of primary chondrocytes during expansion, and possible need for open surgery. These techniques are also limited by low tissue availability (8). Hence, there is a pressing demand for novel treatments capable of regenerating tissues and restoring functionality, enabling horses to resume physical activities and athletic performance (1, 4, 7, 9).

Dedicated research in regenerative medicine has led to the creation of novel therapeutics targeting cartilage and bone pathologies. These advancements focus on modulating and inhibiting disease progression while promoting tissue regeneration, ultimately aiming for a return to anatomical and physiological function closely resembling the original tissue properties (10, 11). The overarching goal is to shift the joint's state from an inflammatory and catabolic nature to an anabolic state, enabling cartilage regeneration.

Implantation of MSCs into cartilage defects has shown great promise in both cartilage and subchondral bone repair (12–16) as they release bioactive factors, promoting repair and regeneration. There is evidence that synovial membrane MSCs (SM-MSCs) present a greater chondrogenic ability among MSCs from other origins, suggesting their superiority in cartilage repair (17–23). These cells have close anatomical contact with cartilage, suggesting a close bias toward the production of cartilage, becoming a good candidate for cartilage tissue engineering (19).

Research also supports the use of umbilical cord MSCs (UC-MSCs) for cartilage regeneration (24, 25). UC-MSCs from Wharton's jelly have high potential for proliferation, differentiation, and lower immunogenicity (12). UC-MSCs can stimulate *in vitro* production of key cartilage components, such as glycosaminoglycans and collagen type II (24). In addition, MSC-conditioned medium (MSC-CM) is emerging as a promising therapy for cartilage repair as a cell-free therapy, presenting all of the advantages of the bioactive factors secreted by MSCs, such as cytokines, chemokines, proteins, extracellular vesicles, and growth factors. These components exhibit both anti-inflammatory and pro-inflammatory effects, influencing chondrocyte processes and enhancing cartilage structure and biomechanical properties (26–30). By modifying the local inflammatory environment, CM may slow down OA progression by reducing harmful metabolites for articular cartilage ECM and chondrocytes produced by activated cells in the joint (27, 31).

In consideration of the aforementioned parameters, a therapeutic product has been developed, combining eSM-MSC with UC-MSC CM. The use of eSM-MSC in treating equine tendonitis and desmitis has already been demonstrated to have very good and promising results in tissue regeneration (32). Additionally, the combined use of eSM-MSC and UC-MSC CM has been previously employed and described in the treatment of a long medial collateral ligament of an equine tarsus, also presenting very favorable results in ligament regeneration, absence of clinical signs, and return to sportive performance and competition (33). The present study introduces the application of this combination in addressing two distinct articular defects: full-thickness and partial-thickness, and intends to understand and describe its regenerative ability.

2 Case description

2.1 Clinical description

A 5-year-old showjumper stallion was evaluated due to an acute lesion of the left forelimb (LF) metacarpophalangeal (MCj) with associated lameness.

On the assessment day, the MCj was swollen, and the horse presented a lameness grade of 4/5 and scored accordingly with the

AAEP lameness grading scale (34). Palpation, manipulation, flexion test, and pain to pressure were performed, and all had a positive reaction (Figure 1, Day = 0).

As complementary diagnostic exams, regional nerve blocks, as well as radiographs (X-rays), arthroscopy, magnetic resonance image (MRI), computer tomography (CT), and computed tomography

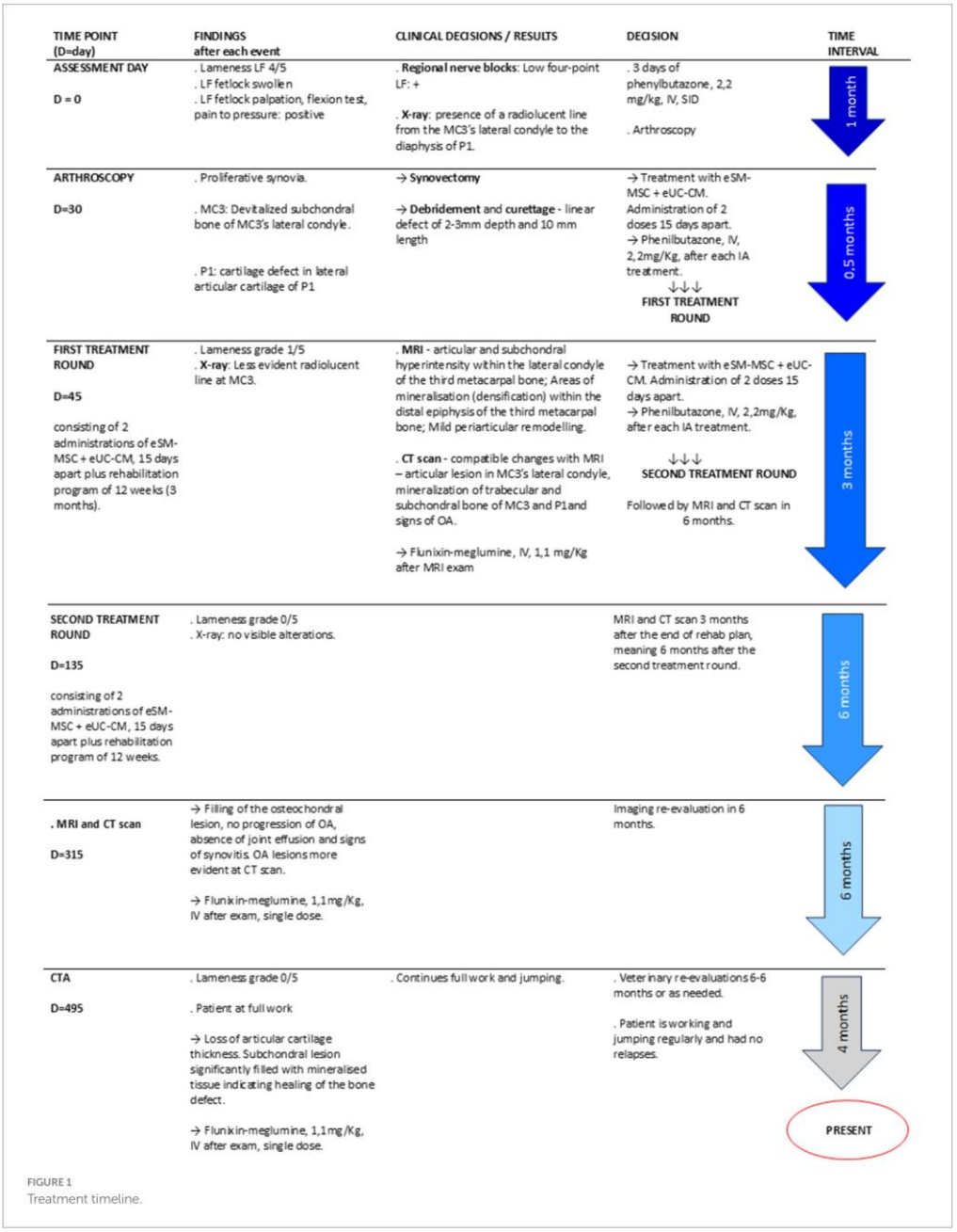

FIGURE 1
Treatment timeline.

arthrography (CTA), were performed at different times of the therapeutical intervention.

