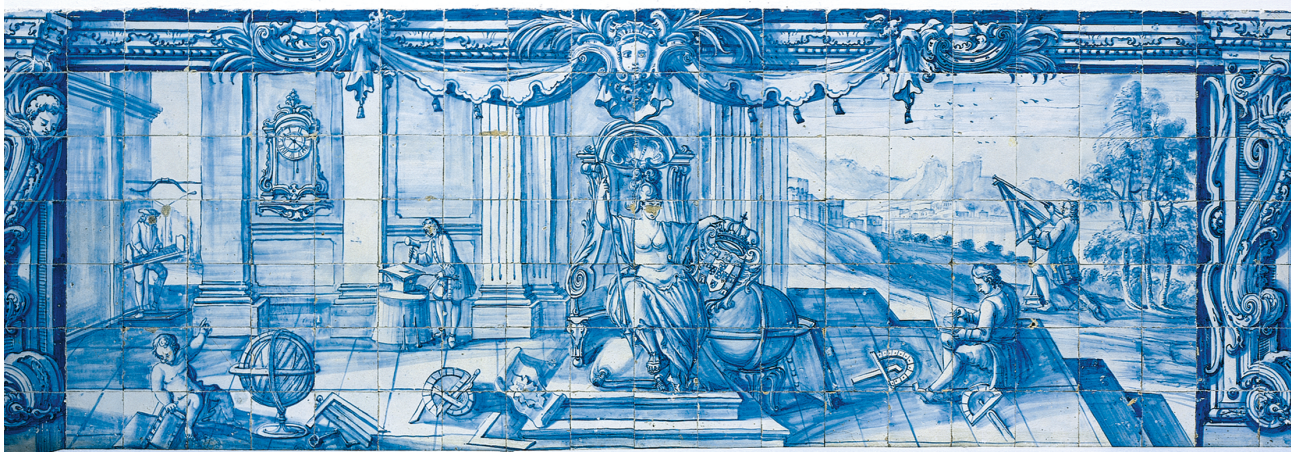
DISTAL LIMB OSTEOARTHRITIS IN THE HORSE

Susana Oliveira Serrano Monteiro

Tese apresentada à Universidade de Évora
para obtenção do Grau de Doutor em Ciências Veterinárias

ORIENTADORES: *Prof.ª Doutora Elisa Maria Varela Bettencourt*
Prof.ª Doutora Maria Manuela Melo Oliveira
Prof. Doutor Olivier M. Lepage

ÉVORA, JANEIRO 2016



INSTITUTO DE INVESTIGAÇÃO E FORMAÇÃO AVANÇADA

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INDEX

Abstract

The aim of this thesis was to study two objective methods of osteoarthritis (OA) diagnosis in horses and use them on the assessment of new intra-articular treatments. The studied methods were a new inertial-sensor based system of lameness detection and cartilage biomarkers in serum. It was found that distal limb flexion is significantly correlated to the presence of metacarpo-phalangeal OA in hind limbs and that inertial-sensors are sensitive in detecting asymmetry in these cases. A positive and significant correlation was observed between Coll2-1 concentration in serum and the presence of joint disease in males and young horses. Fib3-2 measurement has good potential to be used since it is not influenced by sex or age. Using an experimental model of OA, adipose stem cells pre-activated with interferon-gamma decreased joint inflammation and radiographic lesions. In clinical cases, a single injection of high-concentrated and high-molecular weight hyaluronic-acid decreased joint inflammation and biomarkers' concentration.

Key words: equine; osteoartrithis; objective diagnosis; intra-articular treatment

OSTEOARTRITE DO MEMBRO DISTAL NO CAVALO

Resumo

A finalidade desta tese foi estudar dois métodos de diagnóstico objetivo de osteoartrite (OA) em equinos e aplicá-los na avaliação de novas terapias intra-articulares. Utilizou-se um sistema de sensores de movimento e foi avaliada a concentração de biomarcadores de cartilagem no soro. Concluiu-se que a flexão distal positiva está correlacionada com OA na articulação metacarpo-falângica nos membros posteriores e que os sensores são sensíveis na detecção de assimetria nestes casos. Existe uma correlação positiva e significativa entre as concentrações de CollI2-1 e a presença de doença articular, sobretudo em machos e jovens. A dosagem de Fib3-2 tem utilidade por não ser influenciada pelo sexo nem idade. Num modelo experimental da doença, a terapia à base de células estaminais reduziu a inflamação articular e as lesões radiográficas. Em casos clínicos, o tratamento com ácido-hialurónico de alta concentração e peso molecular provoca uma diminuição da inflamação articular e dos biomarcadores no soro.

Palavras-chave: equino; osteoartrite, diagnóstico objetivo; tratamento intra-articular

Dedication

To my daughter Marisa

Funding acknowledgments

- Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM) supported the 3 months externship in the VetAgro Sup – Veterinary Campus of Lyon (France).
- This work was funded by Luso-French integrated actions (LF-IA) 2013/2014 and 2014/2015:
  - Travels and Lameness locator transportation between Lyon and Lisbon was funded by LF-IA: JLS/2013.210 and CT R08 (Vetagro Sup – Veterinary Campus of Lyon).
  - Participation in the 24th Annual Meeting of ECVS (Berlin 2015) was funded by LF-IA: TC nº14/14 (Universidade de Évora).
  - Participation in the First European Stem Cell Symposium for vets (Maastricht 2015) was funded by LF-IA: 2014-C17 (Universidade de Évora)
- Part of this work (biomarkers analysis) was funded by FEDER Funds through the Operational Programme for Competitiveness Factors - COMPETE and National Funds through FCT - Foundation for Science and Technology under the Strategic Projects PEst-C/AGR/UI0115/2011, PEst-OE/AGR/UI0115/2014 and through the Programa Operacional Regional do Alentejo (InAlentejo) Operation ALENT-07-0262-FEDER-001871/ Laboratório de Biotecnologia Aplicada e Tecnologias Agro-Ambientais.
- The lameness locator and the study about the use of allogeneic pre-activated mesenchymal stemcell was funded by the GREMERES (Groupe de Recherche en Médecine Et Rééducation des Equidés de Sport), VetAgro Sup – Veterinary Campus of Lyon (France).
- ARTIALIS Laboratory collaborated in biomarkers' analysis study.
- AZEVEDOS Laboratory collaborated in the hyaluronic acid clinical study.

Personal acknowledgments

To Dr. Elisa Bettencourt for being my supervisor, she is the main responsible for this part of my academic life, always transmitting energy and optimism, essential for this work.

To Dr. Olivier M. Lepage for accepting to be my co-supervisor, adding intellectual and scientific stimulus, sharing the innovative techniques I have learned and used during this work. I would also like to thank all other colleagues and staff of the VetAgro Sup - Veterinary Campus of Lyon and Sanofi-aventis, Montpetlier, for the multidisciplinary work we did together.

To Dr. Manuela Oliveira for being my co-supervisor and for having taught me everything I know about statistics and adding criticism to the data collection and analysis performed, which was very important to this research.

To all my colleagues and students at Universidade de Évora for their assistance, a special thanks to Dr. Liliane Damásio, Dr Luís Antunes and Dr Sandra Branco.

To Dr. Maria José Correia and Eng. António Saraiva for sharing the Unidade Clínica of Fundação Alter Real, Alter do Chão, and to be willing to contribute with the genetic background of these animals.

To Dr. Rui Martelo, Dr Ricardo Romão, Dr Joana Rua and staff of the Vetal Veterinary Clinic, Portalegre, for sharing their patients and be willing to participate in this project.

To Dr. João Borges, Dr. Gonçalo Paixão and the Hospital Militar de Equinos – Escola de Armas, Mafra, for sharing their patients, knowledge and interest in equine lameness evaluation and osteoarthritis treatment.

To Dr Kevin Keegan for assistance in interpretation of lameness locator results.

To António Bettencourt and Graciete Rabaça for language assistance.

To Dr. Dulce Gomes for assistance in part of the statistical analysis.

To my family, for believing and allowing me to follow my dreams, nothing would be possible without them and in particular to my mother for being such an extraordinary person.

To my husband Pedro for his support at all moments and assistance in the graphic part of the work and my daughter Marisa that was born and grew together with this project, both have brought love and a different meaning to my life.

To all my friends…

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List of abbreviations

Abbreviation	Unabbreviated	Abbreviation	Unabbreviated
AAEP	american association of equine practicioners	Hmin	difference in head minimum height between left and right
ADAMTS	a desintegrin and metalloproteinase with thrombospondin motifs	HMW	high molecular weight
ANOVA	analysis of variance	IA	intra-articular
APMVE	portuguese association of equine veterinarians	ICAAM	mediterranean agricultural and environmental sciences institute
ASCs	adipose tissue- derived mesenchymal stem cells	IFN-γ	interferon-gamma
ASCs-γ	ASC activated with interferon-gamma	IL	interleukin
AUC	area under the curve	IM	intramuscular
CD	clusters of differentiation	IQR	interquartile range
cDNA	complementary deoxyribonucleic acid	IV	intravenous
cm	centimeters	κ	kappa agreement
Coll2-1	type II collagen peptide (108HRGYPGLDG116)	kDa	kilodalton
Coll2-1NO_2	nitrated form of Coll2-1	kg	kilogramme
CO_2	carbon dioxide	L	liter
COX	ciclooxygenase	MAD	median absolute deviation
Da	daltons	MC3	third metacarpal bone
DIP	distal interphalangeal	MCP	metacarpophalangeal
DMEM	dulbecco's modified eagle's medium	µg	microgramme
ECM	extracellular matrix	µL	microliter
ECVDI	european college of veterinary diagnostics and imaging	mg	miligramme
ECVP	european college of veterinary pathology	min	minutes
ECVS	european college of veterinary surgeons	mL	mililiter
EDTA	ethylenediamine tetraacetic acid	mm	milimiter
ELISA	enzyme like immunosorbent assay	mM	milimolar
Fib3-1	fibulin 3 fragment 1	MSCs	mesenchymal stem cells
Fib3-2	fibulin 3 fragment 2	MPA	methylphrednisolone acetate
g	gramme	MMP	metalloproteinase
ga	gravitational force	mRNA	messenger ribonucleic acid
G	gauge	*n*	number
GAG	glicosaminoglycan	ng	nanogramme
GLM	generalized linear model	nM	nanoMolar
HA	hyaluronic acid	NSAIDs	non-steroidal anti-inflammatory drugs
Hmax	difference in head maximum height between left and right	OA	osteoarthritis

OARSI	osteoarthritis research society international	qRT-PCR	quantitative real-time polymerase chain reaction
OCD	osteochondrritis dissecans	r	pearson's correlation coefficient
OSM	oncostantin-M	r^2	coefficient of determination
P	calculated probability	RNA	ribonucleic acid
P1	first phalanx	ROC	receiving operating characteristic curve
PBS	phosphate buffered saline	rpm	rotation per minute
PDmax	difference in pelvis maximum height between left and right	SAS	statistical analysis software
ΔPDmax	PDmax change before and after flexion	SCB	subchondral bone
PDmin	difference in pelvis minimum height between left and right	SD	standard deviation
ΔPDmin	PD min change before and after flexion	SEM	standard error of the mean
pg	picogramme	SF	synovial fluid
PGE2	prostaglandin E2	SPSS	statistical package for the social sciences
PIP	proximal interphalangeal	TNF-α	tumor necrosis factor alpha
pM	picomolar	TA	triamcinolone acetonide
PRP	platelet rich plasma	TP	total proteins
PSB	proximal sesamoid bones	VS	vector sum (total head asymmetry)
PSGAG	polisulphate glycosaminoglycan	ΔVS	vector sum change change before and after flexion

INTRODUCTION

Lameness, and particularly osteoarthritis (OA), is still one of the main reasons for early retirement of horses and economic losses in the equine industry. Articular cartilage is the main component of a joint and is responsible, together with synovial fluid (SF), for the frictionless movement. OA is characterized by degeneration and loss of cartilage, which occurs when catabolic pathway overcomes the anabolic process (Frisbie, 2012). The general objective of this thesis was to advance further the diagnosis of OA and at treatment knowledge in horses. We used two objective diagnostic methodologies of OA: lameness detection with intertial sensors technology and analysis of serum cartilage biomarkers in combination with traditional subjective lameness assessment and radiographic examination. We used these methods to assess two novel intra-articular (IA) treatments: adipose tissue-derived allogeneic mesenchymal stem cells (ASCs) pre-activated with interferon-gamma (IFN-γ) on experimental induced OA and a high concentrated and high molecular weight (HMW) hyaluronic acid (HA) on naturally occurring disease.

Osteoarticular pain is the key of OA disease assessment but it is difficult to quantify in horses. A recent method appeared in order to objectively determine the amount of asymmetry of the four limbs. This system uses wireless transmission of data from small inertial sensors attached to the horse's body. Keegan *et al*. first introduced this technology in 2002 and its repeatability and sensitivity has been demonstrated (Keegan *et al*., 2011; McCracken *et al.,* 2012). In this research we have objectively evaluated lameness and response to IA treatments using this technology, owned by the VetAgro Sup – Veterinary Campus of Lyon. The PhD student spent 3 months in this institution, learning how to use this technology while testing pre-activated ASCs with IFN-γ (ASCs-γ) IA treatment.

Mesenchymal stem cells (MSCs) have reparative effects, due to their differentiation properties and to paracrine actions, but they need to be activated by inflammatory mediators released from immune cells. Pre-activation with IFN-γ enhances the therapeutic activity of MSCs in animal models of colitis and graft versus host disease (Polchert *et al*., 2008; Duijvestein *et al.,* 2011). Therefore, pre-activation of MSCs could lead to a more efficient therapeutic activity than unstimulated cells, initiating regeneration and repair of hyaline cartilage. Murine collagenase induced OA models showed that a single injection of autologous ASCs reduced synovitis, enthesophyte formation and decreased score of cartilage lesions (ter Huurne *et al*., 2012). Furthermore, ASCs have recently shown a protective effect in human cartilage *in vitro* by preventing chondrocyte apoptosis and fibrosis (Maumus *et al*., 2013). Our objective was to study the effects of INF-γ pre-activated or "primed" allogeneic ASCs in an experimental OA model in horses. The preliminary results of this experiment were orally presented at the I congresso da Associação Portuguesa de Médicos Veterinários de Equinos (APMVE), Lisboa 2014, in the 24th Annual Meeting of the European College of Veterinary Surgeons (ECVS), Berlin 2015 (Appendix A: abstract) and at the 1^{rst} European Stem Cell Symposium for vets, Maastricht 2015.

To achieve early recognition of disease, equine research, as a valid model for human OA, has focused on biomarker analysis. Verwilghen *et al.* (2011) correlated higher OA advanced degenerative

lesions with increased type II collagen 108HRGYPGLDG116 peptide (Coll2-1) in SF and severity of radiological lesions with increased Coll2-1 nitrated form (Coll2-1NO_2) in horse blood (Verwilghen *et al*., 2009). In humans, Fibulin 3 peptide fragment 1 (Fib3-1) and fragment 2 (Fib3-2) have recently shown to be elevated in serum of OA patients and differentiate between OA and normal populations (Henrotin *et al*., 2012) but have never been studied in horses. In this project we have studied Coll2-1, Coll2-1NO_2 and Fib3-2 in naturally occuring OA in 51 Lusitano horses. The abstract have been accepted at the poster session of the 25th Annual Meeting of the ECVS (Lisboa): Susana Monteiro, Olivier M Lepage, Luís Antunes, Liliane Damásio, Sandra Branco, Manuela Oliveira, Elisa Bettencourt. Relationship between biomarkers of cartilage in serum and degenerative joint disease in Lusitano horses. Using the same Lusitano horse population, subjective lameness assessment was compared to objective lameness detection and related to radiographic distal limb OA findings. The results have been presented at the V Congreso Annual de La Asociación de Veterinarios Especialistas en Équidos de España (AVEE), WEVA Intermadiate meeting España-Portugal (Madrid): Cómo están relacionados los hallazgos radiográficos de la extremidad distal con la evaluación objectiva de cojera.

Finally, a group of eight horses presenting clinical OA were treated with a single injection of HMW-HA and objectively assessed for lameness and serum biomarkers' concentration. Hyluronic acid is injected due to viscosupplementation, light anti-infllammatory properties (Takahashi *et al*., 1999) and disease-modifying effects (Marshall *et al*., 2000). On the other hand, Coll2-1 and Coll2-1NO_2 biomarker have been shown to be significantly decreased in serum of OA human patients after HA treatment (Henrotin *et al*., 2013). High molecular weight HA is reported to have superior cartilage-sparing properties in OA models (Asari *et al*., 1998), to enhance *in vitro* lubricant function (Antonacci *et al*. 2012) as well as to induce better clinical improvement (Philips, 1989) when compared to the lower molecular weight.

This thesis is divided in seven chapters. Chapter I contains a brief review on distal limb OA pathogenesis, diagnosis and conventional OA treatment. An extensive review of IA biologic therapies was published: Monteiro SO, Bettencourt EV, Lepage OM. Biologic strategies for intra-articular treatment and cartilage repair. J Eq Vet Sci 2015; 35:175-190 (Appendix B). Chapter II includes the comprehensive research work, which presents the results of a survey to Portuguese equine veterinarians about OA (Appendix C: questionnaire) and the preliminary results of the effects of equine allogeneic ASCs-γ *in vitro* (a collaborative study). The fundamental research work is presented in the following four chapters and consisted in testing ASCs-γ treatment on experimental OA *in vivo* (Chapter III), evaluating cartilage biomarkers in serum (Chapter IV) and studying lameness detection (Chapter V) in a Lusitano horse population and finally assessing the effect of a single injection of HA treatment on clinical cases of OA using both objective methodologies (Chapter VI). A general conclusion is presented at the end of the thesis.

We believe that veterinarian researchers should focus their work on objective methodologies and that biologic strategies will play an important role in equine OA therapy. This PhD thesis is just a piece of the puzzle.

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CHAPTER I – Review of literature

1. Articular biology of equine distal limb joints

Joint health is of major importance for horses' performance and survival. The equine distal limb is made up of diarthrodial joints that allow smooth articulation of 2 bone ends. The joint capsule supports the synovial structures and is made up of a fibrous tissue surrounded by tendons and colateral ligaments. Below the capsule there is a thin synovial membrane with 3 types of cells: synovial type A cells that have a phagocytic function; type B cells that produce hyaluronic acid (HA) and an intermediate celular type (C cells). The gaps between synoviocytes, the absence of a basal membrane and the presence of subsynovial blood vessels allow exchange of molecules under 10 kilodalton (kDa) between plasma and sinovyal fluid (SF) (Todhunter, 1996b). Synoviocytes are also able to produce inflammatory mediators, making the synovial membrane an important structure during joint inflammation (Briston *et al.*, 2010).

Hyaline cartilage covers the bone extremities and is made up of a small population of chondrocytes distributed in the extracelular matrix (ECM). This avascular connective tissue is especially developed for shock absorption and weight bearing (Palmer & Bertone, 1996), being highly dependent on the macromolecular arrangement (Kempson, 1980). The collagen molecules make a cross-linked network together with HA molecules. HA is connected by link proteins to proteoglycan aggrecans, which are negatively charged by their polyanionic glycosaminoglycan (GAG) sidechains: keratan and chondrotin sulphate (Ogston, 1970). This allows an osmotic swelling pressure, attracting water and expanding the collagen network, which results in a highly stiff but resilient tissue (Ogston, 1970; Eyre & Wu, 1995; van Weeren & Brama, 2001). The articular cartilage has a non-calcified section, which is divided in a superfitial tagential zone, where chondrocytes and collagen fibrils are horizontally oriented, an intermediate and deep zone, where they are vertically oriented and consequently less resistant. The underlying calcified section is separated by a tidemark from the subchondral bone (SCB) that consists of a dense cortical bone plate followed by a trabecular bone. SCB provides a structural support to the joint and vascular supply to the immature cartilage.

Viscosity of SF is due to high HA concentration, acting like a lubricant that reduces friction, improves joint motion and plays a role in joint homeostasis. This joint component is excellent for joint health assessment both in clinical and research settings; moreover, the correlation between concentration of small molecules in SF and plasma encourages research based on the evaluation of these molecules in the blood (van Weeren & Firth, 2008).

Equine distal limb has 3 synovial joints, 2 high-motion: metacarpo/tarsal-phalangeal (fetlock) and distal interphalangeal (DIP) joint and 1 low-motion: proximal interphalangeal (PIP) joint (Sisson, 1986).

2. Pathogenesis of equine OA

OA is the most frequently used term for joint disease and is specifically defined as degeneration and loss of articular cartilage, it is also known as osteoarthrosis or degenerative joint disease. The terminology debate is present because an inflammatory feature is not always present. However, inflammation is considered to be the key of OA pathogeny and the term osteoarthritis will be consistently used in this text. Equine degenerative arthritis was first reported in 1938, after comparing human and equine cartilage (Callender & Kelser, 1938). Although not life-threatening, OA is a disabling disease representing a very important economic burden.

The joint is a complex organ and there are several ways in which traumatic damage can occur, eventually resulting in cartilage degradation. Nowadays, it is recognised that OA can be the consequence of numerous disorders affecting not only the cartilage (Brandt *et al.,* 2006) but other joint-associated tissues, like synovial membrane and subchondral bone (SCB). OA can also be defined as a "failed repair of damage that has been caused by excessive mechanical stress (defined as force/unit area) on joint tissue" (Brandt *et al*., 2009). This disease can be triggered by several factors, including genetics, molecular and biochemical changes of cells and ECM, or be secondary to conditions like osteochondritis dissecans (OCD), synovitis/capsulitis, desmitis and subchondral bone disease. Cartilage can also be disrupted by intra-articular (IA) fractures. Direct trauma can cause ulcerative lesions and cyclic fatigue damage can be responsible for collagen network injury. It is currently accepted that synovitis and capsulitis contribute to the degenerative process through the release of enzymes, inflammatory mediators and cytokines (Mcllwraith & Van Sickle, 1981; Briston *et al.*, 2010). Subchondral bone disease can also lead to secondary cartilage damage due to the loss of support and the release of cytokines or the decrease of shock absorption, in case of bone sclerosis (Lories & Luyten*,* 2011; Pan *et al.,* 2012).

OA is primarly trigerred by interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNFα), identified as the major pro-inflammatory cytokines in joints (Smith *et al*., 1997; Kappor *et al.,* 2011). Secretion of inflammatory mediators like substance P and prostaglandin E2 (PGE2) will induce further release of other pro-inflammatory cytokines (IL-6, IL-17, IL-18), chemokines and other inflammatory mediators such as nitric oxide, oncostatin-M (OSM) and leukemia inhibitory factor (Goldring, 2000). Trauma can, in fact, initiate the degradation process or cause a direct physical defect. This will initiate both IL-1β and (TNFα) release, up-regulating metaloproteinase (MMP) production by synoviocytes. MMP1 interstitial collagenase, MMP3 stromelysin and MMP13 (collagenase 3), disintegrin-like and metalloproteinase with thombospondin type 1 motifs 4 and 5 (ADAMTS 4 and 5) are the most important catabolic MMPs that are responsible for ECM degradation (Lefebvre *et al.*, 1990; Mengshol *et al*., 2000; Tortorella *et al.,* 2001). Together with down-regulation of synthesis, articular cartilage matrix components, like collagen II, aggregan and small proteoglycan are destroyed (Chadjichristos *et al*., 2003; Seguin & Bernier, 2003).

3. Diagnosis of equine OA

Cartilage lesions can be characterized by local splitting and fragmentation (fibrillation) of cartilage and are usually accompanied by synovitis and joint effusion (Jaffe, 1972). Degenerative lesions were at first not well correlated with the evidence of pain but cartilage wear lines were commonly seen (Nilsson, 1973). In horses, any joint can be affected, but, OA is more commonly found in distal limb joints (interphalangeal joints and metacarpo/tarsophalangeal joints), carpus, tarsus and stifle. A single joint can be affected but frequently several joints are affected at the same time (Kidd *et al*., 2001).

As previously mentioned, the equine distal limb has three synovial joints and there are differences between high and low-motion joints. High-motion joints frequently exhibit synovitis, cartilage erosion, subchondral sclerosis and capsular fibrosis. Low-motion joints usually show little evidence of synovitis but frequently present full thickness cartilage necrosis with little erosion but subchondral lysis, due to resorption of the subchondral bone by osteoclasts, generally progreding towards ankylosis (Pool, 1996).

Despite OA affecting most prominently the cartilage, clinical signs are essentially related to joint inflammation and pain. The inflammation role is controversial but it is accepted to be present at least in the initial stages of the disease. The most commonly complaints presented by the owner are slowly progressive lameness, often bilateral, and poor performance. Besides clinical history, it is essential to perform a thorough physical examination. It is common to find variable joint effusion and, at advanced stages of the disease, bone remodelling is sometimes associated to limited range of joint motion (Kidd *et al*., 2001). To confirm the origin of pain veterinarians should perform a complete lameness examination. The following section briefly describes some of the limitations of subjective assessment and introduces a new objective technology for lameness detection in horses.

3.1 Lameness evaluation

Lameness scales have been developed in order to improve communication among veterinarians, with their clients and to better assess and quantify the clinical improvement (Ross, 2003). Despite the routine application of these scales by equine practitioners, several confounding factors can influence their application in practice, namely the horse temperament and the veterinarians' experience (Keegan, 2007). In recent years, subjective evaluations have been assessed and compared to new objective techniques. It has been found that subjective agreement for mild lameness is low, so, detection and evaluation of the most lame limb should not be performed by more than one evaluator, when doing clinical trials or when studying other lameness evaluation methods. Moreover, agreement is not improved by lungeing and flexion test compared to straight line alone, nor by videotaped recordings compared to real time evaluation (Keegan *et al*., 2004).

For an objective detection of lameness, we can use two different approaches: the kinematics, that describes motion using a treadmill, stationary cameras and passive markers and the kinetics, that studies the action of forces using stationary force plates or force-measuring treadmills (Keegan, 2007). The wireless, inertial sensor-based system device was first described as a method of continuous

lameness quantification, that could be used in the field (Keegan *et al*., 2002). This device consists of 2 single-axis acceleration sensors, one attached to the head and another between the tubera sacrale, and 1 single-axis piezoelectric, gyroscopic sensor, attached to the right front pastern (Figure 1). Lameness is detected and quantified by analysing the patterns of vertical head (Figure 2A) and pelvic (Figure 2B) movement during trot movement (Keegan, 2004). Data is then transmitted wirelessly and analysed using commercially available software (Lameness Locator®, Equinosis).

Figure 1: Horse instrumented with 3 inertial sensors during a lameness exam. There is a uni-axial accelerometer attached to the head bumper; one uni-axial accelerometer placed between the tubera sacrale on the midline of the most dorsal aspect of the pelvis with tape and a third uni-axial gyroscope wrapped to the dorsal aspect of the right forelimb pastern (VetAgro Sup - Veterinary Campus of Lyon).

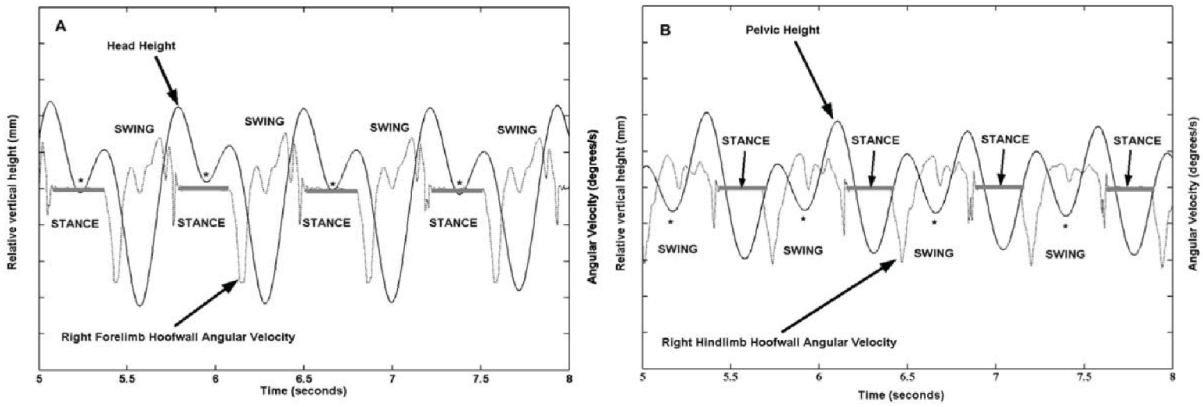


Figure 2: Illustration of data used for detection and quantification of lameness in horses using inertial sensor system (Keegan 2004). A: There is less downward movement of the head during the stance phase of the right forelimb (righ forelimb lameness) and B: more downward movement of the pelvis during the stance phase of the right hind limb (left hind limb lameness).

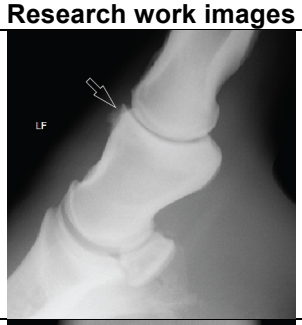
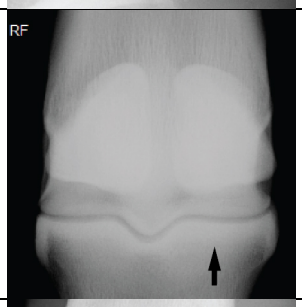
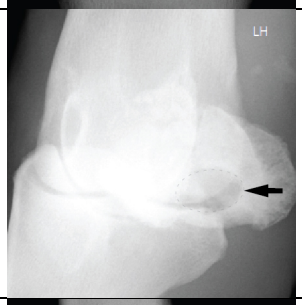
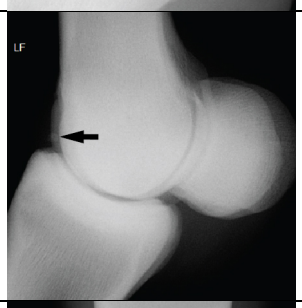
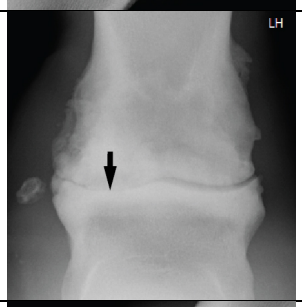
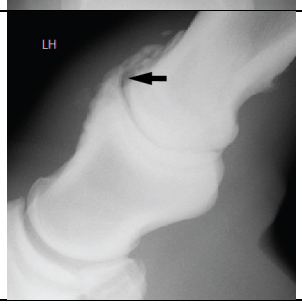
Its repeatability was assessed (Keegan *et al*., 2011), compared to a stationary force plate (Keegan *et al.,* 2012) and sensitivity compared to subjective evaluations (McCracken *et al*., 2012). In the latter study the inertial sensors detected lameness sooner than the consensus of 3 experienced evaluators, regardless the induced pain location or fore/hind limb lameness in the straight line. Furthermore, this device has been evaluated for proximal hind limb response to flexion (Marshall *et al*., 2012). It was found that subjective positive response to flexion resulted in significant changes in objective measurements of pelvic asymmetry. However, this was never studied for distal limb flexion in forelimbs and hind limbs.

3.2 Imaging techniques

Several imaging techniques, like scintigraphy, can be useful particularly if there is multiple limb lameness or no response to local analgesia. Standing magnetic resonance imaging (MRI) is available for horses and may be an option for distal limb problems particularly if soft tissue structures are a concern (Schramme, 2015). However, both exams are expensive and not available in every country nor every equine hospital. Eventually, ultrasound can detect different degrees of synovitis and periarticular fibrosis, however it is not frequently used for OA diagnosis. The gold standard method to detect OA is the radiographic examination. This method, noninvasive, portable and cost-effective, is very useful when lameness examination is performed thoroughly and regional analgesia is able to localise the source of pain. Despite being indispensable to evaluate joint status, there is, however, a poor correlation between radiographic changes and clinical signs (Widmer & Blevins, 1994). Baccarin *et al*. (2012) have more recently reported no correlation between radiographic scores and the presence or absence of lameness.

Joint inflammation, arthritis, can be seen radiographically as evident capsule distension without apparent new bone formation. Osteoarthritis or osteoarthrosis are characterized by bone involvement with or without an inflammatory soft-tissue component, respectively. If a primary cause is known, such as in OCD or IA fracture, the term secondary joint disease can be used. Immune mediated joint disease should be considered if several joints are affected and infection can be excluded. Degenerative joint disease is used to refer to any condition that affects the joint and its supporting structures. It is more frequently encountered in adults but can be present in young horses sometimes due to poor conformation and/or hard use (Butler, 2008). Radiographic abnormalities associated to joint disease include several features that can be related to different pathogenic mechanisms (Table 1).

Table 1: Radiographic features and pathogenic mechanism of OA (adapted from Frisbie, 2012).

Radiographic feature	Pathogenic Mechanism	Research work images
Periarticular ostophyte formation	Endochondral ossification occurring at bony margins of unknown cause. Possible repair attempt modulated by altered cytokine *milieu*.	
Subchondral bone sclerosis	Deposition of new bone as a response to changes in force transmission and from healing of trabecular microfractures. Corresponds to areas of maximum stress. Significant sclerosis often corresponds to full-thickness cartilage loss.	
Subchondral bone lysis	Less-common change of uncertain pathogenesis. Possibly pressure necrosis from synovial fluid, gaining access to subchondral plate via fissures, or related to pressure necrosis from trauma to bone.	
Osteochondral bodies	Disintegration of joint surfaces or fractured osteophytes.	
Joint space narrowing	Cartilage degeneration and loss. Usually areas of weight bearing or high stress. May be absent when focal cartilage loss occurs.	
Advanced remodelling / ankylosis	Articular response to advanced degeneration.	

One or more features can be associated with joint disease. Periarticular osteophytes should be differentiated from entheseophyte formation (bone production at ligament insertion) and, as discussed before, are not necessarily synonymous of clinical signs. Conversely, absence of radiographic changes does not preclude the existence of cartilage degeneration.

In order to quantify and compare radiographic evaluations semi-quantitative scales were developed allowing radiographic lesions' classification. In humans, the Kellgren & Lawrence (1957) scale (0-4) is commonly used. In horses, metacarpophalangeal lesions may be classified from 0-4 depending on the severity of osteophytes, subchondral sclerosis and narrowing of the joint space (de Grauw *et al.*, 2006).

Radiographic lesions are frequently present bilaterally and may only be found in latter stages of OA. Therefore, in order to find a method for earlier recognition of the disease, other techniques have been studied like the measurement of cartilage biomarkers in SF and serum.

3.3 Cartilage Biomarkers

Intensive research has been developed on equine cartilage degradation and inflammation biomarkers, which are described as more dynamic and more suitable signal-to-noise ratio than imaging techniques (Ameye *et al*., 2007). Ideally, they should be able to predict disease, differentiate OA affected from non-affected joints, quantify the degree of cartilage degradation and evaluate response to therapy (Mcllwraith, 2005; de Grauw *et al*., 2006). Direct biomarkers from cartilage metabolism are breakdown products like type II collagen (e.g. Coll2-1, Coll2-1NO_2) and proteoglycan fragments (e.g. GAG), interesting for clinical purposes. Indirect biomarkers can be produced secondarly to stress or damage, including inflammatory mediators (e.g. PGE2) proteolytic enzymes (e.g. MMPs); link proteins (e.g. cartilage oligomeric matrix protein) and pro-inflammatory cytokines (e.g. IL-1), being more interesting for research purposes. These molecules have their epitopes identified by specific immunoassays, that are commercially available for humans and increasingly present in the market for horses.

There are additional factors that should be considered when choosing the type of sample to be analysed. Repeated SF samples can be difficult to obtain in equine athletes. Moreover they are considered responsible for the increase of GAG and PGE2 concentrations (Frisbie *et al*., 2008) and have associated infection risk. Blood samples are better tolerated and it has been described as having significant correlation with the concentration of the molecules in SF (Catteral *et al*., 2010). Several factors can influence biomarkers in serum and SF. Exercise alone can induce significant increase of collagen metabolism biomarkers in serum (Billinghurst *et al*., 2003) and GAG in SF (Frisbie *et al*., 2008). This was not the case for PGE2, which was significantly increased in SF of experimental OA-joints compared to exercise alone-joints. This difference was not evident in the serum (Frisbie *et al*., 2008).

Collagen type II molecules (Coll2-1 and Coll2-1NO_2) were studied as potential biochemical markers for cartilage metabolism in humans (Deberg *et al*., 2005) and in mice (Ameye *et al*., 2007). Type II collagen peptide (Coll2-1) is located in the collagen triple helix, requiring multiple enzymatic digestion

of the collagen molecule. Coll2-1NO_2 is the nitrated form of Coll2-1, requiring oxidative stress in articular cartilage and may indicate the level of inflammation in the synovium, being higher in rheumatoid human patients compared to OA patients (Deberg *et al*., 2005). Coll2-1 and Coll2-1NO_2 are expressed in the ECM around superficial lesions and in tendon insertion sites but absent from healthy and intact cartilage (Ameye *et al*., 2007). In this study mices were followed for 5 months, Coll2-1 was found to be constant but Coll2-1NO_2 increased with age, suggesting different biologic processes and providing complementary information. In humans, type II collagen biomarkers are also expressed in superficial cartilage lesions and Coll2-1 decresases to reference values 3 months after hip replacement (Deberg *et al.,* 2008). Moreover, Coll2-1 biomarker is significantly lower in patients responding to viscosuplementation (Henrontin *et al*., 2013) and has been shown to be significantly decreased in serum of OA human patients after systemic curcumin treatment (Henrotin *et al*., 2014). In horses, Verwilghen *et al*. (2009) studied these two molecules as diagnostic tools and reported a tendency towards an increase in severity of radiological class lesions with increased Coll2-1NO_2. Significantly higher plasmatic levels of Coll2-1 were found in degenerative joint disease group (Verwilghen *et al*., 2009) and higher SF and plasmatic values were associated to more severe degenerative cartilage lesions of tarsocrural joints assessed by arthroscopy (Verwilghen *et al*. 2011).

Fibulin 3 is a member of extracellular matrix proteins that has been found to be elevated in OA cartilage (Wu *et al*., 2007). More recently, fibulin 3 fragments (Fib3-1 and Fib3-2) were shown to be elevated in urine and serum of severe OA patients compared to healthy controls (Henrotin *et al.,* 2012). In particular, Fib3-2 was not modified by sex, aging or hormonal changes and has never been studied in horses. Also, there are no studies in equine medicine reporting the serum profile of these molecules after applying any type of treatment.

4. Treatment of equine OA

The articular cartilage is incapable of an adequate self-repair and full-thickness cartilage defects are replaced by less functional fibrocartilage, predominantly composed by type I instead of the original type II collagen. The depth, size and location of the defect and age of the patient affect endogenous repair (Frisbie *et al*., 2012). In OA treatment, the target is not only to abolish pain effectively, but also, to re-establish its function. The additional factor is the constant pressure on equine veterinarians to successfully treat performance-limiting joint problems, preferably with something inexpensive, effective and risk-free (Richardson & Loinaz, 2007).

OA treatment has not yet a gold standard therapy and despite all efforts it is still accepted that established OA cannot be cured. Nevertheless, clinicians have several options between clinical signs or disease modyfing drugs available on the market, the latter drugs are supposed to decrease pain but also revert the disease evolution. Both groups have systemic and IA administration options. The most commonly used systemic drugs of the first group are the non-steroidal anti-inflammatory drugs (NSAIDs) like the non-selective cyclooxygenase (COX) inhibitors: phenylbutazone, suxibutazone, flunixin meglumine and ketoprofen. They are inexpensive but frequently associated to secondary

effects, like gastric ulcers and renal failure if long-term therapies are applied (Erkert *et al*., 2005; Goodrich & Nixon, 2006). More recently, selective COX-2 inhibitors, like meloxicam and firocoxib have been studied with encouraging results in equine synovitis and OA (de Grauw *et al.,* 2009; Orsini *et al*., 2012). Systemic disease-modyfing drugs are becoming increasingly common and consist of polisulphate glycosaminoglycan (PSGAG), HA and nutraceutical agents like glucosamine and chondroitin sulphate. These therapies have limited evidence of efficacy (Richardson & Loinaz, 2007; Vandeweerd *et al*., 2012) and were not under the scope of this thesis. We did a review on IA drugs used to manage lameness in the equine athlete and focused in the most recent biologic strategies (Monteiro *et al*., 2015).

4.1 Intra-articular synthetic drugs

Corticosteroids

The most commonly used groups of drugs in IA treatment are corticosteroids, HA and PSGAG. Among these medications the corticosteroids have the most potent anti-inflammatory and pain relief effect, by inhibition of phospholipase A2 and COX-2 expressions (Masferrer & Seibert, 1994). They are also capable of supressing the most important cartilage degradation mediators, IL-1 and TNF-α (Laufer *et al*., 2002), and have cartilage-sparing effects at low-doses, without marked effects on chondrocyte health (Richardson & Dodge, 2003). A recent study showed that IA corticosteroid treatment, associated to systemic NSAIDs, have better owner-reported return to performance, in particularly in the fetlock joint and negative prognostic if DIP joint was involved (Brommer *et al*., 2012). Adverse effects may follow repeated injections of high doses of corticosteroids in vigorously exercised horses. Methylprednisolone acetate (MPA), particularly, inhibits proteoglycan synthesis and unfavorably influences the structural organization of collagens within cartilage *in vitro* model (Todhunter *et al*., 1996a) and *in vivo* model (Frisbie *et al*., 1998). Negative effects were not observed in subsequent *in vitro* and *in vivo* studies about MPA (Kawcak *et al*., 1998; Murphy *et al*., 2000; Murray *et al*., 2002; Raynauld *et al.,* 2003). Neverthless, in a 2010 review made by McIlwraith he described that betamethasone esters have no deleterious side effects, triamcinolone acetonide (TA) is chondroprotective and MPA has deleterious effects and therefore should be used juditiously.

Hyaluronic acid (HA)

Hyaluronic acid is also frequently used as an IA drug. This glycosaminoglycan is an important component of articular cartilage and SF. Antonacci *et al*. (2012) reported that equine SF from acutely inflamed joints presented significantly lower HA concentration, but, when concerning chronic inflammation cases, there was no difference compared to controls. The same authors reported poor boundary lubrication in acute post-injury, probably due to diminished concentration and molecular weight of HA. In clinical practice exogenous HA is injected due to viscosuplementation properties but also for the anti-infllammatory (Takahashi *et al*., 1999) and disease-modifying effects (Marshall *et al*., 2000). High molecular weight (HMW) HA is reported to have superior cartilage-sparing properties in OA models (Asari *et al*., 1998), to restore *in vitro* lubricant function (Antonacci *et al*., 2012) as well as

better clinical improvement (Philips, 1989) when compared to the lower molecular weight. Also, higher concentrations (20mg and 40mg) have been described to better improve lameness after a single injection, when compared to lower doses (Richardson & Loinaz, 2007). In humans, clinical efficacy has been described to be higher in mild cases of OA (Wang *et al*., 2004). In horses, there is still a lack of scientific evidence on clinical efficacy. A study on experimental OA revealed that 3 injections of 22mg HA together with 125mg amikacin, didn't improve lameness but reduced cartilage fibrillation (Frisbie *et al*., 2009a). Some other studies also suggest that the natural time course of lubrication is important (Antonacci *et al*., 2012) and this could explain disappointing results in chronic cases. Corticosteroids are frequently combined with HA in order to combine potent anti-inflammatory with chondroprotective action (McIlwraith, 2010). However, it has been found that a single IA injection of TA-HA association compared to the steroid alone, had worst clinical results 3 weeks post treatment and the same outcome 3 months post-injection (de Grauw *et al*., 2014).

Polysulfated Glycosaminoglycan (PSGAG)

PSGAG is a semisynthetic preparation, from bovine trachea, composed mainly of chondroitin sulphate, a glycosaminoglycan found in articular cartilage. Its intra articular efficacy has been reported in horses (Hamm & Jones, 1988). However, due to risk of joint infection (Gustafson *et al*., 1989), injections should be accompanied by an antibiotic administration (e.g. amikacin, 125 mg). HA together with sodium chondroitin sulphate and N-acetyl-D-glucosamine was compared to IA saline and amikacin alone (placebo). In this study, there was 16% improvement in lameness scores, slight improvement on radiographic bone proliferation and less cartilage erosion compared to placebo treated joints (Frisbie *et al*., 2013).

In conclusion, controversy about efficacy and recommended doses and protocols for intra-articular HA and PSGAG still exist. Their application in OA prevention also needs further clarification. Conversely, these drugs do not present limitations in competing events and detection times, as doping, like for systemic NSAIDs and corticosteroids. Therefore, these therapies still play a role for the treatment of OA.

In the last decades, a different category of disease modifying drugs has gained popularity both in human and equine medicine. These are based on biological and natural organism ability to repair and try to amplify their effects, the so-called regenerative or biologic therapies.

4.2 Intra-articular biologic strategies

These therapies may be classified into two main groups: the cell and the blood derived therapies (Appendix B: Biologic strategies for intra articular treatment and cartilage repair)**.** Few studies on the effect of biologic therapies on equine cartilage regeneration have been published, most being *in vitro* or on disease-induced protocols, and scarce studies were done on natural occurring disease. Blood derived therapies are widely available because there are no restrictions and few legislation regulating their use. They are based on autologous products, so readily available from the patient, and supposed to be safe for the same reason. There are two main products being used in equine patients,

autologous conditioned serum and platelet rich plasma (PRP). The main interest associated with these products is based on their anti-catabolic and anabolic effects. As performance enhancement has not been demonstrated, these therapies are not included in the prohibited list of doping controls and, consequently, competing horses can benefit from their effects. They have been adapted from human medicine, with little supporting investigation. Nevertheless, they are being widely applied to equine patients.

Mesenchymal stem cells

Stem cells are unspecialized cells, which have the ability to renew themselves indefinitely and, under appropriate conditions, give rise to a variety of mature cell types (Caplan, 1991). Multipotency means they can differentiate into two or more cell lines (Spencer *et al*., 2011). Autologous multipotent cells are normally used because they have good differentiation capacity and require simple procedures to be collected from the patient. Mesenchymal line, containing undifferentiated cells from bone marrow and adipose adult tissues are the two populations most frequently used for cartilage repair (Caplan, 2007). Some studies report a significant higher chondrogenic potential of bone marrow-derived stem cells, synovial membrane and peri-articular fat (Mochizuki *et al*., 2006; Vidal *et al*., 2008; Vinardell *et al*., 2012). However, adipose subcutaneous tissue has gained popularity because it is more readily available and allows obtaining a high number of cells in the initial yield, which means little need of expansion and culture time (Vidal *et al*., 2008).

In horses, two studies using a carpal osteochondral fragment model of osteoarthritis and focused on clinical assessment of pain besides radiographic lesions, SF and histologic evaluation, did not found significant improvement in pain score after MSCs therapy (Frisbie *et al.,* 2009b; McIlwraith *et al.,* 2011). These studies in experimental osteoarthritis models give little support to the use of MSCs at the acute phase. Even so, a prospective multicenter study was designed on naturally occurring OA. Treated joints were mostly stifles with advanced fibrillation and loss of soft tissue structures and received bone marrow MSCs and HA. In an average of 21 months follow-up, authors reported 76% return to work, from which 38% of horses returned to their primary intended use. Authors also found a superior long-term outcome, when treatment was performed in at least one month after diagnosis (Ferris *et al.,* 2009). More recently, this has been published in 33 clinical cases of stifle lesions using autologous bone marrow derived MSCs (Ferris *et al.,* 2014) and allogeneic peripheral blood-MSCs added with PRP, in 91 clinical cases of OA in different joints (Broeks *et al.,* 2014). Both studies reported high rates of horses returning to work, 75% at 24 months and 78% at 6 months follow-up, respectively. Based on these reports, IA administration of stem cells seems an interesting and straightforward therapy.

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CHAPTER II – Comprehensive research work

This chapter consists of two sections. The first section presents the results of a survey to Portuguese equine veterinarians about diagnosis and treatment of OA, which helped understanding the Portuguese reality and the clinical practice needs. The subsequent section presents the *in vitro* work about the effects of different doses of equine allogeneic ASCs with or without IFN-γ pre-activation before application in an experimental model of the disease *in vivo*.

1. Survey to Portuguese equine veterinarians

A survey (Appendix C: Questionnaire) was prepared for equine veterinarians in order to understand the most common clinical features of OA and most common treatments used in equine field practice in Portugal.

1.1 Materials & Methods

Contact with 106 members of Associação Portuguesa de Médicos Veterinários de Equinos (APMVE) from April 2013 to April 2014 was attempted electronically, sending them a link for a web-based survey (Google Drive form) via email. Moreover, a form was distributed at the I Congress of the Associação Portuguesa de Médicos Veterinários de Equinos (APMVE), on the 17th April 2014, about sport horse medicine and all answers were inserted online.

1.2 Results

Sample characterization

A total of 24 surveys were analysed: 7 forms were submitted online and 17 written forms were completed. The majority of respondents (87.5%) indicated to have more than 75% of their activity in equine practice. There were 3 young veterinarians (less than 5 years of experience), 45.8% (11/24) between 6 and 10 years of experience and 41,7% (10/24) with more than 11 years of experience. Most of the veterinarians (37.5%) worked primarily with show jumping horses.

Diagnostic responses

Regardless of the veterinarians' experience and the main equine sport discipline, OA was most commonly diagnosed in 10 to 14 year-old horses (58.3%), followed by 5-9 year-old (16.7%) and 15-19 year-old (12.5%). Horses presenting OA were described to work at moderate level (more than 5h/week) by 41.7% of the veterinarians. The majority (87.5%) of the answers reported to find OA clinical signs without radiographic lesions, which were most frequently seen in the fetlock and DIP

joints. Moreover, 87.5% of the answers reported to find radiographic lesions without OA clinical signs, which were most frequently seen in the PIP joints and distal intertarsal and tarso-metatarsal joints.

Treatment responses

The most common first choice treatment was IA injection (11/24, 45.8%) followed by corrective shoeing (6/24, 25%). The alternative approach to OA treatment was systemic NSAIDs (4/24, 16.7%) and food/work management (3/24, 12.5%). The less frequently recommended options, as the first choice, were other systemic drugs: oral supplements, intramuscular (IM) PSGAG followed by endovenous HA. Tetracyclines were rarely choosen.