The patient did not receive any other medical treatments for at least 2 months before and after the cell-based treatment (including non-steroidal anti-inflammatory drugs, intra-articular corticosteroids, hyaluronan, glycosaminoglycans, hemoderivative treatments, and other MSCs preparations), except for phenylbutazone (Phenylarthrite®, 2,2 mg/kg, IV, Vetoquinol, France), SID for 3 days on the assessment day, a single dose of phenylbutazone after every intra-articular (IA) treatment, and a single dose of flunixin-meglumine (Flunixin 3E®,1.1 mg/kg, IV, Norbrook Laboratories, Ireland) at MRI and CTA examinations.

Figure 1 presents a schematic timeline of the clinical case.

3 Diagnostic assessment

3.1 Regional nerve blocks

Three regional nerve blocks were performed on the LF: digital palmar, abaxial, and low four-point nerve block. Lidocaine 2% (Anestesin® 20 mg/mL, Orion Corporation, Finland) was injected medially and laterally (2 mL per injection site) in each assessment with 25G needles. These localized the pain to the fetlock region (Figure 1, D = 0).

3.2 Diagnostic imaging

3.2.1 Radiographs

Radiological examination (X-ray) of the LF MCj was performed with a digital system—CareRay Cw series® (CareRay, Suzhou, China), radiological constants: 72 Kv, 0.8 mA. The distance between the X-ray generator (Orange 1,060 HF, EcoRay, Seoul, Republic of Korea) and the flat panel was approximately 66 cm. Seven standard views of the MCj were obtained, namely lateromedial (LM), dorsopalmar (DP), Latero-medial flexed (LM Flex), oblique dorsolateral-palmaromedial (DLPMO), oblique dorsomedial-palmarolateral (DMPLO), oblique palmarolateral-dorsomedial oblique (PLDMO), and Palmaromedial-Dorsolateral oblique (PMDLO). The radiological examination was performed on assessment day (Figure 1, Day = 0), and those views that presented alterations were repeated during the rehabilitation program, as described in Table 1.

LM and DP did not evidence abnormalities (Figures 2A,B). The LM flexed projection exhibited slight remodeling of the dorsoproximal aspect of the first phalanx without evidence of a loose intra-articular fragment (Figure 2C). The DMPLO projection revealed a faint radiolucent area over the lateral condyle (Figure 2D).

3.2.3 Arthroscopy

Arthroscopy was performed to explore the LF MCj, to eliminate the possibility of a proximal P1 osteochondral chip fracture, to precisely assess the nature and extent of the radiological findings at MC3 and P1, and therapeutically address the pathological evidence (Figure 1, Day = 30). The patient underwent premedication with penicillin procaine (Depocilina 300 mg/mL®, 12 mg/Kg, IM, MSD Portugal), phenylbutazone (Phenylarthrite®, 2.2 mg/kg, IV, Vetoquinol, France), and detomidine (Domosedan®, 0.02 mg/kg, IV, Orion Corporation, Finland). The arthroscopic examination was performed under general anesthesia with the patient in dorsal recumbency. Induction was performed with ketamine (Ketamidor®, 2.2 mg/Kg, IV, Richter Pharma, Austria) and midazolam (Dormazolan®, 0.02–0-08 mg/kg, IV, Dechra, United Kingdom), and maintenance was under gas anesthesia with isoflurane (Isovet®, Piramal Critical Care Limited, Netherlands) and a continuous infusion of romifidine (Rominervin®, 0.025 mg/Kg, IV, Dechra, United Kingdom).

TABLE 1 Physical rehabilitation program.

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 + X-ray
5	Handwalk: 15 min
6	Handwalk: 20 min VET-CHECK
7	Handwalk: 25 min
8	Handwalk: 30 min VET-CHECK + X-ray
9–10	Handwalk: 30 min + 5 min trot
11–12	Handwalk: 30 min + 10 min trot VET-CHECK + MRI + CT scan (first treatment round)

A standard dorsal approach was performed to the left MCj, and an 18 cm length x 4.5 mm diameter scope (KARL STORZ® SE & Co. KG, Tuttlingen, Germany) was introduced into the joint.

Following mild synovectomy to enhance visualization and lesion assessment, the following lesions were identified:

(1) A linear partial thickness lesion extending distally through the axial aspect of MC3 lateral condyle (Figure 2E blue); a (2) semilunar-shaped partial thickness lesion dorsoproximal aspect of P1 (Figure 2E red); a (3) circular partial thickness lesion on the lateral side of the sagittal ridge (Figure 2F pink). With slight flexion of the joint, the main lesion was visible on the middle aspect of the lateral condyle, and this was a (4) full-thickness linear lesion of approximately 7 mm in length and covered with fibrillated fibrin (Figure 2G green). This lesion was debrided using a combination of bone curettes and a synovial resector down to the healthy subchondral bone plate at a depth of 2 mm (Figure 2H green). Immediately adjacent to this lesion, there was a similar (5) linear lesion in the first phalanx, suggesting that these lesions were correlated and that the MC lesion had led to abrasion of the adjacent P1. The latter was debrided in a similar fashion, but its depth did not extend further into the subchondral bone (Figure 2H yellow).

The time gap between the first assessment of the patient and the arthroscopic examination was based on reasons other than clinical principles, as this was an outpatient ambulatory clinic.

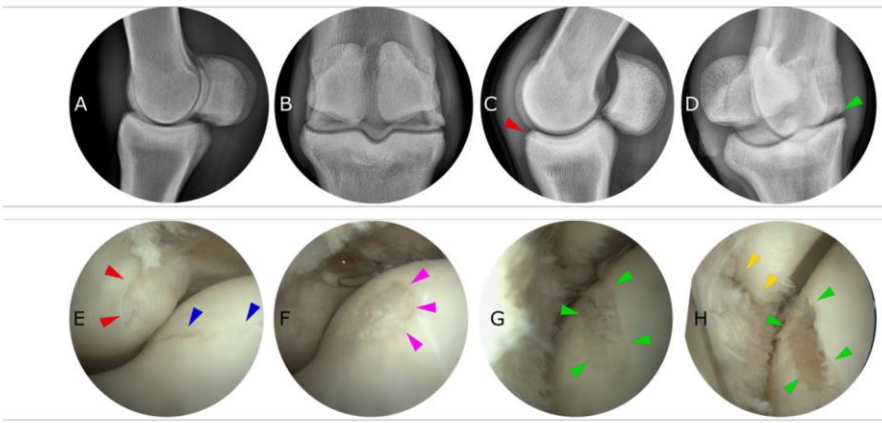

FIGURE 2
Pre-treatment diagnostic imaging and surgery: Radiographs and arthroscopy. Four radiographic projections **(A)** Lateromedial (LM), **(B)** Dorsopalmar (DP), **(C)** LM flexed, and **(D)** DMPLO (dorsomedialpalmarolateral Oblique) are presented. The slight radiological alterations are highlighted with a red (first phalanx) and green (MC3) arrow. Arthroscopic images of the LF metacarpophalangeal joint. **(E)** Red arrows present a semilunar partial thickness lesion on the dorsoproximal of P1 and blue arrows delimit a partial thickness liner lesion on the lateral aspect of the sagittal ridge of MC3. **(F)** Pink arrows present a circular partial thickness defect further proximal to the lateral aspect of the sagittal ridge of MC3. **(G,H)** show the main lesions on the dorso-distal aspect of the lateral condyle of MC3 (green arrows) before and after debridement of the lesion. Yellow arrows indicate an adjacent lesion on the dorsoproximal aspect of P1 (note that the joint is in slight flexion to allow visualization).

3.2.4 Magnetic resonance image

MRI of the MCj was performed under the same general anesthesia protocol previously described at arthroscopy. A single dose of flunixin meglumine (Flunixin 3E®, 1.1 mg/kg, IV, Norbrook Laboratories, Ireland) was administered. The protocol included sagittal—Time weighted image (TW) 2*W and Short Tau Inversion Recovery (STIR), dorsal—T1W GRE and STIR, and transverse—T1W multiecho gradient recalled echo (GRE), T2W fast spin echo (FSE), and STIR. Two MRI exams were performed approximately 6 months apart (Figure 1, D = 135 and D = 315).

3.2.5 Computed tomography scanning

Cone-beam CT scans were conducted simultaneously with each MRI session (Figure 1, D = 135 and D = 315).

3.2.6 Computed tomography arthrography

A CTA was performed 6 months after the second CT scan described above to assess cartilage thickness. In brief, under standing sedation with detomidine (Domosedan®), 10 mL of iohexol solution (Omnipaque™ 300, GE Healthcare, United Kingdom) was injected into the MCj, and a CT scan was performed (Figure 1, D = 495). A single dose of flunixin meglumine (Flunixin 3E®, 1.1 mg/kg, IV, Norbrook Laboratories, Ireland) was administered.

4 Treatment protocol

Following clinical examination and diagnosis, the patient underwent a treatment protocol comprising two intra-articular administrations of a novel therapeutic combination of eSM-MSCs with eUC-MSC CM at the left MCj, with 15 days intervals, followed by a rehabilitation plan (Figure 1, D = 45).

The therapeutic protocol began with the patient's sedation with detomidine (Domosedan®), followed by clipping of the left MCj area. The skin was aseptically prepared. The therapeutic combination was prepared and loaded into a 2-ml syringe and subsequently injected with a 20G needle, ensuring homogeneity of the solution and MSCs viability (35) through a dorsal approach into the MCj. The intra-articular therapeutic solution comprised allogenic eSM-MSCs suspended in eUC-MSC CM.

In brief, the eSM-MSCs donor was a young and healthy 6-month-old foal whose cause of death was a field accident. Collection preparation and procedures were previously described (32). After collection, the equine synovial membrane was prepared at the Laboratory of Veterinary Cell-based Therapies, ICBAS-UP. The isolation protocol of eSM-MSCs had been developed by Regenera® (32). eSM-MSCs were characterized through trilineage differentiation, immunohistochemistry, and karyotype analysis. eSM-MSCs CM preparation and analysis were also performed as previously described (32). eUC-MSCs were isolated from equine UC—Wharton's jelly—and the connective tissue surrounding UC. It was expanded to form the culture of adherent cells with fibroblastic morphology. Trilineage differentiation, immunophenotype, and bacteriological control were previously performed and described (33). CM preparation and analysis were previously described (33). This process is a patented technology owned by Regenera® (33).

The therapeutic solution for intra-articular clinical application was a combination of allogenic eSM-MSCs suspended in eUC-MSC CM. Prior to the preparation of the final therapeutic combination, eSM-MSC and UC-MSC CM were produced and preserved as described above. Cryopreserved P3 eSM-MSCs batches were

suspended in the treated animal's autologous serum. Approximately 2 mL of eSM-MSCs (1×10^7 cells) solution was suspended in UC-MSC CM, final concentration 1:1, and transferred to a perforable capped vial and preserved on ice until the moment of administration. This therapeutic combination is currently a patented technology owned by Regenera® (PCT/IB2019/052006, WO2019175773). This preparation was previously described (33).

After each intra-articular administration, the limb was bandaged for 24 h, and the horse received a single dose of phenylbutazone (Phenylarthrite®, 2.2 mg/kg, IV, SID) to avoid any joint flare reaction after MSCs administration. The horse was closely monitored for 48 h post-treatment. After the first intra-articular administration, a 12-week rehabilitation program was initiated, beginning with 2 days of stall rest followed by 13 days of 10-min hand-walking sessions with increasing times of exercise (Table 1) (5, 33, 36–39). This set of procedures resumes the first treatment round (Figure 1, D = 135). After undergoing the prescribed program and veterinary clinical reassessments, advanced diagnostic imaging, namely MRI and CT exams, was performed to better analyze the healing of the defects and to decide the patient's readiness to resume full work (Table 1).