Considering the scope of this thesis we further asked which drugs and protocols were currently performed for the IA treatment at high motion joints. The first choice protocol was a single injection of TA alone (12/24, 50%) or together with HA (6/24, 25%). The doses of TA varied from 4 to 12mg per joint. Reported doses of HA, low molecular weight, were 20-25mg per joint, with 4,2% (2/24) of the practioners performing a second injection 2 weeks after. Betamethasone sulphate was the first choice for (5/24, 20.8%) of the veterinarians (6-12mg per joint) and MPA the first choice for (4/24, 16.7%) of the veterinarians (20-40mg per joint). Antibiotic injection together with other treatments was considered by 50% (12/24) of the respondents, most frequently in the less than 10 years of experience group (8/12) compared to the more than 10 years of experience group (4/12). Biologic therapies like IRAP, PRP and MSC were the least used therapies together with PSGAG. There were 37.5% (9/24) of the veterinarians considering to use at least one of the biologic products, most frequently in clinical cases not responding to other therapies. This was not correlated with years of experience (r = 0.430).

1.3 Discussion

The number of participating veterinarians was lower than expected but interesting answers were found. The results concerning presence of OA without radiographic changes in DIP and fetlock joints did not fit the previous findings of Baccarin *et al*. (2012), which found significantly lower scores in distal limb joints in sound horses compared to lame horses. However, the results about small tarsal joints are in agreement. In the same study there was a low correlation between radiographic scores and lameness. Therefore, it would be interesting to find if lameness and, eventually, flexion-test response assessed objectively would show higher correlation with distal radiographic findings, compared to subjective evaluation.

The second point that was supposed to be addressed was using IA injection as a first line treatment. Almost half of the enquired veterinarians treated OA this way. This is probably due to the pressure imposed by owners to return animals back to work as soon as possible. Neverthless, NSAIDs are still the second choice after corrective shoeing, commonly used as support the treatment. Systemic PSGAG, HA and nutraceuticals are not a common choice probably due to their low efficacy and associated high price. One should agree that in terms of price/efficacy corticosteroids are the gold standard but veterinarians should limit their doses (Richardson & Dodge, 2003). In fact, some veterinarians are using MPA and TA at doses under the recommended guidelines (Caron, 2005). On

the other hand, for the last decades MPA has shown detrimental effects in the cartilage of high-motion joints (Frisbie *et al.*, 1998) and that is probably why it is the least frequently used steroid. An American equine veterinarian survey confirmed that TA is most frequently used in high motion joints but MPA is still used in low motion joints (Ferris *et al.*, 2011). Similarly to this study, the most common combination with steroids was HA and the use of antimicrobials, regardless of the injected product.

Finally, veterinarians are faced with particular OA cases in which they don't recommend steroids. American practioners reported to apply HA products alone more frequently in acute disease affecting high motion joints, followed by chronic cases with radiographic indication of OA and chronic maintenance cases (Ferris *et al.*, 2011) but they were not asked about injection protocols. In Portugal, veterinarians reported HA therapy use on a single injection protocol, which supported our interest in further exploring this protocol. Biologic therapies were not used but they were considered, most commonly, in cases not responding to steroids any more.

In conclusion, alternative therapies to steroids are needed in particular cases. The problem is the lack of clinical and scientific evidence. Therefore, our goal was to objectively study the effects of new IA therapies. Before *in vivo* application, adipose stem cells activation with INF-*γ* was studied *in vitro*. A part of the results about the effects of this treatment on a model of osteoarthritis in equine cartilage explants are presented in the following section.

2. Effects of allogeneic ASCs pre-activated by INF-γ on experimental OA *in vitro*

Abstract

The objective of this part of the work was to study the dose-effect of allogeneic adipose mesenchymal stem cells (ASCs) and preactivation with interferon-*γ* (INF-*γ*), using an equine fetlock OA model *in vitro*. Equine fetlock cartilage explants were OA-induced with 1 ng/mL of interleukin-1β (IL-1β) and 10 ng/mL of oncostatin-M (OSM) and tested with or without a low (25 x10^3) and high dose (100 x10^3) of ASCs. OA induction increased cartilage degradation genes (n=2) and significantly increased GAG release (n=10) ($P < 0.001$), these effects being reverted by the high dose of ASCs. Subsequently we tested both concentrations with or without IFN-*γ* preactivation (100 ng/mL for 24h) for GAG release during 12 days. The high dose of ASCs decreased GAG release and preactivation shortened the onset and the necessary dose to reach the effect.

2.1 Introduction

The immunomodulatory capacity of MSCs has been the focus of allogeneic treatments and immuno-mediated diseases. The rationale would be to control host exacerbated response to the "non-self" therapy (Polchert *et al.*, 2008). IFN-γ was first reported to stimulate MSCs production of indoleamine 2,3-dioxygenase, which in turn inhibited the proliferation of activated T or natural killer cells (Krampera *et al.,* 2006). In recent years the stimulation of MSCs has been studied and several

studies support better immunomodulatory properties after preconditioning of cells in inflammatory conditions (Crop *et al.*, 2010; Carrade *et al.,* 2012). This preactivation can affect ASCs morphology, proliferation and citokine expression but their differentiation capacity seems to be preserved (Crop *et al.*, 2010). Therefore, IFN-γ preactivation of ASCs could improve clinical efficacy after IA treatment in horses with osteoarthritis, even if the immune features are less well understood than in human patients.

2.2 Material & Methods

Isolation, characterization and pre-activation of cells

Adipose tissue was retrieved from the subcutaneous gluteal fat from a 4-year-old French Standardbred donor horse owned by Sanofi-Pasteur. The procedure was performed standing, under sedation and local anesthesia, using tail head standard technique (Frisbie *et al.,* 2009). Tissues were kept at 4-10ºC in a dissection medium, Dulbeco's phosphate buffer saline (PBS, Gibco, Life Technologies), during transportation and processed within 3 hours. Fat was digested with 0.25% collagenase 1 (Worthington Biochemical Corp.) under agitation at 37ºC during 1h30 and the homogenate was centrifuged during 10 min at 300 ga using routine laboratory techniques. The stromal vascular fraction was collected, cells were filtered successively through a large sterile filter, a 100 and a 70 µm porous membrane filters, in order to remove fat. To avoid red cells, a hemolysis step was done briefly by adding a lysis buffer for less than 5 min. Single cells were seeded in 10 cm Nunclon Petri dishes (passage 0) and amplified in a 5% CO_2 incubator at 37ºC. After 2-3 days, cultures were washed with PBS before changing the medium. After one week, cells were trypsinized (passage 1) and split for another amplification step. Following cell growth and checking for colony formation cells were trypsinized (passage 2), counted and stocked at 3 to 15 million cells per mL in freezing buffer at -80ºC or -160ºC. ASCs were characterized by their immunophenotype (positive for CD44, CD73, CD90, CD105 and negative for CD34 and CD45) and their trilineage potential.

ASCs preparation for preactivation consisted in thawing cells at 37ºC, transferring to prewarmed culture medium and plated (passage 2) at 1 million cells for a 10 cm Nunclon petri dish. Medium was changed every 2-3 days for 10 days. At day 9, cells had an IFN-γ pretreatment (100ng/mL) during 24h. ASCs were then trypsinized (passage 3) counted and centrifuged during 5 min at 300 ga and resuspended in injection medium (vehicle) without IFN-γ (Stem Alpha 7900).

Cartilage explant model and OA induction

Equine joint specimens were retrieved from 4-8 year-old French trotters, euthanized for any other reason than osteoarthritis (n = 10). Macroscopically healthy fetlock joints were processed within 3 hours and allowed retrieving five cartilage explants per well using 3 mm biopsy punch (Harris Uni-core). Cartilage explants were cultured with 1mL buffered Dulbecco's modified Eagle's medium (DMEM), high Glucose-glutamax (Gibco, Life Technologies), Hepes 20 mM, ascorbic acid 5 µg/mL (Carlo Erba Reagents), 0.5 µg/mlL Insulin, 0.5 µg/mL Transferrin, 0.5 ng/mL Selenium (2.5 µL) ITS-

Supplement (Sigma-Aldrich) and 50µg/mL Gentamycin for preliminary two days of stabulation. OA condition was induced by injection of two cytokines: 1 ng/mL IL-1β (R&D Systems) and 10 ng/mL OSM (Invitrogen, Life Technologies) to the culture medium (Pelletier & Martel-Pelletier, 1989; Barksby et al., 2006)

Cell Coculture and GAG release

Cartilage explants (*n* = 10) were cultured with DMEM (control), DMEM/ IL-1β /OSM (OA condition) alone and together with one of the 4 different ASCs treatments: 25 $x10^3$ or 100 $x10^3$ cells, both concentrations with or without IFN-γ. GAG release speed was assessed for each condition in order to test the dose and the preactivation effects. Culture medium was assessed in triplicate and changed every 2-3 days until day 12 after treatment. Microplate was read by spectrometry at 656 nanometers (Flexstation, Molecular devices) after quantitative dye-binding method, for analysis of sulfated proteoglycan and glycosaminoglycans (Blyscan®, Biocolor). The median value obtained from the triplicate measurements was divided by the number of days in order to obtain the GAG degradation speed. Each cartilage explant showed similar weight, 8 ± 0.19 mg (mean ± SEM) which precluded a normalization step. Mean GAG values for each condition were calculated at days 2, 5, 7, 9 and 12 and compared to OA condition baseline.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Additionally, cartilage explants from two horses (*n* = 2) were used in order to test the *in vitro* OA model (OA condition *versus* control) as well as the two ASCs concentration effects on culture explants (ASCs *versus* OA condition) by presenting the fold-change in gene concentration. After a 10 day-treatment the explants were digested with collagenase II for subsequent RNA extraction. Total cellular RNA was isolated from 45mg of cartilage together with 1mL of TRIzol (Invitrogen). Each sample (0.06µg) was reverse transcribed using High capacity cDNA Reverse Transcription kit (Applied Biosystem). Quantitative real-time PCR (qRT-PCR) was carried out to determine the mRNA levels of cartilage matrix synthesis genes (collagen type 2 and 9 and aggrecan) and cartilage matrix degradation genes: ADAMTS-4, ADAMTS-5 (disintegrin and metalloproteinase with thrombospondin motifs 4 and 5), matrix metalloproteinase-1, 3 and 13 (MMP-1, MMP-3 and MMP-13) with miRNEasy Kit (Qiagen) according to manufacturer's instructions. After amplification, target genes were normalized against the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The following probes (Applied Biosystem) were used: Collagen II (ref EC03467411); Collagen IX (ref EC03470076); Aggrecan (ref EC03469667); ADAMTS-4 (ref EC03469180) ADAMTS-5 (ref EC03470666); MMP1, MMP3, MMP13 (ref EC03468020, ref EC03468674 and EC03467796).

Statistical Analysis

Genetic study (qRT-PCR) was performed in cartilage explants from two horses precluding statistical analysis. The rest of the data were tested for normality and the homogeneity of variances by using the Shapiro–Wilk and the Levene tests, respectively. *In vitro*, GAG values were analyzed with two-way analysis of variance (ANOVA) with the Dunnett's method for multiple comparisons. Statistical

analysis was performed using software package EverStat V6 under SAS v9.2. Significance was set at $P \leq 0.05$.

2.3 Results

GAG release and gene expression on OA-induced cartilage explants

OA induction by IL-1β and OSM treatment significantly increased the GAG release speed (μg/mL/day) at all times compared to culture medium controls ($P < 0.001$) (Figure 3).

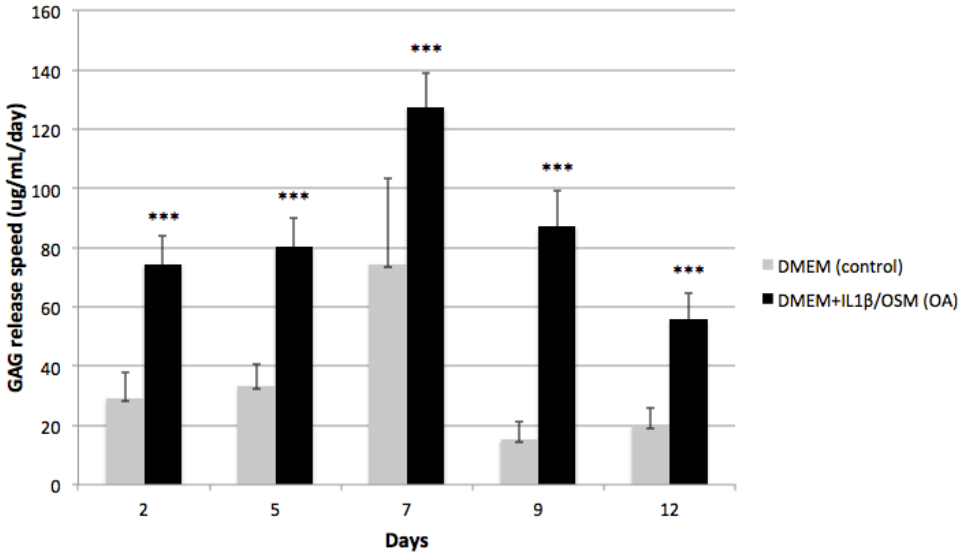

Figure 3: Mean (± SD) glycosaminoglycan release speed over 12 days of culture ($n = 10$). Explant model - culture medium controls compared to OA condition. Significantly different results are represented by *** $P < 0.001$.

The OA model was further validated by decreased expression of cartilage matrix synthesis genes and increased expression of cartilage degradation genes compared to medium control in both cartilage explants. Aggrecan was 87 and 100-fold less expressed after OA induction in cartilage explants 1 and 2, respectively. Collagen 2 was less expressed in both cartilage explants and collagen 9 was not expressed after OA induction. Concerning degradation genes, ADAMTS-5 was 4.4 and 2.2-fold more expressed in cartilage explant 1 and 2, respectively. ADAMTS-4 was not detected in controls but was expressed in both cartilage explants after OA induction. MMP1 was 63 and 56-fold more expressed; MMP-3 was 217 and 330-fold more expressed and MMP13 was 217 and 413-fold more expressed after OA induction, in cartilage explant 1 and 2, respectively.

Effects of ASCs on cartilage OA-model

Cartilage synthesis and degradation genes were assessed in cartilage explants of two horses for the low dose (25 x10^3) and high dose (100 x10^3) of ASCs and compared to OA condition. The most consistent results from cartilage synthesis genes were observed for collagen 2, more expressed with the high dose in the 2 horses (39 and 1.63-fold) and collagen 9, which was not detected in any

condition. Concerning the degradation genes, the first cartilage explant presented less expression of ADAMTS-4 with low (7-fold) and high dose (20-fold) and less expression of ADAMTS-5 with low (4.3-fold) and high dose (14.3-fold) of ASCs. In the second cartilage explant, there was increased expression of ADAMTS-4 and ADAMTS-5 at low dose, 1.32 and 2.82-fold, respectively. However, decreased expression of both genes was observed with higher dose, 3.5 and 2.4-fold, respectively. A similar tendency was found in the first cartilage explant, presenting a better reduction on expression of MMP1 (2.6-fold), MMP3 (50-fold) and MMP13 (25-fold) with high, compared to low dose, of ASCs. In the second cartilage explant there was increased expression with both concentrations of MMP1 and with the low dose of MMP-3 and 13. There was decreased expression of MMP-3 (3.7-fold) and MMP-13 (2.4-fold) with the high dose. These results show more collagen 2 expression and less degradation by MMP and aggrecanases in cultures using the highest dose of ASCs in both cartilage explants.

Effects of INF-γ pre-activated ASCs on GAG release

Allogeneic ASCs treatments revealed significant differences in GAG release compared to OA condition (Figure 4), showing a transient increase in GAG mean values at day 2 and 5. Cartilage explants treated with 100 x10^3 ASCs significantly decreased GAG release at day 9 ($P < 0.001$) and IFN-γ preactivation extended the significant period from day 7 ($P < 0.001$) to day 12 ($P < 0.001$). Treatment with 25 x10^3 ASCs alone didn't show significant GAG reduction but with IFN-γ pre-activation, induced significant reduction, presenting the lowest values at day 7, at day 9 and at day 12 ($P < 0.001$). These results show that high dose ASCs decreased GAG release and preactivation shorten the onset and the necessary dose to reach the effect.

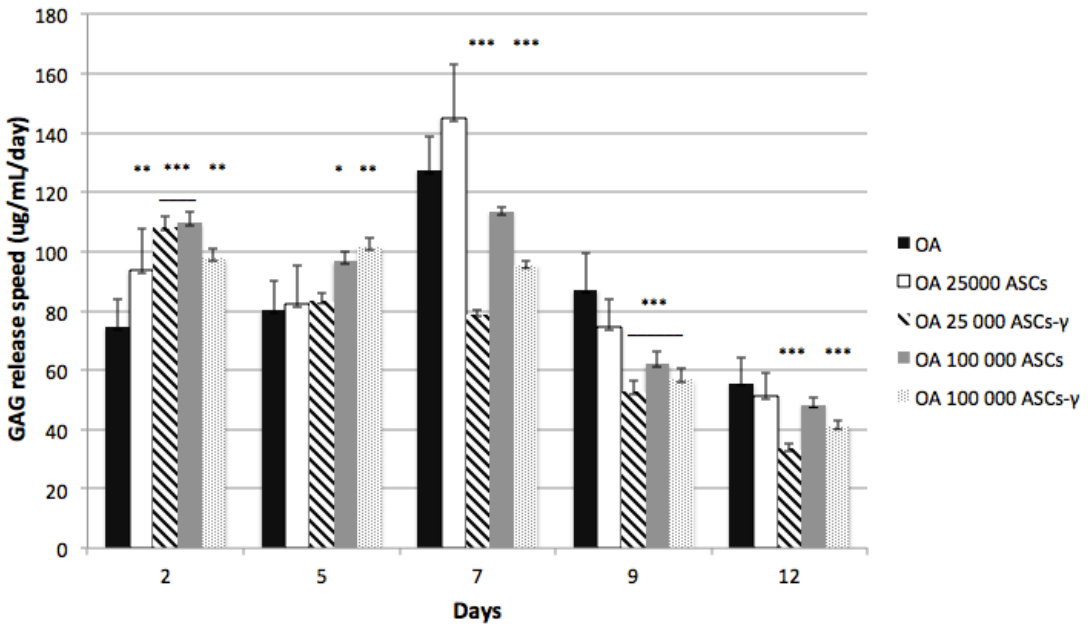

Figure 4: Mean (± SD) glycosaminoglycan release speed over 12 days culture (n = 10). Coculture - ASCs low (25x103) and high dose (100x103) ASCs, with or without IFN-γ pre-treatment, compared to OA condition alone. Significantly different results are represented by * $P < 0.05$ ** $P < 0.01$ and *** $P < 0.001$.

2.4 Discussion

The role of IFN-γ in the immunosuppressive function of MSCs has been reported (Krampera *et al*., 2006) but it was never studied in equine experimental OA. The *in vitro* part of the study was performed in nearly safe cartilage explants and IL-1β and OSM treatment was effective in producing experimental OA. This was shown by significant increase of GAG release, which is similar to a human cartilage coculture model (Fearon *et al*., 2006). Moreover, qRT-PCR results further confirmed the cartilage degradation with increased expression of cartilage degradation genes (ADAMTS-4, ADAMTS-5, MMP1, MMP3 and MMP13). The OA-baseline for GAG release allowed the evaluation of the two ASCs concentrations but more importantly the INF-*γ* preactivation effect. All ASCs treatments induced transient increase followed by decreased GAG release into different culture conditions. The initial GAG release could be explained by transient cartilage degradation or ASCs production (Tapp *et al.,* 2008) and proteomics analysis could be interesting to clarify this question. Without preactivation, higher dose had decreased GAG release speed compared to lower dose, which is similar to a dose-dependent study that reports better efficacy of higher concentration of autologous ASCs (Jo *et al*., 2014). This was further supported in our study by the reduced expression of cartilage degradation genes with high dose of ASCs. With preactivation, both ASCs concentrations decreased GAG release during an extended period of time, which reflects an additional protective effect. However, this was more evident with lower dose (25 x 10^3) of ASCs. It is possible that at higher concentrations ASCs preactivation could induce cluster formation too quickly and subsequent cell death, stimulating cytokine production that would induce GAG release. This could also be due to an effect on the degradation/synthesis balance showing the importance of knowing the correct dose for optimal effect.
In conclusion, high concentration of ASCs is important to reach an anti-catabolic effect of cartilage *in vitro* and IFN-γ preactivation allows reduction of the number of needed cells, extending the effective period of action. Further research *in vivo* is necessary to clarify these findings and better understanding the clinical potential of this therapy.

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CHAPTER III – Effects of allogeneic ACSs pre-activated by IFN-γ on equine groove model of metacarpo-phalangeal osteoarthritis

Susana Monteiro[1], Ugo Maninchedda[2], Gautier Roussignol[3], Bernard Remandet[3], Emilie Segard[2], Sandrine Hilairet[3], Géraldine Pénarier[3], Régis Steinberg[3], Manuela Oliveira[4], Christian Jorgensen[5], Olivier M. Lepage[2]

[1] Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Departamento de Medicina Veterinária, Escola de Ciências e Tecnologia, Instituto de Investigação e Formação Avançada, Universidade de Évora, Évora, Portugal

[2] GREMERES-ICE Lyon Equine Research Center, VetAgro Sup, Veterinary Campus of Lyon, University of Lyon, Marcy l'Etoile, France

[3] Sanofi-aventis Recherche, Montpellier, France

[4] Departamento de Matemática, Escola de Ciências e Tecnologia, Universidade de Évora, Évora, Portugal

[5] CHU Lapeyronie University Hospital, Montpellier, France

Abstract

The aim of this work was to study the effects of allogeneic adipose mesenchymal stem/stromal cells activated with INF-γ (ASCs-γ) in equine experimental osteoarthritis (OA) *in vivo*. We used the metacarpophalangeal groove model in one forelimb while the contralateral joint was sham operated. The study involved ten Standardbred horses divided in two groups. Group A (*n* = 6) received an intra-articular (IA) injection of 15 million ASCs-γ in 3 mL of vehicle and Group B (*n* = 4) received the vehicle alone (3 mL). After surgery and 2 weeks stall-rest, horses respected an exercise protocol for 8 weeks. Animals had objective lameness evaluation, SF biochemical analysis and radiographic examination. In the end, horses were euthanized for macroscopic and microscopic joint assessment. The groove model was validated by group B results. It was observed a significant increase in total proteins (TP) at week 1 and 2, significant increased in the radiographic score at week 6 and 10 as well as significant increased microscopic lesion score adjacent to groove in OA-joints compared to sham-joints. The allogeneic cell treatment effects were represented by results in group A: significant decrease in prostaglandin E2 (PGE2) values in OA-joints compared to sham-joints at week 10 was observed. One horse from group A presented a flare reaction and had a chondroid-like cell focus inside one of the grooves. Another cell-treated horse presented mesenchymal-like cell focus inside two grooves. The latter horse was not lame, had no radiographic increased score and presented normal TP in the OA-joint throughout the study. There were no significant differences between OA-joints of both treatment groups but significant increased radiographic scores of OA-joints compared to sham-joints were present in group B but not in group A and could demonstrate an ASCs-γ effect. Our results confirmed the efficacy of the groove model. The IA allogeneic adipose-derived stem/stromal cell treatment showed possible modifying disease effects that should be further explored before application in clinical OA in horses.

1. Introduction

Mesenchymal stem/stromal cells (MSCs) have shown protective effects by preventing chondrocyte apoptosis and fibrosis due to their differentiation properties but also to paracrine actions (Maumus *et al.*, 2013). In a murine collagenase induced OA model, a single intra-articular injection of autologous adipose derived-tissue mesenchymal stem cells (ASCs) reduced synovitis, osteophyte formation and decreased score of cartilage lesions (ter Huurne *et al.*, 2012) and could depict a potential therapeutical effect in other species. MSCs need to be activated by local inflammatory mediators released from activated immune cells (Ghannam *et al.*, 2010). However, while inflammatory conditions are required for the immunosuppressive function of MSCs, they do not enhance their capacity to survive *in vivo* (Toupet *et al.*, 2015). IFN-γ was first reported to stimulate MSCs' production of Indoleamine 2,3-dioxygenase, which in turn inhibited the proliferation of activated T or natural killer cells (Krampera *et al.*, 2006). More recently, human ASCs have been showed to increase immunosuppressive capacity under inflammatory conditions *in vitro*, which affected gene expression of cytokines as well as ASCs morphology and proliferation while their differentiation capacity was preserved (Crop *et al.*, 2010). A

different study found that non-stimulated MSC did not change lymphocyte proliferation, but, when activated there was an increase in the secretion of PGE2 and Interleukin-6 as well as an inhibitory effect on T-cell proliferation and cytokine production (Carrade *et al*., 2012). Additionally, pre-treatment with IFN-γ enhances the therapeutic activity of MSCs (Duijvestein *et al*., 2011) and IFN-γ alone have shown protective effects on osteoarthritic animal models *in vitro* and *in vivo* (Page *et al*., 2010).

Most equine studies use an autologous source of stem cells (Frisbie *et al*., 2009; McIlwraith *et al*., 2011). This is probably due to the fact that the use of allogeneic source increases the occurrence of rejection and induces faster elimination of cells (Zani *et al*., 2009). Moreover, the allogeneic source can induce a loss of activity and have associated risks of spontaneous tumor transformation or differentiation in other cell types (Sivanathan *et al*., 2014). Conversely, autologous source supposes a delay from harvesting cells to their production and the use of allogeneic stem cells would have the advantage of being readily available and allow to standardize protocols. In a murine model, allogeneic MSCs were able to suppress graft to host disease after donor T-cell recognition of antigen only after IFN-γ pre-activation. In this study, it was sustained that allogeneic MSCs required a minimal threshold of IFN-γ and that more exposure amplified MSCs suppression potential. Therefore, *in vivo*, cytokine levels and time of MSCs injection would be critical for effective anti-inflammatory effects (Polchert *et al.*, 2008). IFN-γ pre-treatment could enhance an ASCs treatment and has never been tested in horses before.

Equine OA diagnosis and the evaluation of treatment efficacy is performed mainly by radiographic examination and subjective lameness evaluation. In recent years, an inertial sensor methodology has been used for objective detection of pain. This technology measures head and pelvic asymmetry (Keegan *et al*., 2004) and its repeatability confirmed (Keegan *et al*. 2011), compared to a stationary force plate (Keegan *et al.*, 2012) and proven to be more sensitive in mild cases of lameness when compared to subjective evaluation (McCracken *et al.,* 2012). Therefore, it could be particularly interesting for therapy assessment both in natural as well as induced OA. An equine groove model of metacarpo-phalangeal (MCP) OA has been developped and under a controlled exercise regime displayed significant radiographic, macroscopic and microscopic degenerative changes, close to naturally ocurring OA and allowing assessement of therapy effects (Maninchedda *et al*., 2015).

The purpose of this study was to evaluate the effects of allogeneic ASCs pre-activated with INF-γ (ASCs-γ) of the equine groove model of MCP OA previously described by Maninchedda *et al.* (2015) while validating the efficacy of this model. We hypothesized that, using the OA model *in vivo,* IA injection of allogeneic ASCs-γ would have protective effects demonstrated by reduced lameness, radiographic lesions, SF inflammation and cartilage degenerative features.

2. Materials & Methods

This study was carried out in strict accordance with the recommendations stated in the ARRIVE (Animal Research: Reporting *In Vivo* Experiments) guidelines. The Ethical Committee of VetAgro Sup-Veterinary Campus of Lyon approved the protocol (Permit Number: RECH-ETIC-P003-E01). All

procedures were performed under general anesthesia or sedation and all efforts were made to minimize suffering.

2.1. Isolation and characterization of ASCs

Adipose tissue was retrieved from the subcutaneous gluteal fat from a 4-year-old French Standardbred donor horse owned by Sanofi-Pasteur. The procedure was performed standing, under sedation and local anesthesia, using tail head standard technique (Frisbie *et al.,* 2009). Tissues were kept at 4-10ºC in a dissection medium, Dulbeco's phosphate buffer saline (PBS, Gibco, Life Technologies), during transportation and processed within 3 hours. Fat was digested with 0.25% collagenase 1 (Worthington Biochemical Corp.) under agitation at 37ºC during 1h30 and the homogenate was centrifuged during 10 min at 300 ga using routine laboratory techniques. The stromal vascular fraction was collected, cells were filtered successively through a large sterile filter, a 100 and a 70 µm porous membrane filters, in order to remove fat. To avoid red cells, a hemolysis step was done briefly by adding a lysis buffer for less than 5 min. Single cells were seeded in 10 cm Nunclon Petri dishes (passage 0) and amplified in a 5% CO_2 incubator at 37ºC. After 2-3 days, cultures were washed with PBS before changing the medium. After one week, cells were trypsinized (passage 1) and split for another amplification step. Following cell growth and checking for colony formation cells were trypsinized (passage 2), counted and stocked at 3 to 15 million cells per mL in freezing buffer at -80ºC or -160ºC. ASCs were characterized by their immunophenotype (positive for CD44, CD73, CD90, CD105 and negative for CD34 and CD45) and their trilineage potential.

2.2. ASCs pre-activation with IFN-γ

ASCs preparation for preactivation consisted in thawing cells at 37ºC, transferring them to prewarmed culture medium and plated (passage 2) at 1 million cells for a 10 cm Nunclon Petri dish. Medium was changed every 2-3 days for 10 days. At day 9, cells had an IFN-γ pretreatment (100ng/mL) during 24h. ASCs were then trypsinized (passage 3), counted and centrifuged during 5 min at 300 ga and resuspended in injection medium (vehicle) without IFN-γ (Stem Alpha 7900).

2.3. Groove model of metacarpophalangeal osteoarthritis

Ten French Standardbreds owned by Sanofi-Pasteur were included. The group consisted in seven geldings and three mares without clinical musculoskeletal abnormalities. Animals were divided into two groups: group A (n = 6), mean age 6.9 ± 1.4 year-old, mean weight 535 ± 54 kg and group B (n = 4), mean age 5.8 ± 0.7 year-old, mean weight 522 ± 38 kg. Animals were numerically identified and had complete MCP joint assessment. This included digital radiographic analysis and lameness evaluation of both front MCP joints.

Before surgery, animals were attributed to 5 left side and 5 right side OA-joints, the contralateral joint being used for the sham procedure. An adapted groove model described in a previous pilot study

was used to create experimental MCP OA (Maninchedda *et al.*, 2015) by the same ECVS surgeon. The dorsal aspect of the joints was assessed for the presence of pre-existing degenerative lesions. Four vertical and horizontal grooves were then created, in a woven pattern on the weight-bearing area of both condyles, with an adapted arthroscopic 2mm hook probe. Grooving was performed under gas (CO_2) distension followed by 3 min of lavage with lactated Ringer's solution. Post–operatively, horses had stall rest for 2 weeks and then were turned out into paddocks while respecting an exercise program that consisted of trotting in a 10-m circle on a sand surface every other day for 15 min in each direction during 8 weeks.

2.4. Intra-articular treatment

One week after surgery viability of cells was evaluated to be above 96% for 24 hours with a Vi Cell Viability Analyzer (Beckmann Coulter Inc.). Up to 5 million ASCs-γ /mL were placed into 5 mL sterile syringes for the injection step. Syringes were kept refrigerated at 4-6ºC and brought to room temperature before treatment. Animals were randomly assigned to 6 ASCs-γ and 4 vehicle injections. IA injection consisted of 15 million allogeneic ASCs-γ in 3 mL of the vehicle (Stem Alpha 7900) in group A and 3 mL of the vehicle alone in group B. Horses were sedated with detomidine (Detogesic®, Fort Dodge Animal) and butorphanol (Turbogesic®, Fort Dodge Animal). The OA-joint was prepared under aseptic conditions, treatments were administered after SF sample collection on the proximo-palmar-lateral synovial pouch and distal limb kept under a sterile bandage for 24h.

2.5. Arthroscopic evaluation

During arthroscopy images were recorded using high-resolution digital video (AIDA, Karl Storz). Arthroscopic exploration videos of OA and sham operated joints were blinded and scored (0-14) for general synovitis (0-1), dorso-distal third metacarpal bone (MC3) chondromalacia (0-1), fissure (0-1), fragment (0-1), wear lines (0-3) and erosion (0-3); and dorso-proximal first phalanx (P1) fragments (0-1) and erosion (0-3). Wear lines were scored from none (0), 1-2 partial thickness wear lines (1), 3-5 partial thickness wear lines or 1-2 full thickness wear lines (2) and more than 5 partial-thickness or 2 full-thickness wear lines (3). Erosions were scored from none (0), less than 5 mm partial-thickness erosion (1), more than 5 mm partial-thickness erosion (2) and full-thickness erosion (3). The other features were scored between absence (0) or presence (1) of the lesion.

2.6. Lameness evaluation

All horses had a clinical lameness examination including distal forelimb flexion tests before OA induction (week 0) and at weeks 6 and 10 post-operatively. The American Association of Equine Practitioners (AAEP) lameness scale was used (0= normal; 1= lameness difficult to observe and not consistently apparent regardless of circumstances; 2= lameness difficult to observe at a walk or trotting a straight line but consistently apparent under certain circumstances; 3= lameness consistently

observable at a trot under all circumstances; 4= obvious lameness at a walk; 5= minimal weight bearing) (Ross, 2003) for general exam and response to distal forelimb flexion classified as negative or positive. Simultaneously, objective lameness examinations were performed using an inertial sensor-based system (Lameness Locator v2014.1.0, Equinosis). Results from Lameness locator were reported as head movement asymmetry (A1/A2 ratio), which reflect the amplitude of the vertical head movement due to lameness divided by the amplitude of the expected normal vertical movement of the head (McCracken *et al*., 2012; Manninchedа *et al*., 2015). These values were analyzed over concrete surface, for trotting sequences of minimum 25 strides in straight line and minimum 10 strides after front distal limb flexion (Marshall *et al*., 2012). One ECVS surgeon performed the clinical and objective evaluations at week 0, 6, and 10.

2.7. Radiographic Examination

Radiographic examination of both front MCP joints was performed under intravenous (IV) detomidine and butorphanol sedation at week 0, 6 and 10. Five radiographic projections (Fuji CR, Fujifilm medical system) including lateromedial, dorsopalmar, flexed dorsopalmar, 45-degree dorsolateral-palmaromedial and dorsomedial-palmarolateral obliques were taken. A blinded European College of Veterinary Diagnostic Imaging (ECVDI) radiologist analyzed all images using a diagnostic workstation (eFilm 3.0, Merge Healthcare). The radiographic changes were semi-quantitatively graded, from no finding (0) to marked finding (2), and median total radiographic score was recorded for each group. A total radiographic score (0-56) encompassed synovial effusion (0-2), joint space width (0-2) and osteophytes (0-24), subchondral bone sclerosis (0-14) subchondral bone lysis (0-14) at different locations (Maninchedda *et al*., 2015).

2.8. Synovial fluid analysis

SF samples, collected in EDTA tubes (Venoject, Terumo), from both MCP joints were assessed for TP concentration using a refractometer before OA induction (week 0), before treatment (week 1) and after treatment at week 2, 6 and 10. Tubes were centrifuged at 4°C, 1500 rpm for 10 min (Jouan XR4i Centrifuge, Thermo Electron Corporation). SF supernatants were stored at -20°C until being transferred to -80ºC for future analysis. Inflammatory PGE2 was analyzed using commercial ELISA kits (R&D Systems).

2.9. Postmortem Examination

At week 10 post-surgery, all horses were submitted to euthanasia with an IV injection of 1g embutramide 2.5g mebezonium and 250 mg tetracaine hydrochloride (T-61®, Intervet).

Macroscopic evaluation

Digital macroscopic photographs of all cartilage surfaces were performed: distal MC3, proximal P1, and proximal sesamoid bones (PSB). One European College Veterinary Pathologist (ECVP) blindly evaluated all photographs. Cartilage findings were semi-quantitatively scored similarly to macroscopic guidelines of the Osteoarthritis Research Society International (OARSI) (Mcllwraith *et al*., 2010). A total macroscopic score (0-21) was obtained from the total MC3 (0-9), P1 (0-6) and PSB (0-6) (Maninchedda *et al*., 2015). The synovial membrane and grooved areas were only evaluated microscopically.

Microscopic evaluation

The distal end of MC3, proximal end of P1 and PSB were sampled with the attached synovial membrane and fixed with neutral buffered formalin (Carbo Erba Reagents). After fixation, latero-medial transverse slices 3 to 4 mm thick of the dorsal and palmar aspect of the two MC3 condyles, of the two P1 articular surfaces and the two PSB were performed (Figure 5). The dorsal MC3 region slices were centered over the grooved areas to allow assessment of microscopic changes surrounding the grooves. All slices were decalcified and processed on histological slides as previously described (Maninchedda *et al*., 2015). One ECVP pathologist blinded assessed the microscopic slides. Each finding was evaluated using modified OARSI histological features and scored from 0 (no finding) to 4 (marked finding). Medial and lateral bone surfaces scores were averaged for any type of finding.

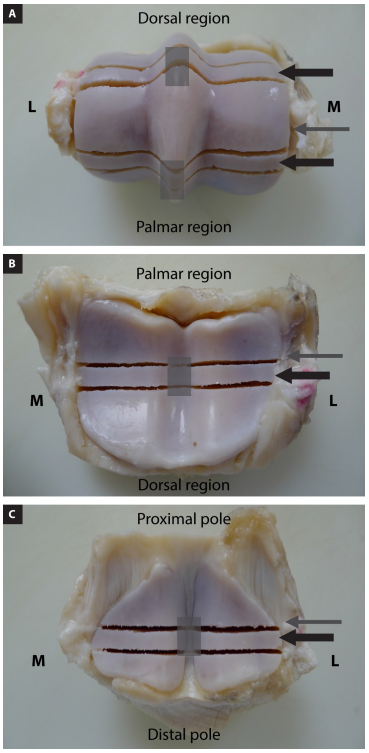

Figure 5: Macroscopic view of the articular surfaces of the metacarpo-phalangeal joint, (A) is the distal aspect of the third metacarpal bone, (B) is the proximal aspect of the first phalanx and (C) is dorsal aspect of lateral and medial proximal sesamoid bones. Black arrows represent the transverse section and grey arrows represent the articular capsule with synovial membrane used for microscopic analysis. Rectangular areas in grey were excluded from examination. L = lateral and M = medial.

Groove and non-groove areas were analyzed separately. Grooved areas consisted of cartilage of the dorsal MC3 and were scored for vertical grooves (0-4) and adjacent to groove cartilage for features of non-calcified cartilage (0-32): chondrocyte necrosis (0-4), chondrocyte clusters (0-4), fibrillation/fissuring (0-4), matrix softening (0-4), horizontal cracking (0-4) and focal ulcers (0-4) and subjacent calcified cartilage: chondrocyte necrosis (0-4) and fissuring (0-4) (Maninchedda *et al*., 2015).

Non-grooved areas consisted of cartilage away from grooves, not only in the distal MC3, dorsal and palmar as previsouly described (Maninchedda *et al*., 2015), but also in the proximal P1 and PSB. The four regions were assessed concerning articular cartilage, subchondral bone (SCB) and synovial membrane. Articular cartilage was scored according to chondrocyte necrosis (0-4), chondrocyte clusters (0-4), matrix softening (0-4) and fibrosis (0-4) adjacent to superficial swelled or depressed areas (0-4). The adjacent features (0-16) to swelled or depressed areas made a sub-total score of 20. The same features were assessed adjacent to superficial (0-4) or deep fissuring (0-4) which made a sub-total score of 24. Together they accomplished a 0-44 range for total non-grooved cartilage score.

SCB was scored (0-20) for fissuring (0-4), osteoblastic margination (0-4), edema (0-4), congestion (0-4) and subacute inflammation (0-4) (Maninchedda *et al*., 2015). Finally, synovial membrane was scored (0-20) for intimal layer thickening (0-4) and associated inflammation (0-4); perivascular inflammation in the sub-intimal layer (0-4) and associated fibrosis (0-4); and for synovial folds (0-4) (Maninchedda *et al*., 2015).

2.10. Statistical Analysis

Data were tested for normality and the homogeneity of variances by using the Shapiro–Wilk's and the Levene's tests, respectively. Group A and B had a different number of observations, therefore, comparisons with non-parametric two-way analysis of variance (ANOVA) were performed for SF (post-hoc using winner), for lameness and radiographic *data* with repeated measures on week and group. Arthroscopic, macroscopic and microscopic data were analyzed with one-way ANOVA within groups and Kruskal-Wallis test between treatment groups. Not normally distributed data were represented by median ± median absolute deviation (MAD) and normal distribution by mean ± standard deviation (SD). Statistical analysis was performed using software package EverStat V6 under SAS v9.2. Significance level was set at $P \leq 0.05$.

3. Results

3.1. Arthroscopic evaluation

At week 0, arthroscopic assessment of the dorsal MCP joint revealed pre-existing lesions (Table 2). The most common degenerative features of the dorsal aspect of the MCP joint were wear lines on the

dorsal MC3 and proximal P1 erosions. OA-joints from group A had significantly higher scores when compared to OA-joints from group B. However, total score differences were not significant between OA and sham-joints, which allowed comparisions within each group.

Table 2: Mean arthroscopic scores (± SD) from dorsal MCP joint at week 0.

	Sham-joint		OA-joint	
	Group A (*n*=6)	Group B (*n*=4)	Group A (*n*=6)	Group B (*n*=4)
Arthroscopic scores				
MC3 (0-9)	1.33 (± 1.211)	1.00 (± 1.414)	1.17 (± 0.983)	0.00 (± 0.000)
P1 (0-4)	0.83 (± 0.753)	0.25 (± 0.500)	1.00 (± 0.632)	0.25 (± 0.500)
Synovitis (0-1)	0.50 (± 0.548) [a]*	0.75 (± 0,500) [a]*	0.00 (± 0.000) [b]	0.00 (± 0.000) [b]
Total (0-14)	2.66 (± 2.066)	2.00 (± 2.160)	2.17 (± 1.472) ‡	0.25 (± 0.500)

Group A received ASCs-γ and Group B received the vehicle injection in OA-joints.
Different letters represent significantly different results between sham and OA-joints within groups. * $P < 0.05$
Significantly different results between OA joint of the two groups are represented by ‡ $P < 0.05$

3.2. Lameness evaluation

There were two horses of group A and one horse of group B with consistently grade 2 lameness and positive distal limb flexion test on the sham-joint. All other horses were consistently grade 2 lameness on the OA-joints at week 6 and decreased to grade 1 at week 10, with positive distal limb flexion at all observations. One horse from group A had transient flare reaction one week after starting exercise, which was managed conservatively by stall rest and compressive bandages during one week. This horse presented lameness visible at walk (grade 4) at week 6, decreased to grade 3 at week 10 and had increased pain after flexion test at week 6.

Objective lameness evaluation (median A1/A2 ratios) revealed increased asymmetry in OA-joints at week 6, decreasing by the end of the study (Table 3). OA-joints of group A had significantly lower asymmetry in straight-line compared to sham-joints at week 0. Significant differences were not observed in OA-joints of group A compared to group B for any circumstance at any time but asymmetry after flexion test in OA-joints at week 6 and 10 was less important in group A when compared to group B.

Table 3: Median A1/A2 ratio (± MAD) for asymmetry of front limbs trotting at straight line and after distal limb flexion.

	Sham-joint			OA-joint		
	Week 0	Week 6	Week 10	Week 0	Week 6	Week 10
Straight-line						
A (*n*=6)	0.7052 [a]** (± 0.1126)	0.2198 (± 0.1531)	0.5978 (± 0.2220)	0.2261 [b] (± 0.0665)	0.7273 (± 0.2263)	0.3944 (± 0.2255)
B (*n*=4)	0.4287 (± 0.2304)	0.1093 (± 0.0555)	0.1648 (± 0.0558)	0.4193 (± 0.2862)	0.6269 (± 0.2032)	0.3482 (± 0.2770)
Flexion-test						
A (*n*=6)	0.5977 (± 0.0833)	0.4182 (± 0.0689)	0.5795 (± 0.2990)	0.1589 (± 0.0573)	0.2873 (± 0.2401)	0.2039 (± 0.1988)
B (*n*=4)	0.5607 (± 0.3901)	0.0730 (± 0.0626)	0.0631 (± 0.0365)	0.2558 (± 0.2558)	0.8468 (± 0.0516)	0.5382 (± 0.2558)

Group A received ASCs-γ and Group B received the vehicle injection in OA-joints.
Different letters represent significantly different results between sham and OA-joints within groups. ** $P < 0.01$
There were no significant differences between OA-joints of the two groups.

3.3. Radiographic examination

Radiographic examination revealed increased median total scores at the end of the study compared to the exam before OA induction, for all groups. There was pre-existing SCB sclerosis in all groups and pre-existing osteophytes in group A. Significantly higher total median radiographic scores in OA-joints compared to sham-joints of group B were observed at week 6 and at week 10. Significant differences were not observed in OA-joints of group A compared to group B at any time (Figure 6). The most common features after OA induction was synovial effusion and increased osteophytes formation at week 10 (Figure 7).

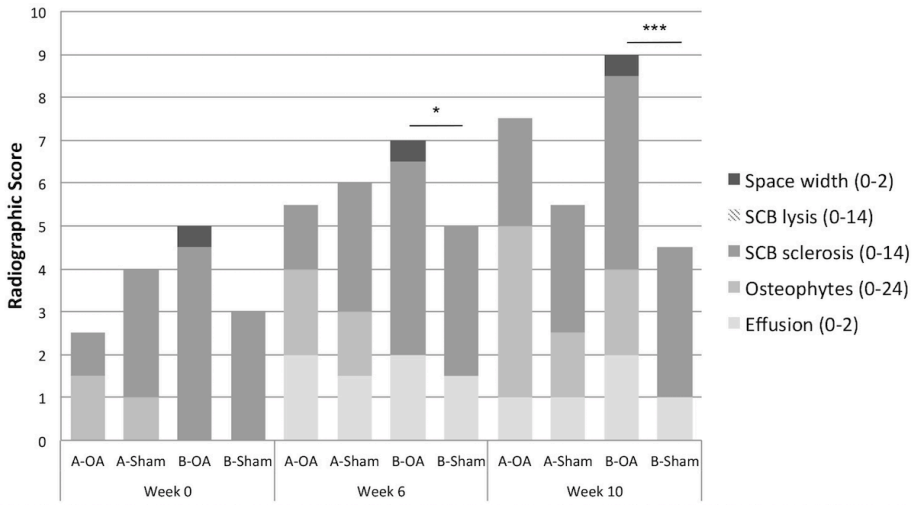

Figure 6: Radiographic scores at week 0, 6 and 10. Median radiographic total scores (0-56) of evaluated features in ASCs-γ treated group (Group A, *n* = 6), vehicle treated group (Group B, *n* = 4) and respective sham-joints. Significantly different results between OA and sham-joints are represented by * $P < 0.05$ and *** $P < 0.001$.

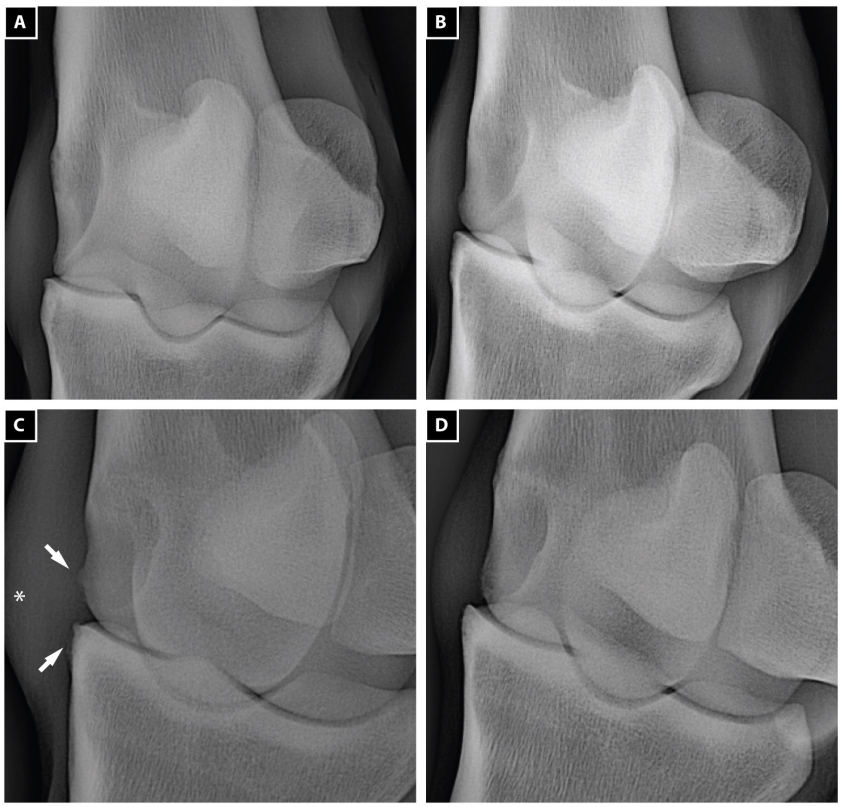

Figure 7: Radiographic views at week 10, the first row (A and B) are 45-degree dorsolateral-palmaromedial oblique views of metacarpo-phalangeal joint from an individual of group A (ASCs-γ) and the second row (C and D) are from an individual of group B (vehicle). The first column (A and C) are the OA-joints and the second column (B and D) are the sham-joints. The arrows show osteophyte formation (grade 2) and the asterisk show the synovial effusion (grade 2) on the OA-joint of the individual of group B.

3.4. Synovial fluid

Mean results from TP analysis are presented (Figure 8A). There were significantly higher TP values in OA-joints compared to sham-joints of group B at week 1 and at week 2. Significantly higher values in SF of OA-joints compared to sham-joints of group A appeared one week after ASCs-γ treatment. There were no differences between OA-joints of group A and B.

Mean results from PGE2 are presented (Figure 8B). There were significantly lower values in SF of ASCs-γ treated OA-joints (group A) compared to sham-joints at week 1 and week 10. There were no differences between OA-joints of group A and B.