After the MRI and CT scan analysis, it was decided to perform a second treatment round using the same protocol (Figure 1, D = 135). Six months later, another set of MRI and CT scans was performed (Figure 1, D = 315).

4.1 Treatment outcome

Clinical improvement was monitored through assessments of lameness, pain response to pressure and flexion, and fetlock swelling/inflammation. During the therapeutical administrations and throughout the course of the physical rehabilitation program, the horse did not present significant clinical signs, such as increased lameness, pain, or inflammation, requiring treatment cessation.

Following the conclusion of the rehabilitation program, the horse exhibited residual lameness (1/5), with the absence of joint swelling and pain upon palpation. At this time, an MRI and a CT scan were performed to assess the healing of the affected tissues. It was demonstrated that the absence of P1's partial thickness lesions was identified at x-ray and arthroscopy, but the full-thickness lesion at MC3's lateral condyle persisted. The following findings were observed at MRI: articular and subchondral hyperintensity within the lateral condyle of the MC3 (Figure 3, "3 months post 1st treatment"); areas of mineralization (densification) within the distal epiphysis of the third metacarpal bone (larger laterally than medially); mild periarticular remodeling at the abaxial margins of the proximal epiphysis of P1; and the proximal articular margins of the proximal sesamoid bones, compatible with mild OA of the MCj. CT scans exhibited compatible changes with MRI images—articular lesion in MC3's lateral condyle, mineralization of trabecular and subchondral bone of MC3 and P1, and signs of OA. As our treatment goal was the achievement of complete clinical recovery as well as imaging improvement of the articular cartilage and subchondral bone, a decision was made to proceed with a second round of treatments performed under the same protocol.

After the second 12-week rehabilitation plan, the lameness grade improved to 0/5, with no pain evident upon palpation, pressure, or flexion. The horse was shod with a thinner lateral branch to mitigate the impact on the lateral side of the joint and resumed normal and full work, including jumping exercises and remaining sound. The MRI performed after the conclusion of the second-round treatment demonstrated the absence of signs of joint effusion/synovitis, stabilization of OA findings, and filling of MC3's osteochondral lesion. Regarding the lesion, it had less defined articular and subchondral hyperintensity, and the areas of mineralization within the distal epiphysis of MC3 (lateral bigger than medial) did not show progression with OA changes not developing (Figure 3, "+ 9 months"). At the second examination, a CT scan revealed more noticeable differences in OA changes than MRI. The articular surface of the lateral condyle was irregular, and a focal hypoattenuating region was seen further palmarly (Figure 3, "+ 9 months"). There was mild periarticular remodeling in both the distal aspect of MC3 and the proximal aspect of P1. These findings did not show progression between the two exams performed 6 months apart.

The horse remained in full work, and 6 months after the last MRI and CT scan, a CTA demonstrated total fulfillment of MC3's lesion with the resumption of bone structure and the presence of a thin layer separating the bone from contrast and no contrast within the lesion confirming lesion fulfillment (healing) and partial cartilage-like tissue recovery (Figure 3, contrast CT).

Twenty months after the first treatment, the horse remains sound and has returned to full athletic performance as a young showjumper.

5 Discussion

Articular cartilage is a challenging tissue to regenerate (40). Assessment of the healing effect of orthobiologics can be challenging in cases of diffuse OA; the occurrence of localized lesions, such as those presented here, provides a unique opportunity to enhance our understanding of the effectiveness of regenerative treatments. This study describes a clinical case of a young horse with a traumatic lesion to the MCj, treated with an innovative therapy combining eSM-MSC and UC-MSC CM.

Using different imaging techniques and clinical follow-up, we were able to assess the safety and outcomes of the treatment. Twenty months post-treatment, the patient resumed jumping activities and competitions, exhibiting no clinical symptoms or relapses detected by diagnostic imaging.

This horse underwent two treatment rounds using the aforementioned combination therapy. Following the initial treatment round, a significant improvement in lameness score indicated a reduction in acute joint inflammation. However, the osteochondral lesion was insufficiently fulfilled, highlighting slow tissue healing. This emphasizes the need for comprehensive monitoring during the healing phase, as decreased pain may tempt an early return to exercise when tissue integrity is suboptimal, risking incomplete healing and potential recurrence (41–45). After the first treatment round, MRI and CT scan exams evidenced the absence of P1's and MC3's partial thickness lesions and increased mineralization in MC3's subchondral bone, suggesting osteochondral regeneration. However, a lesion at MC3's lateral condyle, approximately 7 mm in depth, was demonstrated. There were significant improvements in both P1 and

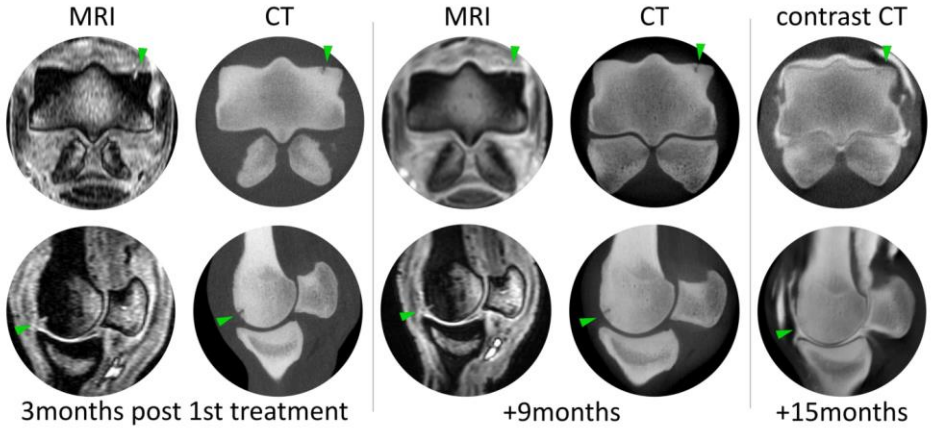

FIGURE 3
Magnetic resonance and CT imaging throughout the post-treatment period at three distinct time points. Lesion progression is evident between each time point, but the majority of the radiographic filling of the lesion occurs between 9 and 15 months post first treatment. Contrast CT allows assessment of cartilage-like tissue thickness, and although the cartilage hallow is slightly thinner than the medial condyle, there is no contrast within the defect.

MC3 lesions but no complete healing of MC3's full-thickness lesion was observed.

At the second treatment round conclusion, 3 months after its beginning, the horse exhibited clinical soundness with no lameness and no radiological abnormalities. MRI and CT scans indicated the absence of lesions in P1's cartilage, with no progression of OA and MC3's osteochondral defect, which was notably less prominent. The absence of OA progression was considered a positive prognostic indicator, suggesting effective pathology control and prevention of further deterioration (35, 46, 47). The observed improvement in lesion fulfillment was interpreted as regenerative, reflecting a significant advantage of this treatment.

Six months later, the CTA confirmed the presence of a thin layer of articular cartilage-like tissue in MC3's condyle. As the horse was sound, in full work, and competing, a histologic examination was not performed to distinguish between cartilage and fibrocartilage. However, the fulfillment of the lesion, the achievement of clinical soundness, and the athletic performance were considered as successful attainment of this protocol treatment.

As it was previously demonstrated, SM-MSCs can delay the progression of cartilage degeneration in osteoarthritis, relieving pain and improving joint function, and are also able to regenerate cartilage and subchondral bone (48, 49), as could be observed in this case. SM-MSCs and UC-MSCs present chondrogenic ability, high proliferative potential, and low immunogenicity (50), being able to modulate inflammatory processes related to musculoskeletal injuries (11, 40, 48, 49, 51) and regenerate osteochondral defects (49). In this case, it is suggested that the combination of SM-MSCs and UC-MSC CM may have had a positive effect on the clinical and diagnostic imaging outcome (52), enabling the patient's return to competition.

The combined MRI and CT assessment provided valuable insight into the case's progression, offering a thorough analysis of osteochondral fulfillment and OA development. This comprehensive evaluation provided essential information, enhancing the understanding of the procedure's success and effectiveness (49).

The rehabilitation program may have played a crucial role in the overall treatment protocol, ensuring that the horse remained consistently active and received a continuous and progressive stimulus on its cartilage, joints, ligaments, and muscles. In contemporary equine sports medicine, it is well-established that physical rehabilitation is a pivotal component, emphasizing the importance of keeping the horse engaged in exercise (37, 38, 53, 54). The incorporation of early mobilizations is recommended as long as rehabilitation protocols are followed carefully. These mobilizations encompass weight-bearing activities, straight-line walking, and strengthening and flexibility exercises (38, 55, 56).

In addition, the importance of physical loading in chondrocyte maturation and phenotype maintenance is widely acknowledged, with diminished biomechanical loading often resulting in atrophy (57–59). Bearing this in mind, a comprehensive understanding of the impact of mechanical stimulation on MSC chondrogenic differentiation can enhance the effectiveness of MSC-based cartilage regenerative therapies, particularly in joints subjected to a mechanically demanding environment (40). Basically, the importance of controlled and purposeful physical activities during rehabilitation contributes to the overall success of the treatment, promoting optimal tissue healing and functional recovery (53).

Comparing the outcomes with the limited existing literature, in this case, the patient was sound and returned to work and competition in a reduced time frame (60–62). In an equine radius articular defect treated with ACI, the horse stood in rest for 4 weeks (vs. 2 days in our protocol) and was sound, having recovered from joint distension after 24 months (vs. 12 months and 3 months with our protocol) (60). In another report describing MSC joint capsule rupture treatment, the horse re-established its athletic performance 1 year later.

Comparatively, a complex joint injury with both partial and full-thickness articular defects of a forelimb fetlock, such as the one herein described, also completely recovered after 12 months and restored athletic performance after 13 months (61). In another group of horses with stifle injury treated with bone marrow-MSCs (BM-MSCs), 43% of the horses returned to previous level work only 24 months after treatment of MSC (62).

Concerning the number of doses and cells applied in this therapeutic protocol, a total of four allogeneic doses of 1×10^7 cells, all from the same donor, were performed. None of them yielded any local or systemic adverse effects. In fact, the number of applied cells is consensual among equine MSCs clinical studies (35, 63, 64). Notably, doses of 1×10^7 cells demonstrated superior clinical improvements compared to higher injected doses (49, 51). The clinical intra-articular application of MSCs is an easy, minimally invasive, and reasonably safe procedure, with no reported serious adverse events (48, 49, 51, 65). These findings emphasize the effectiveness, safety, and easy-to-use characteristics of the applied MSC therapy in this particular case.

Since there are no well-defined protocols for applying MSC-based therapies in horses, the administration scheme herein applied was decided based solely on the patient's clinical outcome. Research involving equine and humans has demonstrated the advantages of increasing the number of intra-articular MSC therapeutic applications (2 administrations and 3 administrations per year) with no clinically relevant side effects (66–68). Therefore, repeated intra-articular administrations are documented and advisable, and in this case, it is believed that it supported treatment effectiveness. Potential limitations regarding repeated intra-articular injections concern the fact they might induce both primary and secondary humoral immune responses. However, MSCs preconditioned with pro-inflammatory cytokines demonstrated an enhanced immunomodulatory capacity. This preconditioning potentially enabled these cells to more efficiently manage inflammation, reducing a primary humoral response upon initial administration (25).

To sum up, the intra-articular administration of this novel therapeutic formulation, combining eSM-MSCs and eUC-MCS CM, demonstrated a successful efficacy in treating both partial and full-thickness cartilage defects in this equine patient. Subsequent assessments revealed notable advancements in clinical and imaging parameters. Remarkably, the patient exhibited complete clinical recovery, restoring athletic activity, and even surpassing the pre-treatment sportive level. Limitations of the current study relate to its observational nature, wherein the diagnostic exams used during the therapeutical follow-up were not employed at the initial diagnosis stage, and, also, after CTA, no arthroscopy and biopsy were performed to guarantee the real nature of the cartilage tissue. However, the horse has completed full function, being in competition, and for that reason, no more invasive exams were performed. Additionally, the absence of a control group further restricts the ability to draw definitive conclusions. The potential benefit of the rehabilitation program should also be considered.

Considering this, further investigations involving a more extensive cohort of equine patients with comparable lesions undergoing meticulous follow-up assessments under standardized conditions are imperative to definitively ascertain the effectiveness of this innovative and auspicious therapeutic protocol.