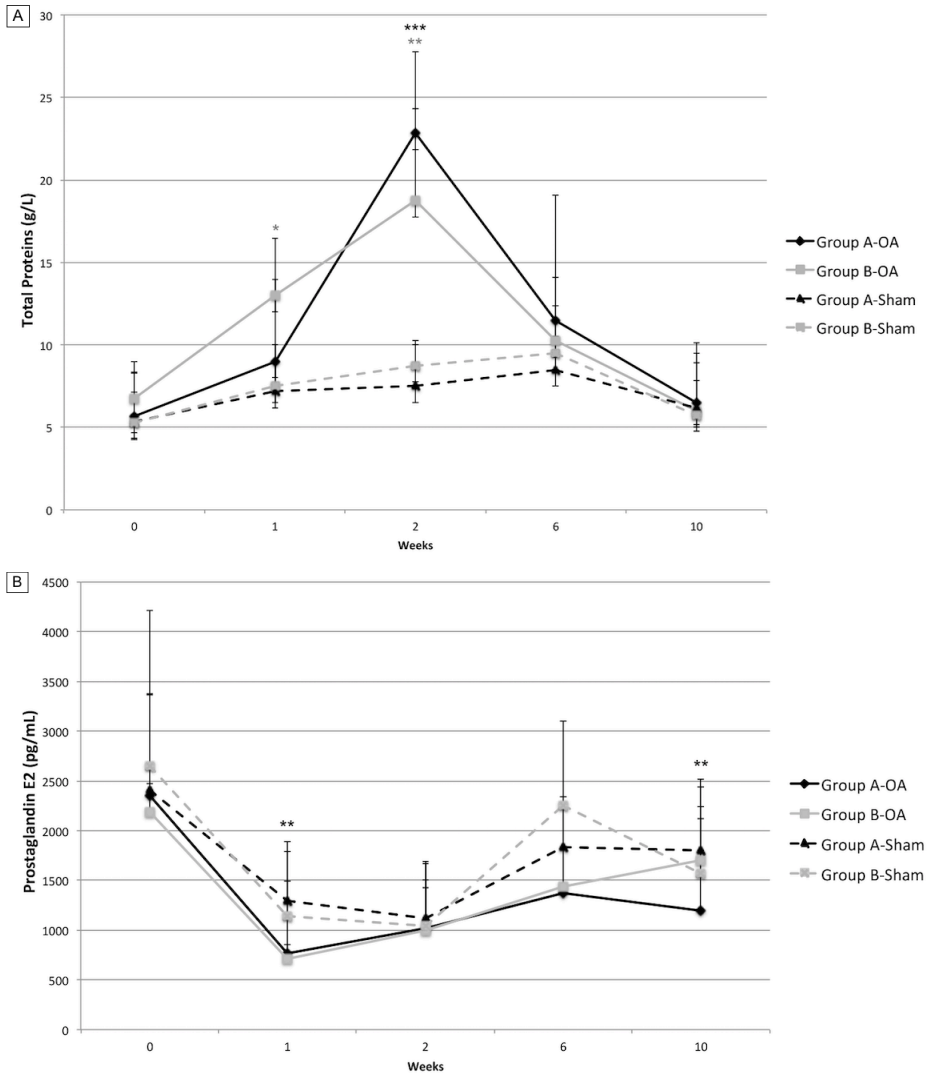

Figure 8: Synovial fluid. (A) Mean (± SD) Total Proteins and (B) Mean (± SD) Prostaglandin E2 values in synovial fluid of the ASCs-γ treated group (Group A, *n* = 6) and the vehicle treated group (Group B, *n* = 4) compared to respective sham-joints (interrupted line). Significantly different results are represented by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (black asterisks: Group A; grey asterisks: Group B).

3.5. Macroscopic and microscopic evaluation

Median results from macroscopic evaluation are shown in Table 4. There were higher but not significantly different total median scores in both OA-joint groups compared to respective sham-joints. Also, there were no significant differences in scores between OA-joints of group A and B.

Median results from microscopic evaluation of grooved areas are also shown in Table 4. There were significant higher values in both OA-joint groups compared to respective sham-joints for groove and for adjacent to groove cartilage scores. Differences between OA-joints of group A and group B were not significant. However, two intra-groove mesenchymal-like cell focus in loose matrix (Figure 9A) and one intra-groove chondrocyte-like cell focus in fibrous matrix (Figure 9B) were observed in OA-joints of two different ASCs-γ treated horses.

Table 4: Median macroscopic and microscopic scores (± MAD) at week 10.

	Sham-joint		OA-joint	
	Group A (*n*=6)	Group B (*n*=4)	Group A (*n*=6)	Group B (*n*=4)
Macroscopic scores				
MC3 (0-9)	2.0 (± 2.0)	2.5 (± 2.5)	5.0 (± 0.0)	5.0 (± 0.5)
P1 (0-6)	1.5 (± 0.5)	2.0 (± 1.0)	2.5 (± 0.50)	2.5 (± 0.5)
PSB (0-6)	1.5 (± 0.5)	1.5 (± 1.5)	1.5 (± 0.50)	1.0 (± 0.5)
Total joint (0-21)	**5.0 (± 4.0)**	**6.0 (± 5.0)**	**9.0 (± 1.50)**	**8.0 (± 1.5)**
Microscopic scores				
Grooved areas				
Grooves (0-4)	0.0 (± 0.00) [a]*	0.0 (± 0.0) [a]*	3.0 (± 0.3) [b]	3.3 (± 0.3) [b]
Adjacent Cartilage (0-32)	0.0 (± 0.00) [a]*	0.0 (± 0.0) [a]*	16.3 (± 1.0) [b]	17.5 (± 0.8) [b]
Non-grooved areas				
Articular Cartilage				
DorsalMC3 (0-44)	2.0 (± 1.5)	2.3 (± 2.0)	2.0 (± 1.3)	3.0 (± 1.5)
PalmarMC3 (0-44)	4.5 (± 2.8)	4.5 (± 4.5)	3.8 (± 2.3)	1.8 (± 1.3)
P1(0-44)	3.0 (± 3.0)	2.3 (± 2.3)	4.3 (± 2.8)	1.8 (± 0.3)
PSB (0-44)	3.0 (± 2.5)	5.5 (± 5.5)	4.5 (± 3.0)	0.0 (± 0.0)
Total cartilage (0-176)	**12.0 (± 10.0)**	**15.8 (± 15.5)**	**19.8 (± 7.3)**	**9.0 (± 3.8)**
Subchondral Bone				
Dorsal MC3 (0-20)	0.0 (± 0.0) [a]**	0.0 (± 0.0) [a]*	2.3 (± 0.8) [b]	2.5 (± 0.5) [b]
Palmar MC3 (0-20)	0.0 (± 0.0)	0.0 (± 0.0)	1.5 (± 0.5)	0.8 (± 0.5)
P1(0-20)	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)
PSB (0-20)	0.0 (± 0.0)	0.80 (± 0.8)	0.0 (± 0.0)	0.0 (± 0.0)
Total SCB (0-80)	**0.3 (± 0.3) [a]***	**1.0 (± 1.0)**	**4.3 (± 0.5) [b]**	**3.8 (± 2.0)**
Synovial Membrane				
Dorsal MC3 (0-20)	0.8 (± 0.3)	1.0 (± 0.5)	1.5 (± 0.5)	1.3 (± 0.8)
Palmar MC3 (0-20)	0.5 (± 0.5)	0.0 (± 0.0)	0.3 (± 0.3)	0.5 (± 0.3)
P1(0-20)	1.5 (± 1.0)	1.5 (± 0.5)	1.0 (± 0.5)	1.8 (± 1.0)
PSB (0-20)	1.5 (± 1.0)	1.3 (± 1.0)	2.5 (± 0.5)	0.8 (± 0.5)
Total synovial membrane (0-80)	**4.3 (± 2.0)**	**3.3 (± 1.3)**	**4.8 (± 0.8)**	**4.8 (± 0.5)**
Total non grooved joint (0-336)	**17.8 (± 12.3)**	**22.8 (± 18.5)**	**28.8 (± 10.3)**	**16.5 (± 3.5)**

The potential score range for each region is listed parenthetically next to each category in the first column.
Group A received ASCs-γ and Group B received the vehicle injection in OA-joints.
Different letters represent significantly different results between sham and OA-joints within groups. * $P < 0.05$ and ** $P < 0.01$.
There were no significant differences between OA-joints of the two groups.

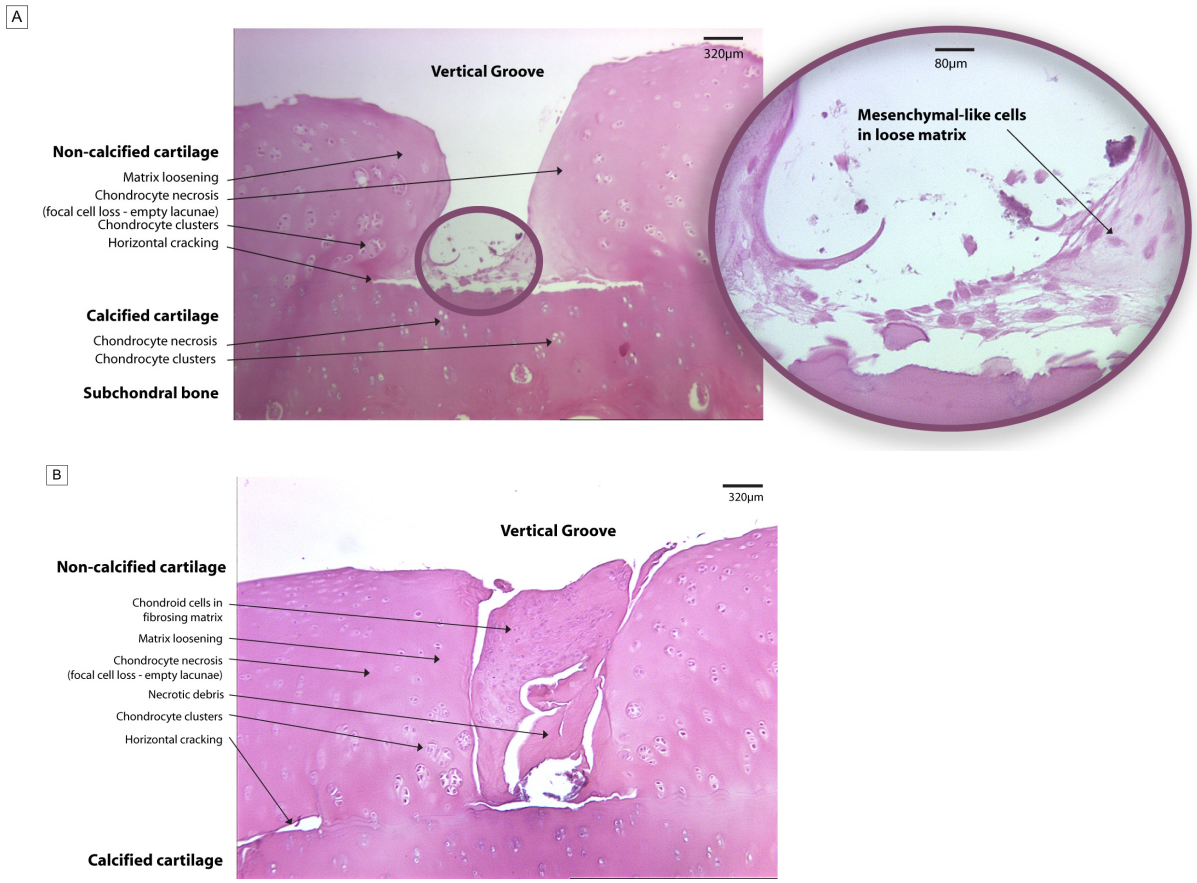

Figure 9: Light micrographs of grooved areas of the dorsal MC3 condylar cartilage (hematoxylin and eosin stained). (A) ASCs-γ treated horse with microscopic lesions adjacent to groove and intra-groove mesenchymal-like cell focus found in loose matrix. (B) ASCs-γ treated horse with microscopic lesions adjacent to groove and intra-groove chondrocyte-like cell focus in fibrous matrix.

Total median microscopic results of non-grooved areas are shown in Table 4. We have found significant differences between dorsal MC3 SCB scores in group A and group B and total SCB score in group A between OA and sham-joints. Most important features found in non-grooved cartilage of both OA and sham-joints were swelled/depressed areas (Figure 10A) and superficial/deep fissuring (Figure 10B). There was an absence of SCB edema and congestion besides absence of intimal inflammation and subintimal fibrosis of synovial membrane.

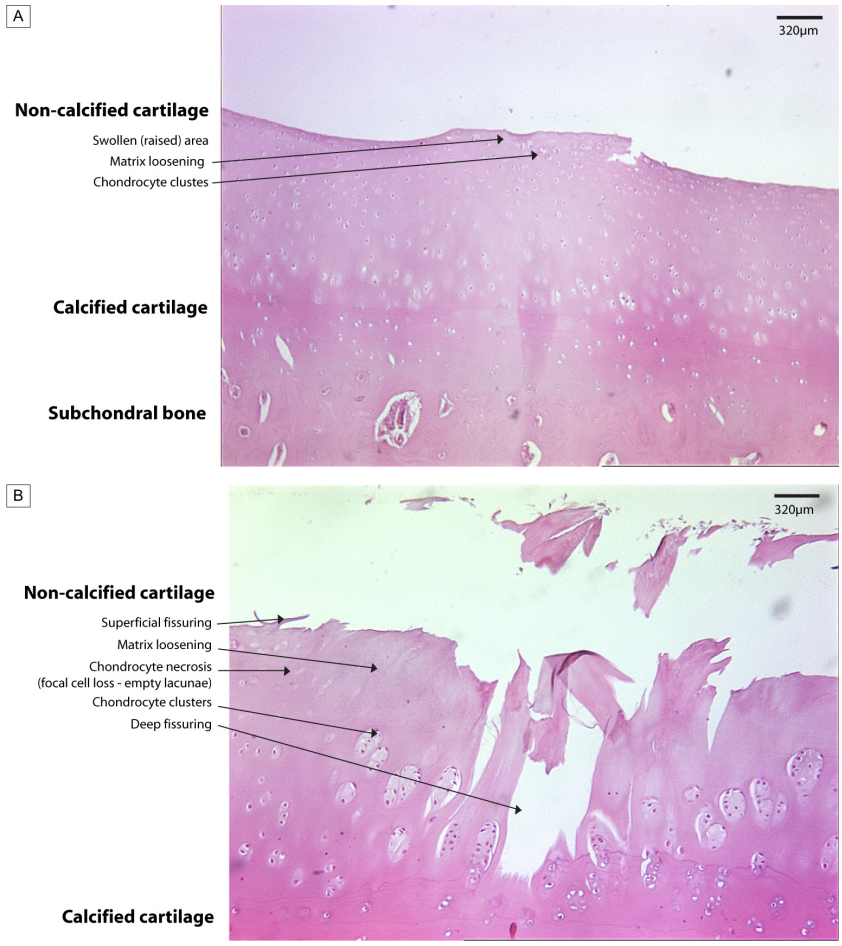

Figure 10: Light micrographs of non-grooved areas of the dorsal MC3 condylar cartilage (hematoxylin and eosin stained). (A) Sham-joint of ASCs-γ treated horse showing a swollen (raised) area of non-calcified cartilage of the palmar MC3. (B) Sham-joint of ASCs-γ treated horse showing a superficial/deep fissuring in non-calcified cartilage of the palmar MC3.

4. Discussion

To the best of the authors' knowledge this is the first study applying allogeneic ASCs pre-activated with INF-γ in horses. The role of IFN-γ in the immunosuppressive function of MSC has been reported (Krampera *et al*., 2006) but it was never studied in equine experimental OA. In the present study, 15 million vehicled allogeneic ASCs-γ were injected into six MCP joints. The protocol treatment aplyed is according with a recent review on IA biologic therapies that describes several *in vivo* clinical and experimental studies in horses using 10-20 million cells per joint, suspended in 2 to 5 mL vehicle solutions (Monteiro *et al*., 2015).

Evaluation of arthroscopic video records revealed pre-existing lesions in all groups, which might have influenced our results. Total score differences were not significant between OA and sham-joints of each treatment group, even if increased synovitis in sham-joints, which allowed comparison within groups. However, the significant higher pre-existent lesions in OA-joints from group A compared to group B may have attenuated a possible ASCs-γ effect. In fact, all the artroscopic scores except

synovitis, were higher in OA-joints of group A compared to group B. Exclusion was not considered before the biginning of the study because pre-evaluation of the overall status of the joint was performed by individual lameness and radiographic examination. In the future, magnetic resonance imaging or a diagnostic needle arthroscope could be used pre-operatively in order to assess both dorsal and palmar compartments in standing horses before OA-induction under general anesthesia (Frisbie *et al*., 2014). In the future, horses with pre-existent signs of OA should be excluded.

At the day of treatment, one week after surgery, there was reduced inflammation in the OA-joints of both treated groups as showed by low TP and PGE2 values. This could be explained by the removal of debris with the lavage performed at the end of the surgery. Osteochondral equine OA model reports no flushing at the end of the surgery and so, apllying this model, TP and PGE2 are supposed to be significantly increased one week and until 7 weeks post-operatively, being associated with increased inflammation and pain (Frisbie *et al*., 2008). The authors have atttributed the high values of PGE2 at week 0, which were similar for all groups, to pre-existent lesions. However, significantly lower PGE2 values at week 10 could support an anti-inflammatory role of ASCs-γ previously described by ter Huurne *et al*. (2012). Allogeneic source of MSCs is described to induce transient TP increase when compared to autologous source (Pigott *et al*., 2013). In the present study, there was increased TP in OA-joints of group A and B at week 2 compared to sham-joints but no significant differences between OA-joint groups were observed. This TP increase is probably due to the surgical model (Maninchedda *et al*., 2015) and the expected allogeneic effect was probably attenuated by IFN-γ pre-activation of ASCs by enhanced immunomodulation (Polchert *et al*., 2008).

Objectively, forelimb asymmetry was more evident after 4 weeks of exercise and decreased at the end of the study, which was similar to the pilot study (Maninchedda *et al*., 2015). Maybe the 3-minute final lavage has removed the inflammatory debris, that are important for perpetuation of pain. In one study, chrondrogenic induced allogeneic stem cells preconditioned with PRP and injected intra-articularly in horses with natural occurring OA, have shown 60% return to work compared to 45% with native MSCs at 6 weeks follow-up and 86% compared to 78% at 18 weeks (Broeckx *et al*., 2014). These authors reported 1.8% (3/165) flare reactions one week after treatment. Therefore, allogeneic treatments can induce flare reactions and IFN-γ doesn't seem to preclude this risk, since this was also observed in our study. Interestingly, in spite of the flare reaction in one ASCs-γ treated joint, the median asymmetry value after flexion was much higher in group B compared to group A at week 6. Although it was not significantly different, this could reflect decreased pain in the ASCs-γ treated group at that time.

Radiographic examination at week 6 and 10 revealed increased median scores in sham and OA-joints of both groups. Interestingly, significant increased scores of OA-joints compared to sham-joints were present in group B but not in group A and could illustrate an ASCs-γ effect. In a caprine mechanical OA knee model, bone marrow tissue-derived MSCs have been described to reduce osteophyte remodeling compared to vehicle treated joints (Murphy *et al*., 2003). In a chemical OA carpal model, in donkeys, x-ray scores of OA were reduced several degrees after MSCs treatment (Mokbel *et al*., 2011). Considering the OA-joints, there was no significant difference between group A and B in the end of the study.

Macroscopic evaluation of the non-surgical damaged areas did not show differences between groups and suggest no influence or treatment effect on pre-existent or secondary cartilage lesions. Microscopically, there was significantly increased in lesion score, adjacent to groove lesions in OA-joints compared to sham-joints in both groups, which reflects the effect of surgical model applied, but not a treatment effect. Significant higher SCB lesions found in dorsal MC3 of both groups were probably secondary to the surgical model and couldn't depict a treatment effect. In the present study, microscopic slides were performed in a perpendicular direction to the ones performed in the previous pilot study (Maninchedda *et al*., 2015), latero-medial instead of dorso-palmar, to better detect wear lines. These macroscopic lesions probably correspond to "swelled and depressed areas" added to the microscopic classification system. The MCP groove model was chosen to be close to the natural disease in the horse (Maninchedda *et al*., 2015). However, as it presents more degenerative than inflammatory features, it is possible that ASCs-γ effect could be more evident in natural occurring disease. Horses had similar age and weight but had different sex and osteoarticular status despite no evident musculoskeletal abnormalities were found before including horses in the study. Moreover, the low number of individuals limited the statistical analysis.

The chondrocyte-like cells found in fibrous matrix in one groove corresponded to the horse presenting a flare reaction and could represent an inefficient repair. On the other hand, the animal presenting mesenchymal-like cell focus in loose matrix, at the bottom of two grooves, could be interpreted as a sign of early healing. Interestingly, the latter horse didn't show OA-joint lameness at any evaluation, no osteophyte formation at radiographic examination and had normal TP throughout the study. The authors didn't expect to find the injected cells at microscopic examination of SF, since systemic clearance of allogeneic cells has been reported to be around 40 days (Zangi *et al*., 2009). However, labelling injected cells would have been important in order to follow exogenous ASCs-γ in the joints, since whitout this we can not guarantee that mesenchymal-like cells found inside the grooves are from the horse itself and not remnants of the injected cells.

In conclusion, the groove-model was validated by the vehicle treated horses (group B) results. We observed significantly increased TP at week 1 and 2, increased radiographic score at week 6 and 10 as well as increased microscopic lesion score adjacent to groove microscopic score in OA-joints compared to sham-joints. Our hypothesis was partially confirmed by the results in group A. The IA allogeneic ASCs-γ treatment significantly decreased PGE2 values in OA-joints compared to sham joints at week 10. Moreover there were no significant differences in radiographic scores between OA-joints and sham-joints at week 6 and 10, which suggest some OA disease-modifying effects. Therefore, the allogeneic ASCs-γ IA treatment is potentially interesting but should be further explored before being recommended for OA treatment in horses.

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CHAPTER IV – Relationship between serum biomarkers of cartilage and osteoarticular disease in Lusitano horses

Susana Monteiro[1], Olivier M Lepage[2], Luís Antunes[3], Liliane Damásio[3], Sandra Branco[1], Manuela Oliveira[4], Elisa Bettencourt[1]

[1] ICAAM - Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Departamento de Medicina Veterinária, Escola de Ciências e Tecnologia, Universidade de Évora, Pólo da Mitra, 7002-554 Évora, Portugal.

[2] University of Lyon; VetAgro Sup, Veterinary Campus of Lyon, GREMERES-ICE Lyon Equine Research Center, Marcy l'Etoile, F-69280, France.

[3] Hospital Veterinário, Universidade de Évora, Pólo da Mitra, Apartado 94, 7002-554 Évora.

[4] CIMA – Centro de Investigação em Matemática e Aplicações, Departamento de Matemática, Escola de Ciências e Tecnologia, Universidade de Évora, 7000 Évora, Portugal.

Abstract

Cartilage degradation biomarkers are a potential tool for early diagnosis of osteoarticular disease. The aim of this research was to evaluate cartilage biomarkers changes in serum of a Lusitano horse population. Fibulin 3-2 has been reported to be higher in serum of humans with osteoarthritis (OA); in young horses, alpha-helical type II collagen peptide (Coll2-1) and is nitrated form (Coll2-1NO_2) have been studied in serum and reported to be useful in the assessment of joint disease. In this study we have evaluated biomarkers' changes with age, sex and exercise and studied the relationship with osteoarticular disease. Subjective and objective lameness evaluations as well as blood and radiographic examination were performed in 51 Lusitano horses. Females presented significantly higher concentrations of Coll2-1 (P = 0.015) and Coll2-1NO_2 (P = 0.014) compared to males. We have found significant influence of low level of work in higher concentration of Coll2-1 (P = 0.001) and significant influence of sex in concentration of Coll2-1NO_2 (P = 0.030). There was no influence of sex, age and work on Fib3-2. All biomarkers were increased in the osteoarticular disease group (n = 35) compared to healthy controls (n = 16). This difference was significant for Coll2-1 (P = 0.015). When sorted by sex and age groups, significant difference in Coll2-1 between disease and healthy controls disappeared in old horses and females. In conclusion, Coll2-1 is a good marker of cartilage degradation in horses with osteoarticular disease, being more specific in young horses and males. Fib3-2 may be further explored to help identify disease in particular cases like female horses.

1. Introduction

OA is a debilitating disease that affects most commonly the metacarpo/tarso-phalangeal (fetlock), interphalangeal and intertarsal joints of old or sportive young adult horses (Baccarin *et al*., 2012). Radiological examination has a major impact on the animal's commercial value and is useful if problems can be treated before horses start or continue training. However, bone radiographic changes are often seen in the latest stages of the disease. For early diagnosis of OA, human and veterinary research has been focused on an alternative tool: cartilage biomarkers analysis detection. Blood sample collection is better tolerated than synovial samples, and serum values have been described as having significant correlation with some molecules concentration in synovial fluid (SF) (Catteral *et al*., 2010). This is probably due to gaps between synoviocytes, absence of a basal membrane and presence of subsynovial blood vessels allowing exchange of macromolecules between plasma and SF (Todhunter, 1996).

Type II collagen is the most abundant protein in extra-cellular matrix from articular cartilage. Coll2-1 is located in the collagen triple helix and Coll2-1NO_2 is the nitrated form of Coll2-1, requiring oxidative stress to be expressed, possibly indicating the level of inflammation in the synovium (Henrotin *et al*., 2004; Deberg *et al*. 2005). Coll2-1 fragment has been described as a valid biomarker for OA in mice (Ameye *et al*., 2007) and in human patients (Henrotin *et al*., 2004; Deberg *et al*., 2005; Deberg *et al*., 2008).

In horses, these biomarkers were first monitored in serum of Ardenner horses with 1.25 to 2.33 year-old (Lejeune *et al*., 2007). Coll2-1NO_2 was significantly higher in females and in the group with degenerative digital osteoarthropathy. Interactions between sex, time and pathology were significant for Coll2-1NO2 and between time and pathology for Coll2-1. Subsequently, a mixed sex and breed population, with a mean age of 3.46 years-old, showing joint effusion and lameness had cartilage assessed by arthroscopy (Gangl *et al*., 2007). Coll2-1NO_2 was significantly higher in the osteochondral lesion group, and no differences in sex and age were reported. In a different study, using warmblood stallions with a mean age of 2.56 years-old, Coll2-1 in serum was reported to be significantly higher in the degenerative joint disease group assessed by radiography (Verwilghen *et al*., 2009). There was a negative correlation between age and Coll2-1 and no correlation between age and Coll2-1NO_2. More recently, a mixed sex and breed population with a mean age of 3.5 year-old was studied by arthroscopy (Verwilghen *et al*., 2011). There were significantly higher blood values of Coll2-1 in the higher degenerative class group. There were no differences in Coll2-1NO_2 concentrations in serum between inflammatory and degenerative classes. These studies involved young horse populations and none evaluated the effect of exercise on the biomarkers' concentration. However, there are several studies in horses reporting higher concentrations of macromolecules in serum from horses submitted to exercise (Billinghurst *et al*., 2003; O'Kane *et al*., 2006; Frisbie *et al*., 2008).

Fibulin 3, a member of a family of extracellular matrix proteins, has been described to be elevated in OA cartilage (Wu *et al*., 2007). It was lately shown that fibulin 3 fragments (Fib3-1 and Fib3-2 peptides) were elevated in urine and serum of human OA patients and could discriminate between OA and normal populations (Henrotin *et al*., 2012). Fib3-2 in serum was not affected by the severity of the disease but presented the advantage of not being affected by sex or age. Therefore, this could be useful in detecting OA alone or in association with other biomarkers. To our knowledge, this is the first study reporting Fib3-2 concentrations in horses. Moreover, No data exists about osteoarticular disease and cartilage biomarkers' concentration in serum of Lusitano horses.

In the present study we evaluated differences in serum biomarkers' concentration for sex, age and exercise level and tried to establish a relationship with joint disease. The first hypothesis was that all three serum biomarkers concentrations change with sex, age and exercise. Secondly, we expected that all three biomarkers would increase in horses affected by osteoarticular disease.

2. Materials & Methods

2.1. Examined horse population, age and exercise groups

The studied population included Lusitano horses presented for lameness examination at three different veterinary clinics between July 2013 and February 2014. Owners signed an informed consent before horses were enrolled in the study. Owners or trainers answered a complete questionnaire and a total of fifty-one horses underwent a subjective and objective lameness evaluation, digital

radiographic examination and blood sample collection. Horses other than Lusitano or with weight bearing lameness were excluded from this study.

There were 37 non-gelded males and 14 females (mean ± SEM age 10.39 ± 0.91 years, range 3-25 years). Two different categories of age were established considering that mean age of horses presenting OA have been described to be 8.4 ± 3.9 year-old (Baccarin *et al.,* 2012). Therefore, 26 animals were included in the young horse category (less than 9 year-old) and 25 animals in the old horse category (9 year-old and more). Regarding exercise, there were 34 horses performing at low level (less than 5h per week) and 17 horses performing at high level (5h per week and more). There were young horses in training (*n* = 6), horses in competition (*n* = 22), field/breeding mares (*n* = 8) and stall/breeding stallions (*n* = 15).

2.2. Lameness examination

All animals underwent a general and orthopaedic examination. Lameness exams were always performed subjectively according to the American Association of Equine Practitioners (AAEP) grading system (Ross, 2003) and recorded by digital video. All horses had a second subjective evaluation through video records visualization. Furthermore, lameness was objectively assessed using an inertial sensor device (Lameness Locator® v2014.1.0, Equinosis). Objective reports from at least 25 strides in a straight-line over concrete surface were reviewed (Keegan *et al*., 2011; McCracken e*t al*., 2012). Horses with “moderate to strong evidence” of lameness were considered positively lame for that limb.

2.3. Radiographic examination and classification

All radiographic examinations were performed according to a standardised procedure. Whenever needed horses were sedated, using 0.01 mg/kg detomidine IV (Domidine®, DFV) alone or combined with 0.02 mg/kg butorphanol IV (Dolorex®, Intervet). Radiographic evaluation was performed for all four distal limbs (metarcarpo/tarso-phalangeal joints; distal and proximal inter-phalangeal joints) and the two tarsal regions (tibio-tarsal, inter-tarsal and tarso-metatarsal joints). Four standard orthogonal projections were taken, including the latero-medial; dorso-palmar/plantar; dorso 45º latero-palmar/plantar medial oblique and dorso 45º medial- palmar/plantar lateral oblique. These joints were chosen based on the most commonly affected OA sites in Lusitano horses (Baccarin *et al*., 2012) but in cases where clinical signs were observed elsewhere (*e.g.* joint remodelling, pain or effusion), supplementary joints were analysed. Radiographs were evaluated by 3 veterinarians and scored from absence (0) to severe (4) radiographic lesions (Figure 11).

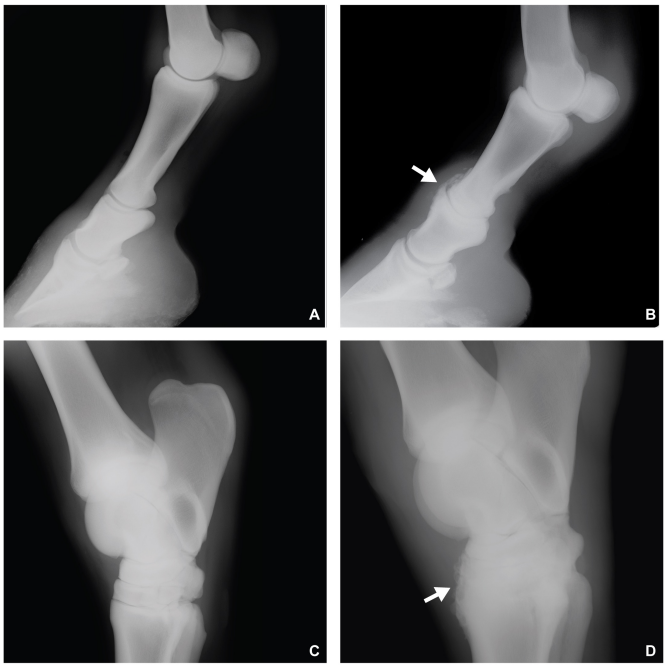

Figure 11: Latero-medial radiographs of equine distal limb region (A, B) and tarsal region (C, D) illustrating grade 0-normal (A, C) and grade 4-severe OA lesions (arrows) of the proximal inter-phalangeal joint (B) and distal inter-tarsal and tarso-metatarsal joints (D).

The OA scale was adapted from previously reported scales (Kellgren & Lawrence 1957; de Grauw *et al*., 2006), in order to obtain a mean grade per joint (0-4) and a mean total score per horse (0-72) (Table 5).

Table 5: Radiographic scoring system (adapted from Kellgren and Lawrence, 1957 and de Grauw et al., 2006).

Grade	Severity of OA	Description of radiographic lesions
0	Normal	Rounded joint margins, no subchondral bone sclerosis.
1	Doubtful	Pointed joint margins or minimal localised subchondral bone sclerosis.
2	Minimal	Small spur(s) on joint margins or mild localized subchondral bone sclerosis.
3	Moderate	Moderate spur(s) or moderate localised subchondral bone sclerosis.
4	Severe	Large spur(s), severe subchondral bone sclerosis or evidence of joint space narrowing.

2.4. Osteoarticular disease

The presence of OA was defined by the presence of a mean radiographic joint grade higher than 1 associated with the presence of one objective lameness report in the same limb or with two positive

subjective lameness reports. Furthermore, osteochondritis dissecans (OCD) lesions were identified separatly, as being a source of OA but potentially leading to a more inflammatory than degenerative joint environment. Horses with presence of OA and OCD were included in the disease group and horses with absence of OA or OCD fragments were used as controls.

2.5. Blood samples

Venous blood samples were collected from the jugular veins in serum tubes (Serum-gel S-Monovette® 4.9 mL, Sarstedt) for the Coll2-1, Coll2-1NO_2 and Fib3-2 peptide measurments. Blood was centrifuged at 1500 rpm for 10 min at 4ºC. The serum was aliquoted and frozen at -20 ºC within 60 min from sampling for three months and then kept at -80 ºC. Serum samples were thawed immediately prior to their use.

2.6. Coll2-1, Coll2-1NO_2 and Fib3-2 assays

Degradation fragments derived from type II collagen and Fibulin-3 are released into horse serum and immunoassays can be used to measure Coll2-1, Coll2-1NO_2, and Fib3-2 sequences. These assays are competitive immunoassays utilizing a synthetic peptide pre-coated onto the ELISA plate for the quantification of the corresponding antigen in serum samples. A binding competition between the immobilized peptide and the peptide contained in the serum samples takes place upon addition of the antibodies. After removal of the unbound peptide, a peroxidase-conjugated antibody is added into each well to detect and quantify the level of competitive binding. After washing the unbound detection antibody, the antibody-antigen complex is detected by a chromogenic reaction. The reaction is stopped and the colorimetric endpoint is determined spectrophotometrically (Verwilghen *et al*., 2011).

The measurement of Fib3-2 was performed in triplicate and adapted from the F3F ELISA kit (Artialis SA), applying a 8-fold dilution of samples. Coll2-1 and Coll2-1NO_2 were quantified in triplicate by competitive ELISA (Artialis SA), applying a 16-fold dilution of samples. Buffers were adapted from previous studies in horse blood (Verwilghen *et al*., 2009).

2.7. Statistical analysis

All data was analysed under SPSS commercial available software (v22). Data were represented by mean ± standard error of the mean (SEM) and significance was set at $P \leq 0.05$. Normality of data was checked using Shapiro-Wilk test.

Relationship between continuous variables was assessed using Pearson's correlation coefficient. Mean values of biomarkers between groups of age, sex and exercise were compared using t-test procedure and disease proportions between groups were studied with Pearson Chi-square test.

The effects of age, sex and exercise on biomarkers were measured using a Generalized Linear Model (GLM) procedure. Binary logistic regression models were applied to evaluate the effect of the biomarkers on the probability of presence/absence of osteoarticular disease and lameness and on the

probability of belonging to a high radiographic category. Finally, sensitivity and specificity of biomarkers' concentration were analysed using the Area Under the Curve (AUC) of the Receiver Operating Characteristic Curve (ROC) analysis.

3. Results

3.1. Effects of sex, age and exercise on serum biomarkers

Significantly higher concentrations of biomarkers were obtained in females compared to males for Coll2-1 (2068.61 ± 56.77 nM compared to 1861.43 ± 58.36 nM; $P = 0.015$) and for Coll2-1NO_2 (10.51 ± 0.49 nM compared to 8.63 ± 0.41 nM; $P = 0.014$). Fib3-2 values were not significantly different but showed higher concentrations in females (605.86 ± 53.85 pM) compared to males (563.53 ± 26.05 pM). Biomarkers presented no significant differences between the two age groups (Coll2-1 $P = 0.0881$; Coll2-1NO_2 $P = 0.995$; Fib3-2 $P = 0.973$). Moreover, we observed a negative, small and not significant correlation between age and type II collagen biomarkers, and a positive, small and not significant correlation for Fib3-2. There were significantly increased concentrations of biomarkers in horses working less than 5 hours compared to horses working more than 5 hours per week for Coll2-1 (1993.09 ± 54.11 nM compared to 1768.71 ± 79.13 nM; $P = 0.022$) and for Fib3-2 (608.43 ± 32.34 pM compared to 508.58 ± 24.51pM; $P = 0.047$). There was higher percentage of females (32.35%) in the low-level compared to the high-level of exercise group (17.65%), this difference was not significant.

Using the GLM procedure we observed that there was significant influence of exercise on Coll2-1 ($P = 0.001$), increasing in the low level of exercise group, there was a significant influence of sex on Coll2-1NO_2 ($P = 0.030$), increasing in females, and that Fib3-2 was not influenced by age, sex or exercise.

3.2. Correlation between biomarkers

There was significant correlation between Coll2-1 and Coll2-1NO_2 ($r = 0.650$; $P = 0.000$) and between Coll2-1 and Fib3-2 ($r = 0.329$; $P = 0.018$). Coll2-1NO_2 and Fib3-2 were not correlated ($r= 0.006$; $P = 0.966$).

3.3. Relationship between Coll2-1, Coll2-1NO_2 and Fib3-2 and clinical findings

Lameness and radiographic examination

Considering subjective grades of lameness and the presence of lameness assessed objectively, there were no significant differences between biomarkers concentrations (Table 6). However, there was a significantly higher percentage of OA horses in the objectively lame group ($P < 0.001$). Binary

logistic regression (backward stepwise method) was used to study the probability of being objectively lame (r^2 = 0.256 with P = 0.938 at the Hosmer-Lemeshow test). The log-odd probability of belonging to the positive objective lameness group compared to the negative lameness group increased by 1.238 per year of age (P = 0.027).

Table 6: Distribution of objective lameness, disease and mean (± SEM) serum biomarkers' values *n*=51.

	OA %	OA + OCD%	OCD %	Without disease	Coll2-1 (nM)	Coll2-1NO_2 (nM)	Fib3-2 (pM)
Sound (*n*=11)	0.00[a] (0/11)	0.00 (0/11)	9.09 (1/11)	90.91 (10/11)	1845.39 ± 83.91	8.72 ± 0.64	553.12 ± 34.08
Lame (*n*=40)	60.00[b] *** (32/40)	20.00 (8/40)	5.00 (2/40)	15.00 (6/40)	1938.35 ± 54.95	9.27 ± 0.41	581.20 ± 29.03

Different letters represent significantly different results between lame and sound groups. *** P < 0.001

There was a positive correlation between total radiographic score per horse among the 3 evaluators (P = 0.001). Total mean radiographic scores had positive, small and not significant correlation with the 3 biomarkers. However, there was a significant influence of more advanced age on the increased radiographic score (GLM procedure; P = 0.003). Moreover, binary logistic regression (backward stepwise method) was used to study the probability of having radiographic score above 14 (r^2 = 0.223 with P = 0.832 at the Hosmer-Lemeshow test). The log-odd probability of belonging to the higher radiographic class increased by 1.002 for each increased unit of Coll2-1 (P = 0.040) and by 1.125 per year of age (P = 0.025)

Osteoarticular disease

Older horses had higher percentage of OA (84.00%) compared to young horses (42.30%) and this was significantly different (P = 0.002). There was higher percentage of OA (69.70%) in lower exercise category compared to horses working more than 5h per week (47.05%) but this difference was not significant (P = 0.101). Considering only males, there was significantly more OA within the lower compared to higher exercise class (P = 0.010).

Biomarkers' concentrations in different groups of age, sex and exercise sorted by presence or absence of disease are presented in Tables 7-9.

Table 7: Distribution of age classes and mean (± SEM) serum biomarkers' values for horses with or without osteoarticular disease *n* = 51.

Disease				Controls			
	Coll2-1 (nM)	Coll2-1NO$_2$ (nM)	Fib3-2 (pM)		Coll2-1 (nM)	Coll2-1NO$_2$ (nM)	Fib3-2 (pM)
Young (*n* = 14)	2049.22 ± 81.79^{a}	9.80 ± 0.69	625.87 ± 71.78	Young (*n* = 12)	1750.47 ± 95.52^{b} *	8.40 ± 0.55	517.69 ± 31.29
Old (*n* = 21)	1956.79 ± 74.35	9.29 ± 0.61	574.83 ± 25.74	Old (*n* = 4)	1761.53 ± 94.75	8.38 ± 0.10	571.69 ± 49.13

Different letters represent significantly different results between disease and control groups. * $P < 0.05$

Table 8: Distribution of sex classes and mean (± SEM) serum biomarkers' values for horses with or without osteoarticular disease *n* = 51.

Disease				Controls			
	Coll2-1 (nM)	Coll2-1NO$_2$ (nM)	Fib3-2 (pM)		Coll2-1 (nM)	Coll2-1NO$_2$ (nM)	Fib3-2 (pM)
Male (*n* = 25)	1964.93 ± 71.22^{a} **	8.98 ± 0.56	578.96 ± 34.63	Male (*n* = 12)	1645.79 ± 71.10^{b}	7.89 ± 0.46	531.38 ± 35.44
Female (*n* = 10)	2075.55 ± 87.13	10.76 ± 0.58	635.95 ± 74.16	Female (*n* = 4)	2065.84 ± 74.18^{a} **	9.89 ± 0.99	530.62 ± 9.40

Different letters represent significantly different results between disease and control groups and between female and male groups. ** $P < 0.01$

Table 9: Distribution of exercise classes and mean (± SEM) serum biomarkers' values for horses with or without osteoarticular disease *n* = 51.

Disease				Controls			
	Coll2-1 (nM)	Coll2-1NO$_2$ (nM)	Fib3-2 (pM)		Coll2-1 (nM)	Coll2-1NO$_2$ (nM)	Fib3-2 (pM)
Low level (*n* = 26)	2028.41 ± 64.08	9.61 ± 0.49	627.93 ± 41.00	Low level (*n* = 8)	1878.31 ± 92.43	8.72 ± 0.77	545.04 ± 26.18
High level (*n* = 9)	1893.66 ± 106.77	9.14 ± 1.08	500.80 ± 22.63	High level (*n* = 8)	1628.15 ± 102.09	8.07 ± 0.56	517.33 ± 47.33

Coll2-1 biomarker values were significantly higher ($P = 0.015$) in horses with joint disease (OA or OCD) compared to control healthy horses (Table 10).

Table 10: Distribution of osteoarticular disease and mean (± SEM) serum biomarkers' values *n* = 51.

	OA%	OA + OCD%	OCD%	Coll2-1 (nM)	Coll2-1NO$_2$ (nM)	Fib3-2 (pM)
Controls (*n* = 16)	0 ^{a}	0 ^{a}	0	1753.23 ± 73.95 ^{a}	8.39 ± 0.47	531.19 ± 26.37
Disease (*n* = 35)	68.57 ^{b} *** (24/35)	22.86 ^{b} * (8/35)	08.57 (3/35)	1993.76 ± 55.09 ^{b} *	9.49 ± 2.67	595.24 ± 32.25

Different letters represent significantly different results between disease and control groups. * $P < 0.05$ and *** $P < 0.001$.

Moreover, binary logistic regression (backward stepwise method) was used to study the probability of having osteoarticular disease (r^2 = 0.169 with P = 0.677 at the Hosmer-Lemeshow test). The log-odd probability of belonging to the osteoarticular disease group increased by 1.002 for each increased unit of Coll2-1 (P = 0.022). Using the area under the ROC curve analysis, the specificity and sensitivity of the 3 biomarkers were tested for osteoarticular disease. The AUC of Coll2-1 was 0.716 (95% confidence interval (CI): 0.570-0.862; p= 0.014). The optimal cut-off was determined as the value corresponding to the greatest sum of sensitivity and specificity of the test for discriminating disease from healthy subjects. At a cut-off point of 1859.73 nM the sensitivity was 71.4% and specificity was 62.5%. Considering males alone, for the same cut-off point was achieved but obtained 68.0% of sensitivity and 83.3% of specificity.

Considering horses with OA separately, there was higher Coll2-1 (P = 0.068) in positive OA (1951.61 ± 70.49 nM; n = 24) compared to negative OA horses (1753.23 ± 73.95 nM; n = 16) being significantly different if considering male horses alone (P = 0.025). There was no difference on OCD distribution between age, sex and exercise groups. Horses with OCD had significantly increased mean values of Coll2-1 (P = 0.007) compared to control horses (2085.73 ± 81.60 nM; n = 11 compared to 1753.23 ± 73.95 nM; n = 16). Also, Fib3-2 presented higher values in horses with OCD (655.11 ± 67.61 nM) compared to control horses (531.19 ± 26.37; P = 0.065).

4. Discussion

The Lusitano horse population presented in this study included different sex, level of work and a large age-range in order to study the influence of these factors on cartilage degradation biomarkers' concentration allowing confirmation of the relationship with the presence of osteoarticular disease. The authors adapted a simple radiographic scale per joint, based on bone sclerosis, osteophyte and new bone formation (de Grauw *et al*., 2006). The distal limb and tarsal regions were chosen based on previous studies reporting these as the most frequent affected joints in horses (Baccarin *et al*., 2012). To our knowledge this is the first time radiographic findings were associated with an objective inertial sensor evaluation of lameness besides the classic subjective evaluation in a biomarkers investigation in horses.

Orthopaedic examination revealed a high prevalence of horses presenting OA lesions (62.74%), which is higher than the 31.75% previously reported by Verwilghen *et al*. (2009) and probably due to the advanced mean age of the studied horses. There were 21.57% of horses presenting OCD lesions, which is lower than the 36.51% in the young horse population described by Verlwilghen *et al*. 2009. In accordance to clinical signs of joint disease in horses (Cantley *et al*., 1999), 80% of lame horses had OA and 25% of lame horses had OCD.

Globally, we have found significantly higher concentrations of Coll2-1 and Coll2-1NO_2 in females compared to males. In particular, Coll2-1 was significantly higher in healthy females compared to healthy males, whereas the difference between the disease groups being only significant in males. Although the number of healthy females was very small, this finding is in disagreement with previously

reported studies in horses from Lejeune *et al*. (2007) and humans from Deberg *et al*. (2005), both authors describing no influence of sex for this specific biomarker. We could hypothesised that the healthy group of females had osteoarticular problems elsewhere. Using a nuclear scintigraphy would be interesting to better differentiate disease from healthy control horses. However, radiographic exam was performed in the most frequent affected joints and the lame limb was objectively identified through an inertial sensor device.

Not surprisingly, age was the most important factor influencing higher radiographic scores and the presence of OA. We have found a negative correlation of age with Coll2-1 that was much lower (r = - 0.053) compared to the previously reported (r = - 0.38) by Verwilghen *et al.* (2009). Interestingly, the difference between the disease groups was only significant for horses less than 9 years-old and could be explained by most of the cartilage breakdown have been completed in chronic stages (Gangl *et al*., 2007).

Exercise alone has been reported to significantly increase collagen metabolism biomarkers in serum (Billinghurst *et al*., 2003). O'Kane *et al*. (2006) also found increased levels of cartilage degradation biomarkers (C2C – carboxy-terminal telopeptide of Type II collagen and CTXII – carboxy-terminal cross-linked telopeptide fragment of type-II collagen) in serum of heavily exercised versus non-exercised horses. Surprisingly, exercise had a negative and significant influence in Coll2-1 in the present study. Higher number of females and horses presenting OA at the low-level of exercise could explain higher concentrations of Coll2-1 in this group.

Similarly to Verwilghen *et al*. (2009) Coll2-1 biomarker value was significantly elevated in horses with osteoarticular disease compared to control horses. There were only eight horses presenting OCD associated to OA and only 3 had OCD alone. In these eleven horses, Coll2-1 was significantly higher compared to healthy controls. This agrees with the significantly higher values in blood described by Verwilghen *et al*. (2011) in higher degenerative classes of horses with OCD evaluated by arthroscopy. Horses presenting OA alone had higher concentrations of Coll2-1 but this was only significant considering males.

Coll2-1NO_2 was significantly higher in females supporting the results of Lejeune *et al*. (2007) and this could be explained by estradiol pro-oxidant action on neutrophil degranulation (Chiang *et al*., 2004). However, difference between OA negative and positive groups was not significant, which differs from the same author results. Coll2-1NO_2 was higher in horses presenting OCD, which is similar to Gangl *et al*. (2007) results. Significance might not have been reached because the small sample size of horses with OCD fragments (n = 11).

Fib3-2 peptide was higher but not significantly different in horses presenting osteoarticular disease, horses with OCD presenting the highest values. Although less significantly than Coll2-1, there was higher Fib3-2 in the low-level of exercise group but significance disappeared when sorting horses by disease. This biomarker presented no significant differences between sex and age groups, which agrees with Henrotin *et al.* (2012) that describes no influence of sex in this biomarker even though oestrogens up regulate Fibulin-3 expression (Chiang *et al*., 2004). These findings, together with significant correlation between Coll2-1 and Fib3-2 concentrations could justify Fib3-2 use to better identify disease in particular cases like in female horses.

The first hypothesis was partially validated. Coll2-1 and Coll2-1NO_2 concentrations were significantly increased in females. Coll2-1 concentrations were significantly correlated with exercise, being significantly increased in the low-level group. There was a tendency for collagen type II biomarkers to decrease with age. Fib3-2 was not different between sex and age but also had a tendency to increase with low-level of exercise. Finally, we have confirmed the second hypothesis. The 3 biomarkers were increased in the osteoarticular disease group. Only Coll2-1 was significantly higher in horses presenting disease, maybe because horses presented a more degenerative than inflammatory process, being more specific in young horses and males. No effect of combined biomarkers was found to predict disease. However, in females presenting higher values of collagen type II biomarkers, disease could be identified by an associated increase in Fib3-2 and should be further explored.