6 Conclusion

Articular cartilage defects in equine joints, particularly the MCj, can lead to significant lameness and inevitably trigger OA development. However, with a precise diagnosis, a positive outcome might be achieved with regenerative treatments, namely with this innovative treatment. The combination of eSM-MSC and UC-MSC CM was demonstrated to be safe and effective, as no adverse signs were reported, and advanced imaging evidenced fulfillment of the osteochondral lesion, absence of the other lesions previously identified at arthroscopy, and OA stabilization. In addition, and no less important, clinically, after a 20-month follow-up period, the equine returned to and progressed in its athletic career without any signs of lameness or clinical relapse, no swelling or pain of the injured area, now competing at a higher level.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal studies were approved by ORBEA – Órgão Responsável em Bem-Estar Animal (ICBAS). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

IR: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. BL: Formal analysis, Investigation, Methodology, Writing – original draft. PS: Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft. AS: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. ARê: Formal analysis, Investigation, Methodology, Writing – original draft. AC: Conceptualization, Investigation, Methodology, Writing – original draft. IB: Investigation, Methodology, Writing – original draft. ARo: Investigation, Methodology, Validation, Writing – original draft. JP: Formal Analysis, Investigation, Methodology, Validation, Writing – original draft. CM: Data curation, Investigation, Methodology, Supervision, Writing – review & editing. JS: Supervision, Writing – review & editing. LL: Formal analysis, Investigation, Methodology, Supervision, Validation, Writing – review & editing. LA: Investigation, Methodology, Supervision, Validation, Writing – review & editing. RA: Data curation, Investigation, Validation, Writing – original draft, Writing – review & editing. AM: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Glossary

AAEP	American Association of Equine Practitioners
ACI	Autologous chondrocyte implantation
BM-MSCs	Bone Marrow mesenchymal stromal/stem cells
CM	Conditioned Medium
CT	Computed Tomography
CTA	Computed Tomography arthrography
D	Day
DLPMO	Oblique Dorsolateral-palmaromedial
DMPLO	Oblique dorsomedial-palmarolateral
DP	Dorso palmar
ECM	Extracellular matrix
eSM-MSC	Equine synovial membrane mesenchymal stem/stromal cell
eUC-MSC CM	Equine Umbilical cord mesenchymal stem/stromal cell conditioned medium
Flex	flexed
FSE	Fast scan echo
G	Gauge
ILGRE	Gradient recalled echo
IA	Intra-articular
ICBAS-UP	Instituto de Ciências Biomédicas Abel Salazar – Universidade do Porto
IL	Interleukins
IV	Endovenous
Kg	Kilogram
Kv	Kilovolt
LF	Left forelimb
LM	Lateromedial
mA	milliampere
MC3	Third Metacarpus
MCj	Metacarpophalangeal joint
mg	milligrams
min	minutes
mL	milliliter
MRI	Magnetic Resonance Image
MSCs	Mesenchymal Stem/Stromal Cell
OA	Osteoarthritis
ORBEA	Organismo Responsável pelo Bem-estar Animal
P1	First phalanx
PLDMO	Oblique palmarolateral-dorsalmedial
PMDLO	Oblique palmaromedialdorsolateral
SID	Once a day
SM	Synovial membrane
SM-MSC	Synovial Membrane Mesenchymal Stem/Stromal Cell
STIR	Short Tau Inversion Recovery
TW	Time weighted image
UC	Umbilical cord
UC-MSC MC	Umbilical cord mesenchymal stem/stromal cell conditioned medium
Vet-check	Veterinary check-up
W	weight
WJ	Wharton Jelly
X-ray	Radiograph

GRAPHICAL PAPER IV

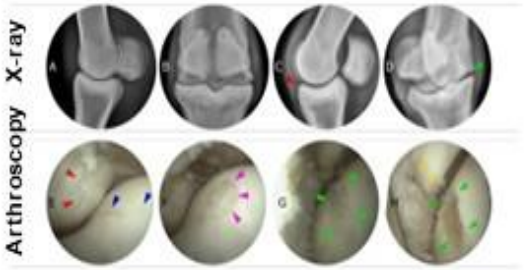
4 Case report: equine metacarpophalangeal joint partial and full thickness defects treated with allogenic eSM-MSC + UC-MSC CM

B eSM-MSCs + eUC-MSC CM

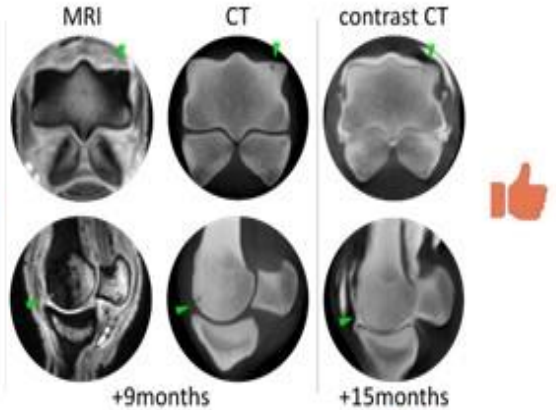
- Stallion, 5 yo, showjumper,
- Acute lesion of the right metacarpophalangeal joint

- Lameness grade 4/5
- Swollen fetlock
- **X-ray** and AINE's for 3 days

- Arthroscopy

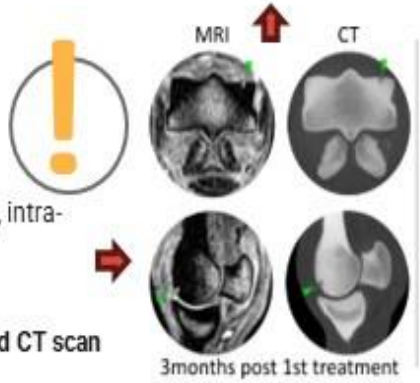


- 4 partial-thickness articular defects
- 1 full-thickness articular defect



Treatment protocol

2nd Tx round



Treatment protocol

1st Tx round

- 2 doses of $1x10^7$ eSM-MSCs + e UC-MSC CM, 2 ml, intra-articular,15 days apart.
- 90 days of physical rehabilitation program.
- Corrective shoeing
- Clinical and imaging assessments : X-ray + **MRI and CT scan**

CHAPTER V

CHAPTER V

- **GENERAL DISCUSSION**
- **CONCLUSION AND FUTURE DIRECTIONS**

CHAPTER V GENERAL DISCUSSION

Musculoskeletal injuries represent a high percentage of wastage among equine and human athletes. The healing of such injuries usually occurs through fibrosis, reducing therefore the tissue structure, affecting its functionality and consequently the well-being, quality of life and performance of our patients. Several conservative therapeutical protocols were developed in order to control pain and inflammation but do not achieve the intended regeneration of these injured structures. Regenerative medicine intends to give a propel in this area, creating new therapeutical answers – the orthobiologics - to fill in the currently existent gap among orthopaedic injuries treatment.

The present work presents the development and the clinical application of, firstly, a product combining equine synovial membrane mesenchymal stromal/stem cells (eSM-MSC) with autologous serum and, in a second phase, the development of a new combination of eSM-MSC and umbilical cord mesenchymal stromal/stem cells conditioned medium (UC-MSC CM). The collection, isolation, culture and characterization of the eSM-MSCs and UC-MSCs was successfully accomplished.

eSM-MSCs have garnered interest among researchers in cellular and cell-based therapies for their ability to enhance tissue regeneration, notably in the repair of articular structures (cartilage and subchondral bone), tendons and ligaments. A significant challenge lies in the comprehensive characterization of eSM-MSCs, as they are less extensively researched compared to mesenchymal stromal/stem cells (MSCs) from other tissues and species, particularly human MSCs. Nonetheless, this study has validated eSM-MSCs and UC-MSCs stemness and origin through various methods, including trilineage differentiation, karyotyping, secretome analysis and immunohistochemistry. The cellular cultures exhibited a monolayer formation, the ability to adhere to plastic, and a fibroblast-like morphology, meeting several of the international society for cell therapy (ISCT) minimal criteria (Fülber et al., 2016; Gale et al., 2019; Murata et al., 2022; Zayed et al., 2021). Successful osteogenic, chondrogenic, and adipogenic differentiation was also confirmed (De Bari et al., 2001). The karyotype of eSM-MSCs presented genomic variations as the number of passages increased, aligning with findings from other studies that observed genomic changes across cell passages (Lupski, 2013; Peterson & Loring, 2014; Rohrback et al., 2018; Thorpe et al., 2020; Vattathil & Scheet, 2016). DNA replication is a critical event for genome duplication. Genome instability, incurred during the process of stem cell isolation, culture expansion, and reprogramming, might be the most critical point of a stem cell-based therapeutic approach as a viable option in the clinical

perspective (Prieto González & Haider, 2021). However, there is very little evidence linking genomic abnormalities, for example, in human Pluripotent Stem Cell (hPSCs) with tumorigeneses (Peterson & Loring, 2014). The frequency and effects of variations is increasing with the development of even more sensitive methods for detecting genomic variation (Lupski, 2013). Thus, it is crucial to be cautious, and minimizing culture times as much as possible is advisable to prevent safety issues (Neri, 2019). More studies must be performed in this area.

As evidenced before, eSM-MSCs are able to promote tendon healing by reducing inflammation and fatty infiltration, stimulating cell proliferation and tenogenic differentiation (Wang et al., 2022). eSM-MScs also present chondrogenic superiority, being considered key contributors to maintaining joint homeostasis, becoming good candidates for cartilage repair (De Bari et al., 2001; Kubosch et al., 2018). In the present work, eSM-MSCs secretome revealed high level presence of pro-inflammatory, pro-angiogenic and fibroblastic factors responsible for tendon, ligament and cartilage regeneration.

Throughout the progression of our research, a novel combination that incorporates a cell-based product – CM - was developed. During the last decade it has been shown that MSCs therapeutic effectiveness is due mainly to the release of paracrine factors, composed of soluble (cytokines, chemokines and growth factors) and non-soluble factors (extracellular vesicles) that are primarily secreted in the extracellular space by MSCs (Mocchi et al., 2020). When in culture, these cells release these factors into the culture medium, constituting the conditioned medium. CM paracrine signaling can be considered as the primary mechanism by which MSCs contribute to healing processes, presenting a promising opportunity for development into medicinal products for use in regenerative medicine (Al Naem et al., 2020; Madrigal et al., 2014). Even though there are very few clinical reports in veterinary medicine, recent acquisitions suggest that MSCs-derived products may have major advantages compared to the related cells, e.g., they are considered safer and less immunogenic (Mocchi et al., 2020; Zhang et al., 2008). The concept of creating a regenerative therapeutic product combining UC-MSC CM with eSM-MSC was particularly attractive, aiming for the synergistic benefits offered by both cell types.

UC-MSCs represent the most studied source of MSCs, being a good candidate for regenerative therapies. Its harvest is easy to perform and non-invasive. In fact, UC-MSC presents a more primitive state, compared with adult MSCs, with unique immunosuppressive properties, evading host rejection and making them valuable tools for cell therapies. This was one of the main reasons why we used their CM. They provide good immunogenic abilities, both cell-to-cell contact and soluble factors, as well as anti-inflammatory properties (Mebarki et al., 2021; Sriramulu et al., 2018; Yea et al., 2023). It has also been demonstrated that UC-

MSC CM has the ability to differentiate cells into various lineages, and their paracrine effects aid in the replacement of damaged cells. Its CM can enhance the efficacy of cell-based therapies as it is enriched with higher levels of growth factors and chemokines, typically creating a chemoattractive environment for the surrounding tissues (Sriramulu et al., 2018).

In light of these principles, a novel therapeutic combination was developed and used in the treatment of desmitis and articular defects.

Building on our findings, multiple studies have shown that tendons treated with MSCs not only heal more effectively but also do so in a shorter timeframe compared to traditional conservative treatments, which often result in the formation of fibrous scar tissue rather than fully regenerated tissue (Shokry et al., 2020). Additionally, regarding articular cartilage, the outcomes suggest that employing this combination might regenerate cartilage. Furthermore, clinical observations indicate that all treated horses returned to full functionality without any instances of reinjury (Cook et al., 2010; Guest et al., 2022; Smith & Webbon, 2005). Osteoarthritis (OA) control also presented as a positive result, with imaging assessments revealing no progression of OA. Along several imaging evaluations, the condition was deemed "stationary." Literature supports this finding as it is demonstrated that MSCs are effective in early pain reduction and had a protective effect on the development of cartilage damage in a murine OA model, by using the regenerative capacities of the MSCs-secreted factors (Khatab et al., 2018).

To summarize, based on our current understanding, the primary benefits of this innovative therapeutic strategy include:

1. The application of eSM-MSCs along with autologous serum yielded highly positive outcomes in the regeneration of tendons and ligaments, as evidenced by excellent imaging and clinical results. However, throughout this research, a new combination was hypothesized to further enhance regenerative capabilities of both cell-types.
2. The synergistic effects between eSM-MSCs and UC-MSC CM, revealed to be important to improve musculoskeletal regeneration, as auspicious results were achieved in lesions very difficult to treat and with poor prognosis to return to athletic performance (desmitis of the tarsus long collateral medial ligament and full and partial thickness articular defects of a fetlock). It must be highlighted that cartilaginous regeneration is still in its most embryonic stage and the literature on this subject is very scarce.