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CHAPTER V – Relationship between subjective and objective assessment of distal limb flexion test in Lusitano horses with osteoarticular disease

Susana Monteiro[1], Olivier M Lepage[2], Luís Antunes[3], Liliane Damásio[3], Kevin Keegan[4], Elisa Bettencourt[1]

[1] ICAAM - Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Departamento de Medicina Veterinária, Escola de Ciências e Tecnologia, Universidade de Évora, Pólo da Mitra, 7002-554 Évora, Portugal.

[2] University of Lyon; VetAgro Sup, Veterinary Campus of Lyon, GREMERES-ICE Lyon Equine Research Center, Marcy l'Etoile, F-69280, France.

[3] Hospital Veterinário, Universidade de Évora, Pólo da Mitra, Apartado 94, 7002-554 Évora.

[4] Departament of Veterinary Medicine and Surgery, College of Veterinary Medicine, University of Missouri, Columbia, MO 65211, USA.

Abstract

The aim of the present study was to compare subjective and objective evaluation after flexion test of distal limb and correlate these responses to the presence of different grades of radiographic lesions in Lusitano horses. Fifty-one Lusitano horses were assessed for distal limb flexion test response (positive or negative) and had radiographic evaluation of the 4 distal limbs. Objective evaluation was performed using an inertial sensor device. Forelimbs were objectively assessed for total asymmetry of the head change (ΔVS). Hind limbs were assessed for change in pelvic maximum movement difference (ΔPDmax) and change in pelvic minimum movement difference (ΔPDmin) between left and right proportions of the stride, before and after flexion. Regarding straight-line lameness there was a moderate agreement between subjective and objective classification (κ = 0.49). Despite low agreement in flexion response, positive correlation was observed between subjective and objective evaluation in hind limbs flexion for ΔPDmax (*P* = 0.017). Also for hind limbs, there was a significant correlation between radiographic scores of the metacarpo-phalangeal (fetlock) joint and subjective response to flexion (*P* = 0.048). Objective (ΔPDmin) evaluation correlated to fetlock joint (*P* = 0.004) and mean distal limb radiographic score (*P* = 0.012). In conclusion, positive response to distal hind limb flexion test is significantly correlated to fetlock joint lesions and asymmetry is better detected objectively and as an impact lameness.

1. Introduction

Lameness examination including flexion test and radiographic examination has become an integral part of the locomotor and pre-purchase evaluation of the horse. Flexion tests are performed to exacerbate the baseline lameness and identify the source of pain, and can be performed in specific regions of one or all four limbs. Most veterinarians perform flexion tests, but they are time consuming and usually subjectively assessed. In a study comparing flexion test response and radiographic findings, using fifty non-lame horses, it was found that response to distal flexion of forelimbs did not correlate with the presence of distal limb radiographic abnormalities (Ramey, 1997).

In the last decades the validity of subjective lameness evaluations have been studied (Keegan *et al*., 1998). Agreement between different observers for identification of mild lameness and detection of the most lame limb is generally low, and therefore it is not reccomended to have more than one experienced observer during clinical trial or when comparing to other lameness evaluation methods (Keegan *et al*., 2010). Evaluation of proximal hind limb flexion test was also reported by Armentrout *et al.* (2012) to have great variability between practitioners. Wireless, inertial sensor-based systems have been described as an objective method of continuous lameness quantification that could be used in the field (Keegan *et al*., 2004). Repeatability was confirmed (Keegan *et al*., 2011), compared to a stationary force plate (Keegan *et al*., 2012) and sensitivity when compared to subjective evaluations (McCracken *et al*., 2012). In the latter study the inertial sensors detected lameness in the straight line sooner than the consensus of 3 experienced evaluators, regardless of the induced foot pain was located on fore/hind limb. Furthermore, inertial sensor devices have been evaluated for proximal hind

limb response to flexion (Marshall *et al*., 2012; Starke *et al*., 2012). Using the same device of the present study, it was found that observers' positive response to flexion was associated with significant changes in maximum pelvic height differences between the right and left hind limbs (PDmax) resulting from pushing off less (Marshall *et al*., 2012). However, this response was never studied for distal limb flexion in forelimbs and hind limbs.

Osteoarticular problems like osteoarthritis (OA) are frequently the cause of lameness. Flexion tests are expected to help localise the affected region (Kidd, 2001) and the fetlock joint is the most common source of pain in equine distal limb (Cantley *et al*., 1999). Although there is poor correlation between the presence of radiographic changes and clinical signs of OA, radiography is the most used method of diagnosis (Widmer & Blevins, 1994) because it is non-invasive, portable and cost effective.

The aims of the present study were: 1) to measure agreement between subjective and objective evaluations, 2) to determine the association between objective and subjective response to flexion, and 3) to determine the association between distal limb flexion test response with presence of osteoarticular abnormalities, both in fore and hindlimbs. We hypothesised that there would be a significantly stronger positive association between the presence of radiographic signs of OA with objective evaluation than with subjective evaluation of a positive response to flexion.

2. Materials & Methods

2.1. Sample population

The studied population included Lusitano horses presented for lameness examination at three different veterinary clinics between July 2013 and February 2014. Owners signed an informed consent before horses were renrolled in the study and a total of fifty-one horses underwent subjective and objective lameness evaluation and digital radiographic examination. There were 37 non-gelded males and 14 females (mean ± SEM age 10.39 ± 0.91 years, range 3-25 years). Horses other than Lusitano or with weight bearing lameness were excluded from this study.

2.2. Lameness examination

All animals underwent a general and orthopaedic examination (Ross, 2003), which was recorded at the same time by digital video. Horses were trotted away and towards the camera, two times, on a concrete surface (straight line). Before flexion-tests, the horse was trotted towards the camera for the forelimbs and away from the camera for the hind limbs, one time, on a concrete surface. Flexion test of the distal limb was then performed during 60 seconds and after flexion the horse was trotted again. The foot was supported in the air by the toe without pressure applied to the back of the fetlock joint or the tendinous structures and with minimum flexion of the proximal region (Ramey, 1997). The same sequence was always used: left and right forelimb followed by left and right hind limb flexion. A single observer subjectively and blindly evaluated all video records, including straight line, before flexion and

after distal limb flexion. This was performed through visualization video records (with sound) at normal speed and at slow motion. Response was given for all limbs during straight line (lame or not lame) and for the flexed limb (positive or negative).

Simultaneously, lameness was objectively assessed using an inertial sensor device (Lameness locator® v2014.1.0, Equinosis) as previously described in other studies (Keegan *et al*., 2011; Marshall *et al*., 2012; McCracken *et al.,* 2012; Rettig *et al*., 2015). Briefly, horses were instrumented with 3 inertial sensors: one uni-axial accelerometer between the ears attached to a head bumper, a second uni-axial accelerometer placed between the tubera sacrale on the midline of the most dorsal aspect of the pelvis with tape, and a third uni-axial gyroscope wrapped to the dorsal aspect of the right forelimb pastern. Data were obtained wirelessly from the inertial sensors for at least 25 strides in straight line and for at least 10 strides before flexion and after distal limb flexion over concrete surface. The same person handled the horse before and after flexion and the same person flexed both forelimbs and both hind limbs.

2.3. Objective lameness measures

The body-mounted inertial sensors provided objective measure of forelimb and hind limb lameness by measuring head and pelvic vertical movement asymmetry. There were 2 situations where at least moderate evidence of asymmetry is reported. 1) when the lameness measure is above threshold and the standard deviation of the lameness measure over all analyzed strides is < 1.2 times the absolute value of the mean over all strides, and 2) when the standard deviation of the lameness measure over all analyzed strides is < 0.5 times the absolute value of the mean of over all strides.

Vertical head movement asymmetry is reported as the vector sum (VS) of maximum (Hmax) and minimum (Hmin) head height difference in millimetres (mm) between the right and left components of each forelimb stride. Lameness side is determined by: sign of Hmin, with positive values indicating right forelimb lameness and negative values indicating left forelimb lameness (Marshall *et al*., 2012; Rettig *et al*., 2015). In the present study, VS change (∆VS) before and after flexion was used to determine positive (more asymmetry) or negative (less asymmetry) response to forelimb flexion, considering that Hmax and Hmin are conceptually related.

Vertical pelvic movement asymmetry is reported as the maximum (Pmax) and minimum (Pmin) pelvis height difference in millimetres (mm) between right and left halves of the stride. Pmin is associated with decreased downward movement (impact lameness) in the first half of the stance and Pmax associated with decreased upward movement (push-off lameness) during the second half of the stance. Positive values of Pmax and Pmin indicate asymmetric pelvic movement due to right hindlimb lameness and negative values indicate asymmetric pelvic movement due to left hindlimb lameness. Pmax and Pmin are conceptually un-related and therefore separately evaluated as dependent variables for hindlimb lameness. For positive and negative response to flexion, left sided pelvic asymmetry values were first standardised my multiplying by -1 to convert to right-sided values (Marshal *et al*., 2012; Rettig *et al.,* 2015). In the present study, before and after flexion change for

Pmax difference (ΔPDmax) and Pmin difference (ΔPDmin) was used to determine positive (more asymmetry) or negative (less asymmetry) response for each component.

2.4. Radiographic examination and classification

All radiographic examinations were performed following a standardised procedure. Whenever needed, horses were sedated, using 0.01 mg/kg IV of detomidine (Domidine®, DFV) alone or combined with 0.02 mg/kg IV of butorphanol (Dolorex®, Intervet). Radiographic evaluation was performed in the four distal limbs (metarcarpo/tarso-phalangeal joints; distal and proximal inter-phalangeal joints). Four classical projections were taken, including the latero-medial; dorso-palmar/plantar; dorso 45º latero-palmar/plantar medial oblique and dorso 45º medio-palmar/plantar lateral oblique. Radiographs were evaluated by 3 veterinarians using a digital software system (Examion® CR-360 system) and scored from absence (0) to severe (4) OA lesions. The OA scale was adapted from previously reported scale (de Grauw *et al.,* 2006), in order to obtain a mean grade per joint (0-4) and mean distal limb score per horse (0-12).

2.5. Statistical Analysis

All statistical analyses were performed using commercially available software (SPSS® v22, IBM corporation). Significance was set at $P \leq 0.05$. Normality of data was checked using Shapiro-Wilk's test. Agreement was estimated by calculation of κ (kappa) between subjective and objective evaluations performed for straight line (lame or not lame) and response to flexion (positive or negative). In the present study, agreement (κ) was considered to be poor if less than 0.2; fair if between 0.21 and 0.4; moderate if between 0.41 and 0.6; good if between 0.61 and 0.8 and very good if between 0.81 and 1 (Altman, 1991). Spearman test was used to correlate objective variables (ΔVS, ΔPmax and ΔPmin) and radiographic scores with response to flexion. Differences between groups were analysed using Mann-Whitney rank sum test.

3. Results

A total of 51 horses were assessed for lameness on a straight line. A total of 102 distal limb flexion tests were performed on 51 left and 51 right forelimbs. One young horse did not allow the hind limb distal flexion; therefore, 100 distal hind limb flexion tests were performed on 50 left and 50 right limbs. Objective parameters (ΔVS, ΔPmax and ΔPmin) and radiographic scores were not normally distributed among different flexion responses, therefore non-parametric tests were used. Data are represented by median and interquartile range (IQR).

3.1. Relationship between subjective and objective evaluations

Regarding straight-line lameness (n = 204), 82.53% of subjectively classified lame limbs agreed with objective classification, whereas 66.19% of subjectively classified non-lame agreed with objective classification (κ = 0.49 – moderate agreement, CI: 0.37-0.61). There was low agreement between subjective response to flexion and objective results for ΔVS (κ = 0.011) ΔDPmax (κ = 0.132) and ΔDPmin (κ = 0.116).

There were lower ΔVS for subjective negative (*n* =74, median 2.2, IQR -1.93, 8.77) compared to subjective positive (*n* = 28, median 2.7, IQR -2.22, 9.37) response to flexion but this was not significantly different (*P* = 0.970).

Spearman correlation test between objective parameters and subjective response to flexion (negative or positive) was significant for ΔPDmax (P = 0.017). There was a significant difference (*P* = 0.018) of ΔPDmax values between negative response (*n* = 54, median 0.79, IQR -0.83, 3.51) and positive response (*n* = 46, median 2.74, IQR 0.66, 6.19). Using the area under the ROC curve analysis, the specificity and sensitivity of ΔPDmax was tested for negative or positive response to flexion. The AUC was 0.638 (95% confidence interval (CI): 0.528-0.747; *P* = 0.018). The optimal cut-off was determined as the value corresponding to the greatest sum of sensitivity and specificity of the test. At a cut-off point of 1.0415 of ΔPDmax the sensitivity was 71.0% and the specificity was 58.4%.

3.2. Correlation between flexion response and radiographic scores

Spearman correlation test between radiographic scores and the forelimb response to flexion was not significant. Regarding the hind limbs, Spearman correlation test between radiographic scores and subjective response to flexion was positive and significant for fetlock joint (*P* = 0.048) but not for the distal mean radiographic score (*P* = 0.077). Correlation between radiographic scores and ΔPDmin response to flexion was significant for fetlock joint (*P* = 0.004) and distal mean radiographic score (*P* = 0.012).

3.3. Forelimb radiographic scores and responses to flexion

In what concerns the forelimbs there were no differences between the mean of the three evaluators' radiographic scores for horses classified with a negative distal flexion test (n= 74, median score 2.5, IQR 1.7, 4.0) or a positive distal flexion test (*n* = 28, median score 2.5, IQR 1.5, 4.2) (Figure 12A, *P* = 0.799).

Concerning objective evaluation of flexion tests in the forelimbs, radiographic scores of distal limb of horses categorised as presenting a negative flexion test (ΔVS negative; n= 34, median score 2.2, IQR 1.7, 4.1) were not significantly different from horses with a positive flexion test (ΔVS positive= 68, median score 2.6, IQR 1.6, 3.9) (Figure 12B, *P* = 0.616). Radiographic scores were not significantly different (*P* = 0.258) in fetlock joints between horses with negative (n = 34, median score 1.3 , IQR 0.7, 2.0) and positive response to flexion (*n* = 68, median score 1.0, IQR 0.7, 1.7).

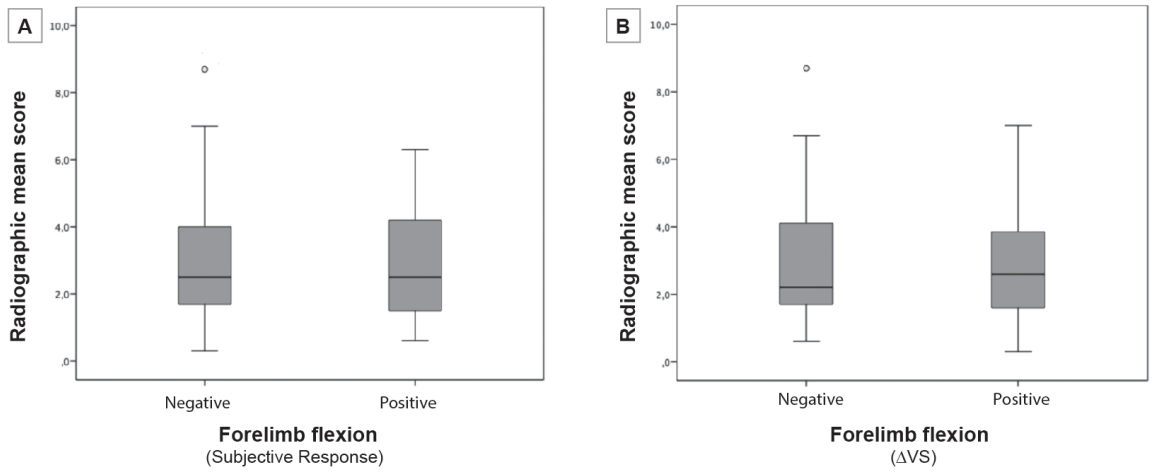

Figure 12: Box plot of distal limb radiographic mean score for positive and negative flexion tests assessed (A) subjectively and (B) objectively, head asymmetry change (ΔVS). There is no significant difference in radiographic scores for positive and negative subjective or objective flexion-test.

3.4. Hind limb radiographic scores and responses to flexion

Concerning subjective evaluation of the hind limbs, mean radiographic scores of the distal limb between horses classified with a negative flexion (*n* = 54, median score 1.9, IQR 1.0, 3.0) were not significantly lower when compared to a positive flexion test (n= 46, median score 2.6, IQR 1.3, 3.7) (Figure 13A, *P* = 0.077). However, when comparing mean radiographic scores of fetlock joints the differences were significant (*P* = 0.048) between horses with a negative flexion test (*n* = 54, median score 0.7, IQR 0.3, 1,0) and a positive response (*n* = 46, median score 0.85, IQR 0.3, 1.7).

Distal limb radiographic scores in the hind limbs showed no significant differences between horses with an objective negative (ΔPDmax, n= 27, median score 2.2, IQR 1.15, 2.95) or positive (ΔPDmax positive, *n* = 73, median score 2.3, IQR 1.0, 3.7) flexion test (Figure 13B, *P* = 0.484). However, when using the objective parameter defined as ΔPDmin, significant differences were observed in horses with a negative flexion test (*n* = 39, median score 1.7, IQR 1.0, 2,8) and a positive flexion test (*n* = 61, median score 2.4, IQR 1.6, 4.1) concerning radiographic score of distal limb (Figure 13C, *P* = 0.013). These differences concerning radiographic scores of the distal limb were associated to significantly different (*P* = 0.005) fetlock radiographic scores between horses with negative (*n* = 39, median score 0.3, IQR 0.3, 1.0) and positive response to flexion (*n* = 61, median score 0.7, IQR 0.3, 1.7).

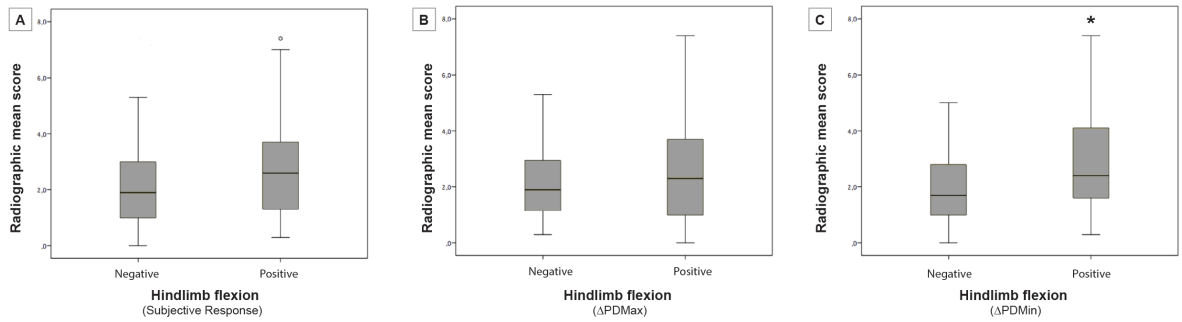

Figure 13: Box plot of distal limb radiographic mean score for positive and negative flexion tests assessed A: subjectively and objectively for B: maximum pelvic height change (ΔPDMax) and for C: minimum pelvic height change (ΔPDMin). There was significant difference in radiographic scores between negative and positive response for ΔPDmin (* P = 0.013).

4. Discussion

In this study the authors have focused on the agreement between subjective and objective evaluation of lameness and flexion test response and their relation with severity of distal limb radiographic lesions. First, subjective and objective agreement was assessed and objective parameters correlated with the subjective response. Secondly, a response to flexion-test was compared to distal limb radiographic scores. It has been described that distal limb flexion of 30 seconds does not always reveal a positive response; while flexion of 2 min could yield a false positive result, optimal duration being between 60 and 90 seconds (Keg *et al.*, 1997). We have decided to perform distal limb flexion for 60 seconds because it is our routine and in order to allow the comparison with previous studies (Ramey, 1997, Marshall *et al.*, 2012). Several aspects have not been addressed in this study, such as the exact source of pain, force of flexion and the probability of a specific limb being called positive.

Previous reports have described an agreement of 76.6% between subjective evaluations (lame or not lame limb) in straight line (κ = 0.44) (Keegan *et al.*, 2010). Similarly, in the present study, 82.5% agreement was achieved for the lame limb between the video observations in straight line and the machine (κ = 0.49). Maybe this was due to the repeated visualisations (with sound) and the use of slow motion mode. Subjective and objective response to flexion had poor agreement as depicted by low κ values. The agreement values are arbitrary but useful as long as limitations are take into consideration. There should be statistical independence of observations and low trait prevalence. Because the orthopaedic and objective exams were performed simultaneously, the subjective classification of lameness was completed through blinded film observation to guarantee independence. Besides, there were horses from different ages and not all the animals were presented with a pain related problem, reducing lameness prevalence (trait prevalence).

We have first compared subjective response to flexion to objective parameters. There was less head asymmetry change for subjective negative response compared to positive response to flexion but these differences were not significant. This could be explained by the fact that on subjective

evaluation, observers classified the flexion as negative, despite increased assymetry within the first steps, which led to increased objective measurements. Moreover, the authors suspect this could be justified by contralateral lameness, which was taken into account by VS but not in the subjective evaluation. In the future, a distinction between flexed or contralateral limb positive responses should be performed for a better analysis of the origin of the pain in the forelimbs.

Regarding pelvic asymmetry, an increase in PDmin difference in response to flexion-test would indicate a decrease in the fall of the pelvis during the stance phase of the flexed limb (impact lameness) and it is surprising that this was not significantly different between negative and positive subjective response. However, the positive subjective response presented significantly increase in PDmax difference. This response to flexion-test would indicate a decrease in the pelvis elevation during the swing phase (propulsion) of the flexed limb (push-off lameness). Similar results have been found previously for proximal hind limb flexion subjective response assessed by the same inertial sensor device in 17 healthy horses (Marshall *et al*., 2012). These authors suggested that the decreased push-off could be observed as an increase in 'hip drop'. In fact, hind limb lameness causes a slight different vertical movement of the pelvis between left and right hind limb strides and a big different vertical movement of the left and right tubera coxae, the pelvis moving less up during and after the last half of stance of the lame limb (Keegan, 2007) and would correspond to the 'hip drop'. Tubera coxae increased amplitude has been recently measured during hind limb lameness evaluation (Starke *et al*., 2015). Our findings show that after a positive flexion-test the observer depicted lower elevation of the pelvis (hip drop) during propulsion of the lame limb. Considering this, we have determined whether PDmax would be useful to differentiate negative from positive flexion test. Using a 1.0415 change of ΔPDmax the sensitivity was 71.0% and the specificity was 58.4%. In this study there was a lower difference of maximum pelvic height for the same sensitivity of a positive response when compared to the previous report on proximal limb flexion (Marshall *et al*., 2012).

In our study there was no significant difference in radiographic scores between horses with negative and positive response to fore limb distal flexion. This agrees with a previous report (Ramey *et al*., 1997) that found that normal horses responding positively to forelimb distal flexion-test had 50:50 chance of having a radiographic abnormality. However, inertial sensor device were associated to lower radiographic scores for negative response and higher radiographic scores for positive response, compared to subjective evaluations. This confirms the utility of the inertial sensor device and suggests higher sensibility to detect asymmetry due to radiographic changes. Regarding the hind limbs, there is significant higher radiographic scores in presence of decreased pelvic downward movement (ΔPDmin), which was mainly due to fetlock changes. This is an interesting finding and confirms assumed osteoarticular problems in association with impact lameness.

In conclusion, agreement between subjective and objective evaluation was moderate for straight-line but low for flexion-test response. This justifies the interest on novel technology for objective assessment of flexion response. Hind limb subjective positive response to flexion-test resulted from significantly less upward movement of the pelvis (push-off lameness). Subjective response to a distal flexion test can be negative in presence of radiographic changes and positive in the absence of lesions. However, objective evaluation was more likely to detect radiographic osteoarticular lesions.

Increased change on minimum pelvic height (impact lameness) was highly correlated to fetlock joint abnormalities. These findings support the inertial sensor system in distal limb flexion-test response evaluation. Objective response in the forelimbs should be further explored to better distinguish a negative from a positive response.

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CHAPTER VI - Effects of a Single Intra-articular Injection of High Concentrated and High Molecular Hyaluronic Acid Treatment on Distal Limb Osteoarthritis – a pilot study

Susana O. Monteiro[1], Elisa V. Bettencourt[1], Sandra M. Branco[1], Olivier M. Lepage[2]

[1] ICAAM - Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Departamento de Medicina Veterinária, Escola de Ciências e Tecnologia, Universidade de Évora, Pólo da Mitra, 7002-554 Évora, Portugal.

[2] University of Lyon; VetAgro Sup, Veterinary Campus of Lyon, GREMERES-ICE Lyon Equine Research Center, Marcy l'Etoile, F-69280, France.