3. The allogeneic composition of the product, enabling an acute clinical treatment. Also, the introduction of the UC-MSC CM was important in this allogeneic scenario due to their recognized immunomodulatory/immunosuppressor effect enabling an universal donor.
4. The availability of a cell and secretome bank, created by this research group, that allows prompt intervention.
5. The straightforwardness of its medical application, resembling intratendinous or intra-articular injections.
6. The low number of cells dosage: $1x10^7$ eSM-MSCs and low number of administrations (2 in tendons and ligaments and 4 in cartilage and subchondral bone).
7. The clinical outcome: all patients recovered their function and achieved pre-injury performance, having some overpassed the previous sportive level;
8. The imagiologic outcomes: structures acquired their original imagiologic appearance.
9. The patient recovery period is shorter than what is reported in the literature for the same musculoskeletal pathologies when treated with other regenerative methods.
10. The follow-up period indicates there were no clinical relapses or injury recurrence.
11. The innovative nature of the therapeutic combination and the obtained clinical outcomes places it in a superior position within the spectrum of orthobiologic treatments.

The weaknesses of this study arise from the small number of horses treated with this innovative combination, indicating a need for more clinical research, in order to obtain more robust, consistent, and reliable results. Also, the findings are based on observational studies, which do not substitute for the rigor of controlled clinical trials with control groups allowing the establishment of statistical results. However, the results accumulated so far, have provided evidence that veterinary patients affected by naturally occurring diseases should provide more reliable outcomes of cell therapy than laboratory animals. (Ribitsch et al., 2020). Additionally, the availability of tissue source presents a challenge to this approach.

While conscious that the presented data is not sufficient to discern the real contribution of the various interveners present in this formulation to tendinous and osteochondral healing and regeneration, this regenerative approach appears as a very promising option for treating

tendonitis, desmitis, osteochondral lesions and preventing osteoarthritis progression in equines.

It must be highlighted that a multidisciplinary approach should always be considered essential. A tailored physical rehabilitation program, specific to the type of injury, must be implemented. Additionally, proper shoeing of the affected limb is crucial for correcting the horse's balance and biomechanics. Equally important is the provision of proper nutritional and vitamin supplementation, alongside with good horsemanship practices.

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CONCLUSION AND FUTURE PERSPECTIVES

The therapeutic combination formulated and developed in this doctoral thesis is unique and was designed considering the distinctive characteristics of each involved cell type. The differences between these cell types and how they could complement each other formed the basis of this formulation, aiming to combine the best musculoskeletal regenerative capacities with the immunomodulatory potential of UC-MSC CM. In our perspective, this is what sets this therapeutic product apart from others currently available.

Also, the goals we set out to accomplish were successfully met, encompassing the laboratorial aspects of collection, isolation, culture and characterization of the two cellular types as well as their cryopreservation and posterior therapeutic product preparation. Also, when applying the developed products, the therapeutical administration to the patients, the rehabilitation programs, the clinical and imaging outcomes as well as the patient follow-up were also successfully accomplished. No adverse effects were reported in any patients. Follow-up monitoring indicated that equine athletes maintained or exceeded their previous performance levels, while leisure horses maintained a quality of life free from pain, inflammation, lameness, and the need for anti-inflammatory medication – **Figure 1**.

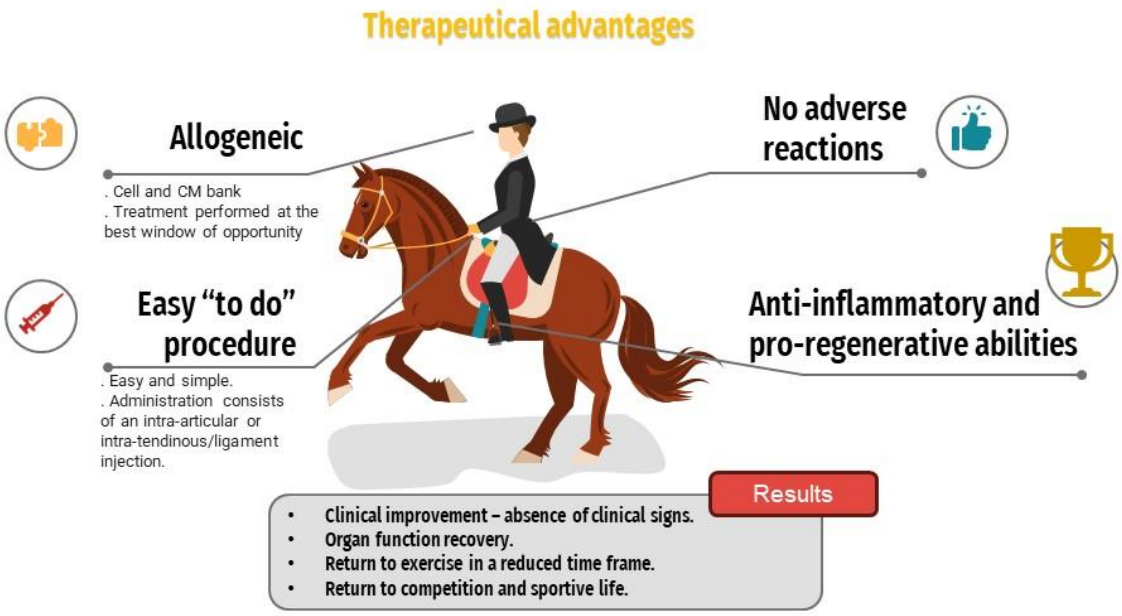

Figure 1. Therapeutical advantages of the regenerative formulations developed during this process.

While conscious that the presented data is not sufficient to discern the real contribution of the various interveners present in this formulation to tendinous and osteochondral healing and regeneration, this regenerative approach appears as a very promising option for treating tendonitis, desmitis, osteochondral lesions and preventing osteoarthritis progression in equines. The patients recovered full functionality and did not need any type of pharmacological control for the treated lesions after the institution of the regenerative treatment.

Currently, the primary hurdle facing regenerative medicine within the domain of sports medicine is the attainment of consistent clinical outcomes. Promising results in the realm of tissue repair and regeneration, particularly due to the advantageous properties of MSCs, are evident in experimental studies with horses. However, further research, clinical trials and randomized studies are imperative to validate efficacy and establish precise guidelines for clinical implementation.