Abstract

The aim of this study was to objectively evaluate the effects of a single intra-articular (IA) injection of high concentrated (88mg/4mL) and high molecular weight (HMW) hyaluronic acid (HA). Lameness examination was performed subjectively and asymmetry measured objectively using an inertial sensor device at week 1, 2, 4 and 8 after treatment. Serum biomarkers' concentration (Coll2-1, Coll2-1NO_2 and Fib3-2) and synovial fluid (SF) analysis (subjective viscosity asesessement, total proteins (TP), and cytological examination) were evaluated before treatment and at the end of the study. Eight horses with clinical osteoarthritis (OA), diagnosed with lameness examination, perineural anaesthetic block and digital radiographic examination, were included. One week after treatment six horses (75%) subjectively improved, being significantly less lame (P = 0.020), but only 25% (2/8) remained clinically better after two months. Subjective flexion test response improved in 50% (4/8) of the horses at the end of the study. Objectively, there was decreased asymmetry at straight line one week after treatment and decreased change in asymmetry after flexion in forelimbs (week 1 and 2) and hind limbs at 2 months (P = 0.016). SF showed significant reduction in TP at the end of the study (P = 0.042). Finally, all serum biomarkers decreased 2 months after treatment, in particular Fib3-2. In conclusion, a single 88mg HMW-HA IA injection has shown to reduce cartilage degradation and inflammation, based on serum biomarkers assessment, besides producing modest subjective and objective improvement in lameness. A randomized, double blind and controlled study should be performed in order to confirm the clinical interest of this therapy.

1. Introduction

OA is a disabling joint disease mainly responsible for premature retirement in sport horses with consequent economic losses. Various medical treatments are available for systemic or IA injection. They include HA reported to have anti-inflammatory (Takahashi *et al*., 1999) and disease-modifying effects on OA joints (Marshall *et al*., 2000). HA is a nonsulfated, unbranched polysaccharide of alternating disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid (Richardson & Loinaz *et al*., 2007). The molecular weight is determined by polymerization and the number of repeating disaccharide units. Commercially molecular weight is usually referred as low ($< 5 \times 10^5$ Da), intermediate (5-10 $\times 10^5$ Da) and high ($>1 \times 10^6$ Da) (Williams, 2007). It has been suggested that HA preparations of HMW have better chondroprotective effects and better clinical results in horses (Philips, 1989). *In vitro*, high HMW-HA (4,000 kd) supplementation restored lubricant function in SF sampled from acute OA joints (Antonacci *et al*., 2012) to normal equine SF values.

A study on experimental OA revealed that 3 injections of 22mg HA together with 125mg amikacin did not improve lameness but reduced cartilage fibrillation (Frisbie *et al*., 2009). More recently, the same authors compared HA together with sodium chondroitin sulphate and N-acetyl-D-glucosamine with IA saline and amikacin alone. In this study, there was 16% improvement in lameness scores, slight improvement on radiographic bone proliferation and less cartilage erosion when compared to placebo treated joints (Frisbie *et al*., 2013). Different concentrations have been studied and better

lameness results, measured by a force plate, were obtained with a single injection of 20mg and 40mg when compared to lower HA concentrations (Richardson & Loinaz, 2007). In a Portuguese equine veterinarian survey we have found that, in field practice, HA (20mg) is most often injected in high motion joints alone or simultaneously with IA corticosteroids in a single injection protocol. Moreover, a recent study compared the clinical efficacy of a single IA injection of 12mg triamcinolone acetonide (TA) alone or together with 20mg HMW-HA and it found lower short-term clinical success with the HA combination but the same outcome 3 months after treatment (de Grauw *et al*., 2014). Therefore, the single injection of 20mg HA is a commonly used protocol but it usually leads to a modest clinical improvement. That is the reason why we decided to investigate a recent product using high concentration (88mg/4mL) of HMW-HA for a single IA injection that has been approved for humans (Goldenberg, 2014). This protocol was never objectively tested in horses, but, if effective, it would present a clear advantage since a single injection of high concentrated HA would be less time consuming and safer.

In order to objectively assess this therapy we have used a body mounted inertial sensor device technology that measures head and pelvic asymmetry in horses (Keegan *et al*., 2004). Its repeatability was confirmed (Keegan *et al*., 2011) when compared to a stationary force plate (Keegan *et al*., 2012) and its sensitivity when compared to subjective evaluations was higher in mild cases of lameness (McCracken *et al*., 2012). Furthermore, as this device would be blind to a drug injection, it will be interesting on objective lameness assessment after treatment.

Several evaluation tests can be used to assess articular status. SF analysis reflects the joint health and can be performed by description of gross appearance, determination of TP, total nucleated cell count but most importantly by cytologic examination (Steel, 2008). Biomarkers are biochemical measures that are indicative of a biologic process or the response to an intervention. Biomarkers of cartilage can be found in SF but also in serum. Type II collagen and Fibulin-3 are abundant in the extra-cellular matrix from articular cartilage. In serum, collagen type II degradation fragments (Coll2-1 and Coll2-1NO_2) (Henrotin *et al*., 2004) and Fibulin-3 fragment (Fib3-2) have been shown to identify joint disease in humans (Henrontin *et al*., 2012). Moreover, Coll2-1 in serum has recently been reported to decrease after OA treatment in humans (Henrontin *et al*., 2014). In horses, type II collagen fragments have been correlated with OA (Lejeune *et al*., 2007; Verwilghen *et al*., 2009) but, as Fib3-2, they have never been used to assess therapy response.

The aim of this study was to objectively test the effect of a single IA injection of high concentrated HMW-HA in horses with clinical signs of OA. The authors hypothesized that lameness, SF parameters and serum cartilage biomarkers would decrease after treatment.

2. Materials & Methods

The care and handling of the animals were in accordance with the provisions of the European Economic Community Council Directive 86/609. Horse owners signed an informed consent for IA treatment and agreed to respect protocol recommendations.

2.1. Animals

Client-owned horses, presenting lameness, with no distinction in sex, age or breed were selected from three different veterinarian practices from February to April 2014. Animals presenting a positive response to anaesthetic block (abaxial sesamoid or distal low palmar/plantar nerve block and DIP or fetlock joint IA anaesthesia) together with radiographic signs of distal limb OA were included in this study. Exclusion criteria consisted of animals receiving any type of systemic or IA treatment 2 weeks before the study, horses presenting osteochondritis dissecans (OCD) lesions in the affected joint or horses with non-weight bearing lameness or signs of systemic disease.

2.2. Lameness evaluation

Lameness examinations were performed routinely and horses subjectively classified according to the pain level from 0 to 5 (Ross, 2003) and according to digital flexion test response: negative or positive, from 1 to 3 (Broecks *et al*., 2014). Simultaneously, objective assessment was performed using a body-mounted inertial sensor system at week 0 (before treatment) and 1, 2, 4 and 8 weeks after treatment. Horses were instrumented with 3 inertial sensors: one uni-axial accelerometer in a head bumper or the crown piece of the head halter, a second uni-axial accelerometer placed between the tubera sacrale on the midline of the most dorsal aspect of the pelvis with tape and a third uni-axial gyroscope in a pastern wrap placed on the right forelimb. Data were obtained wirelessly from horses trotted in straight line (at least 25 strides), before and after flexion (at least 10 strides) (Marshall *et al*., 2012; Rettig *et al*., 2015).

Vertical head movement asymmetry is reported as the vector sum (VS) of maximum (Hmax) and minimum (Hmin) head height difference in millimetres (mm) between the right and left components of each forelimb stride. The side of lameness is determined by sign of Hmin during right forelimb stance minus Hmin during left forelimb stance, with positive values indicating right forelimb, and negative values indicating left forelimb lameness (Marshall *et al*., 2012; Rettig *et al*., 2015; Maliye *et al*., 2015). In the present study, VS at different time points was used to compare straight-line asymmetry. VS change (ΔVS) before and after flexion, at different time points was used to determine response to forelimb flexion, considering that Hmax and Hmin are conceptually related.

Vertical pelvic movement asymmetry is reported as the maximum (Pmax) and minimum (Pmin) pelvis height difference in millimetres (mm) between right and left halves of the stride. Pmin is associated with decreased downward movement (impact lameness) in the first half of the stance and Pmax associated with decreased upward movement (push-off lameness) during the second half of the stance. Positive values of Pmax and Pmin indicate asymmetric pelvic movement due to right hindlimb lameness and negative values indicate asymmetric pelvic movement due to left hindlimb lameness. Pmax and Pmin are conceptually un-related and therefore separately evaluated as dependent variables for hindlimb lameness. Left sided pelvic asymmetry values were first standardised my

multiplying by -1 to convert to right-sided values (Marshall *et al*., 2012; Rettig *et al*., 2015). In the present study, Pmax difference (PDmax) and Pmin difference (PDmin) at different time points were used to study straight-line asymmetry. Before and after flexion change for PDmax (ΔPDmax) and PDmin (ΔPDmin) between the right and left part of the stride was used to determine flexion response in hind limbs.

2.3. Synovial fluid analysis

SF analyses were performed before and 2 months after treatment. Affected joints were clipped and aseptically prepared. Sedation was performed using detomidine hydrochloride (0.01 mg/kg) and butorphanol tartrate (0.02 mg/kg). SF sample collection was performed using an 18-21 G needle. TP concentration was measured using a portable refractometer (ref: 50303020), concentrations less than 20 g/L were considered normal. Viscosity was assessed as decreased (SF string less than 2.5 cm) or normal (SF string between 2.5 -5 cm) as a gross measure of HA content. Subsequently, SF was stored in EDTA tubes and centrifuged at 1500 rpm for 10 min. The sediment was resuspended after which the smear was performed and air-dried for cytological examination. Smears were stained with May-Grunwald (MGG) and microscopically examined for cellularity (normal or increased), percentage of neutrophils (less than 10% was considered normal) and for mucin pink material on the background of smears (decreased or normal) (Steel, 2008).

2.4. Treatment protocol

Product contained 88 mg of lightly cross-linked HMW-HA in 4 mL of phosphate buffered saline. Horses were restricted to box rest for 48h after injection. Exercise protocol consisted of 10 min hand walking exercise for one week followed by lounge exercise with increased trot periods during the second week. Owners were told that no other medications should be given for 2 months.

2.5. Serum biomarkers analysis

Biological assays using specific ELISA kits were performed according to manufacturer's recommendations. Coll2-1 (nM), Coll2-1NO_2 (nM) and Fib3-2 (pM) were measured in the serum of OA patients before and 2 months after treatment (Henrotin *et al*., 2012; Henrotin *et al*., 2014).

2.6. Statistical analysis

The effect of treatment on subjective and objective lameness, SF parameters and serum biomarkers were determined using Wilcoxon signed rank test or t-test related samples. Statistical significance was set at $P \leq 0.05$ and data were presented by median and interquartile range (IQR).

3. Results

3.1. Animals

Included horses consisted of 3 Lusitano, 3 Portuguese Crossbreed and 2 Hanoverian horses, from 6 to 18 years of age (median 9.50 years). There were 2 females, 1 gelding and 5 males (*n* = 8).

One horse had two affected joints and the others had a single affected joint. Therefore, there were 7 fetlock joints (2 forelimbs and 5 hind limbs) and 2 distal inter-phalangeal joints (2 forelimbs) treated with HA (*n* = 9). Six horses presented acute signs of the disease (less than 1 month with mild to moderate radiographic lesions) and two horses presented chronic signs (more than 1 month with severe radiographic lesions). No severe adverse reactions were noted after treatment.

3.2. Lameness examination

On a straight line, horses presented median grade 3 subjective lameness at all times except at week 1 (median grade 2) and this was significantly different compared to what was found before treatment (*P* = 0.020). Six horses were clinically improved one week after treatment and 2 horses remained less lame at the end of the study. In what concerns the flexion test, horses presenting median grade 1 positive response to subjective flexion at all times. At the end of the study 4 horses treated for fetlock OA had flexion response improvement, but this was not significantly different. One horse from the forelimb group improved from grade 1 to grade 0, from the hind limb group one horse improved from grade 2 to 0 and two horses improved from grade 3 to 1.

Objective lameness evaluation was performed in 4 the forelimbs and 4 the hind limbs, one horse (hind limb fetlock) being excluded due to high outlier values. Examinations had at least 2 animal evaluations at each time point for forelimb and hind limbs. Results were presented as head asymmetry (VS) and pelvic asymmetry (PDmax and PDmin). Straight-line evaluations presented lower VS at week 1 (median 7.15 IQR: 6.22, 15.80) but there were no significant differences compared to week 0 (median 15.98 IQR: 7.24, 21.63) (Figure 14A). Regarding hind limbs (Figure 15A and Figure 16A), straight-line evaluations presented lower values at week 1 (PDmax median 6.27 IQR: 1.86, 10.54; PDmin median 4.43 IQR: 1.76, 8.78). Differences between week 0 (PDmax median 9.72 IQR 4.72, 13.53; PDmin median 11.61 IQR: 5.29,14.06) and week 1 were not significant for PDmax or PDmin.

Asymmetry before and after flexion-test was presented as change on head asymmetry (∆VS) and on pelvic asymmetry (∆PDmax and ∆PDmin). Forelimbs presented negative median ∆VS values at week 1 (median -2.96 IQR: -6.53,1.21) and at week 2 (median -0.31 IQR: -7.33, 1.78), but there were no significant differences compared to week 0 (median 0.59 IQR: -0.28, 22.53) (Figure 14B). Hind limbs, presented positive ∆PDmax values at the first four time points but decreased from week 2 to negative values at week 4 (median -5.60 IQR: -8.71, -2.48). Furthermore differences between week 0 (median 4.56 IQR: 0.99, 16.16) and week 8 (median -5.60 IQR: -8.71, -2.48) were significant (*P* = 0.016) (Figure 15B). Change in PDmin was positive at all time points, decreasing from week 1

(median 6.37 IQR: 2.51, 10.60) to week 8 (median 1.97 IQR: -3.64, 7.58), but differences were not significant (Figure 16B).

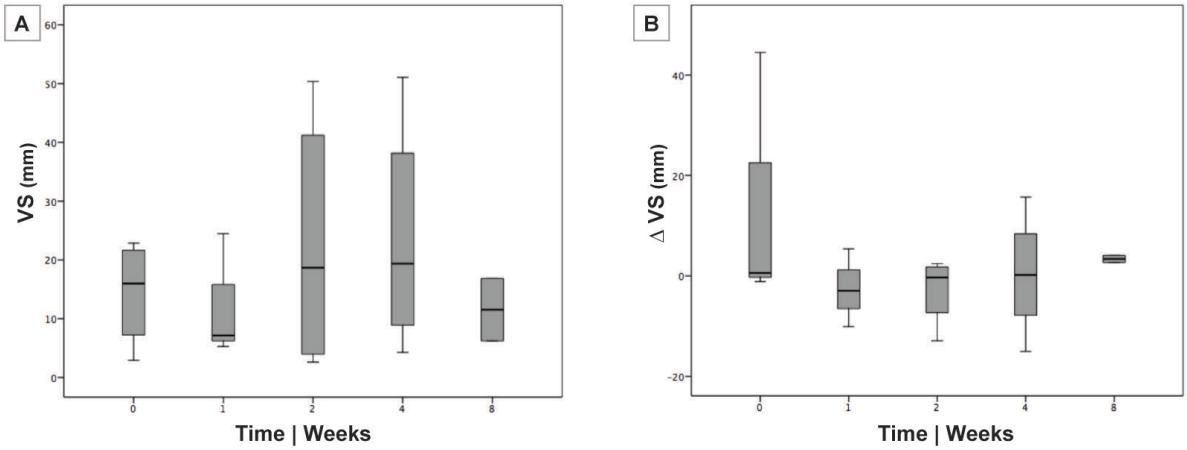

Figure 14: Box plot of objective lameness evaluations in fore limbs (A): head asymmetry (VS) at straight line and (B): head asymmetry difference before and after flexion (ΔVS). There were no significant differences.

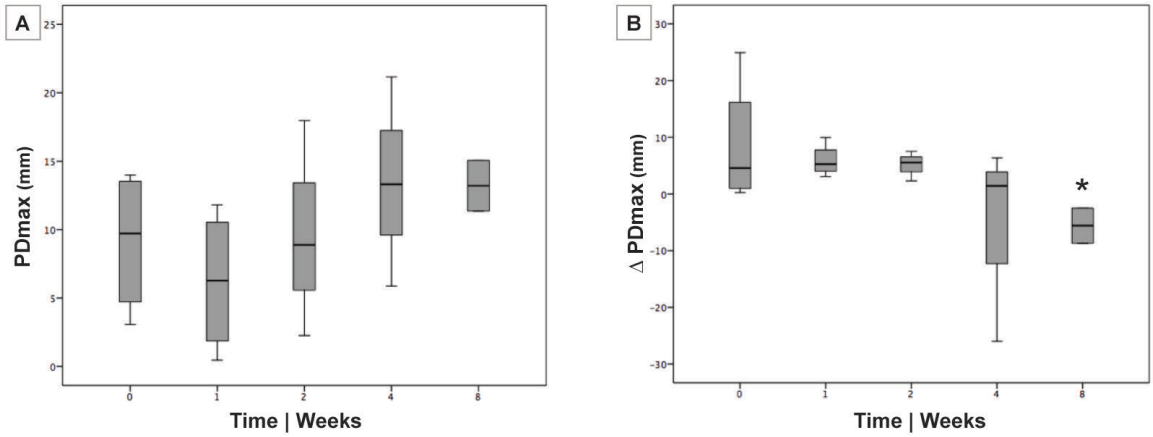

Figure 15: Box plot of objective lameness evaluations in hind limbs (A): maximum pelvic asymmetry (PDmax) at straight line and (B): difference between before and after flexion asymmetry (ΔPDmax). Significant differences between ΔPDmax at week 0 and at week 8 are represented by * $P < 0.05$.

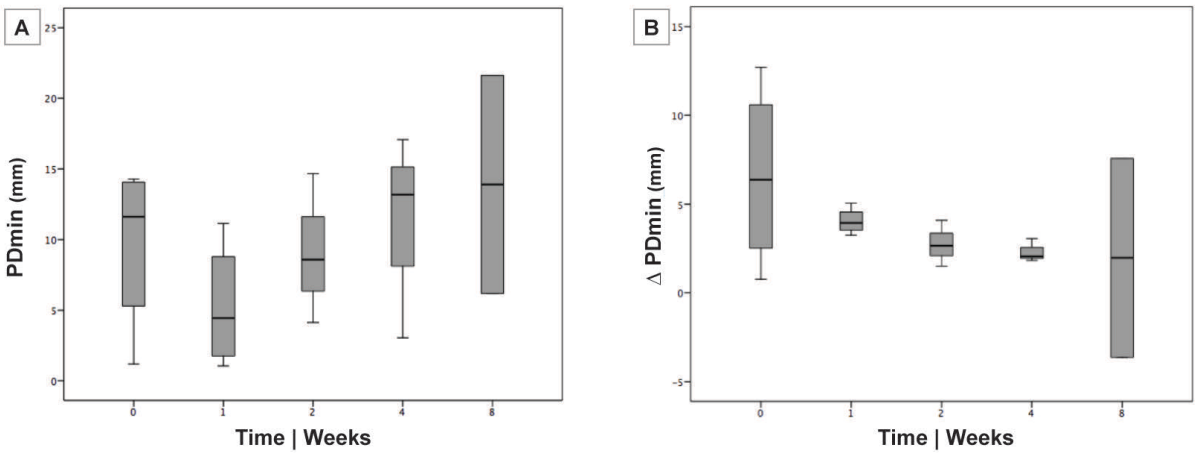

Figure 16: Box plot of objective lameness evaluations (A): minimum pelvic asymmetry (PDmin) at straight line and (B): difference between before and after flexion asymmetry (ΔPDmin). There were no significant differences.

3.3. Synovial fluid analysis

It was possible to retrieve SF samples from all joints before the IA injection (*n* = 9). However, 2 months after treatment 2 owners were not available to allow for the sample collection procedure (*n* = 7).

TP concentration was higher before treatment (median 22 g/L, IQR: 10, 29) than after treatment (median 8 g/L, IQR: 6, 30) and related samples analysis were significantly different (*P* = 0.042). Five horses presented abnormal elevated concentrations before treatment. The two horses presenting persisting elevated values at the end of the study were associated with iatrogenic haemorrhage at collection time. Subjective macroscopic viscosity and cytological mucin assessment did not differ before and after treatment. Cytological examination showed increased cellularity in 5 horses with no significant differences 2 months after treatment injection. Percentage of neutrophils was elevated in a single horse and decreased to normal values after treatment.

3.4. Biomarkers analysis

Biomarkers concentrations in serum of treated horses (*n* = 8) are presented graphically for Coll2-1, Coll2-1NO_2 and Fib3-2 in Figure 17A-C, respectively.

There were higher median concentrations of all biomarkers before treatment compared to 2 months after treatment: Coll2-1 presented median 1752.2 nM (IQR: 1650.9, 1862.0) compared to median 1696.9 nM (IQR: 1559.9, 2013.2); Coll2-1NO_2 presented median 3.63 nmol/L (IQR: 3.26, 5.03) compared to median 3.39 nM (IQR: 3.08, 4.09); Fib3-2 presented median 523.1 pM (IQR: 402.7, 567.3) compared to median 484.6 pM (IQR: 459.7, 558.9). However, related samples analysis showed no significant differences.

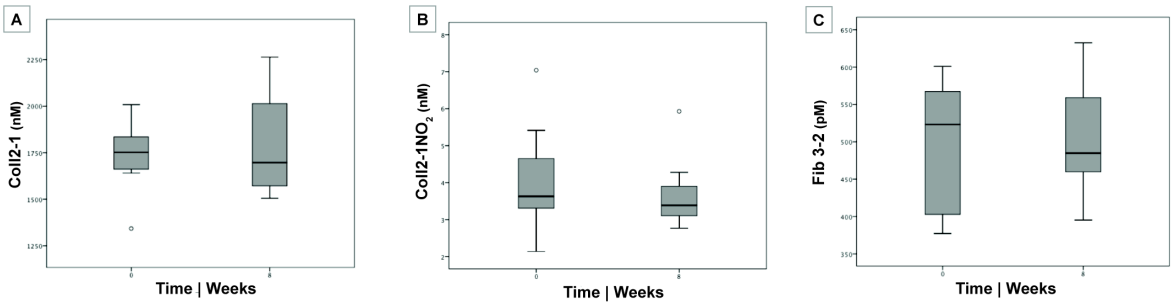

Figure 17: Box plot of serum biomarkers' concentration (A): Coll2-1 (B): Coll2-1NO_2 and (C): Fib3-2 before (week 0) and 2months after HA treatment (week 8). There were no significant differences.

4. Discussion

HA is a ubiquitous component of extracellular matrices, and performs important functions in cartilage. By binding and immobilising aggrecan, HA mediates the formation of massive aggregates of fixed negative charge, that retain water, it is a critical extracellular matrix component and assures cartilage mechanical properties. This molecule is produced by synoviocytes and chondrocytes and is responsible for SF viscoelasticity, playing an important role in cartilage lubrification (Todhunter, 1996).

The aim of this study was to test a high concentrated single HMW-HA injection on distal limb OA joint treatment. Besides subjective methods an objective assessment was used at the short-term outcome and 2 months after treatment. Body mounted inertial sensor device was used for lameness evaluation. Serum biomarkers' concentration was measured in order to assess cartilage degradation and joint inflammation. As far as we know, these methodologies have never been used to evaluate therapeutic response in horses. Eight horses presenting clinical signs of the disease were injected intra-articularly. One horse had two treated joints making a total of nine distal limb high-motion joints assessed. The treatment was well tolerated and there were no adverse reaction reported.

Median grade of subjective lameness on a straight line was significantly reduced one week after treatment regardless acute or chronic OA. The objective evaluation proved asymmetry reduction but it didn't confirm the significant difference. However objective evaluation of impact lameness (PDmin) showed a clear reduction almost aproaching significance.

We did not include a placebo-treated group as it was considered practically unfeasible to get owners to consent to withholding treatment of their lame horses. We can not exclude that clinical improvement during the first week in 75% of horses could be associated to the restricted walking protocol that was recommended. However, a recent cohort double-blinded study reported only 10% (1/10) improvement in acute and chronic OA groups after IA placebo (Spadari *et al*., 2015). In the present study there were 50% of the horses with decreased response to subjective flexion test at the end of the study in particular for hind limbs. This could be explained by the presence of two DIP joints in the forelimb group, that showed no improvement in our study. In fact another study associated DIP joint OA to a worst outcome (Brommer *et al*., 2012). Objective improvement of flexion response was

only evident during the first two weeks after treatment in forelimbs. Gradual improvement was observed in hind limbs, presenting significantly difference between week 0 and week 8 for PDmax, and meaning less push-off lameness or pain during the swing phase of the stride. Surprisingly, the asymmetry improvement in hind limbs flexion response had different evolution from straight-line, where asymmetry increased from week 1 until the end of the study. One horse was excluded from the objective analysis due to extremely high asymmetry values in spite of bearing weight on the OA limb. This probably reflects the disappointing results often encountered in chronic cases. However, the second chronic clinical case also had important asymmetry reduction at week 1.

Viscosity of SF is directly related to the quantity and degree of polymerization of HA (Pearson, 1971). The authors expected significant improvement of viscosity after treatment but this was only seen in one horse both macroscopic and histologically. Maybe differences existed sooner after treatment but samples were performed only once, 2 months after HA injection. It would also have been interesting to biochemically analyse the concentration of HA using an ELISA-kit assay (Antonacci *et al.*, 2012). Beside increased lubricant function in SF from acute cases after HMW-HA supplementation, these authors reported that SF from acutely inflamed joints presented significantly lower HA concentration compared to controls but difference did not exist in chronic cases. The other parameter tested before cytology was TP concentration. TP increase in case of joint trauma and inflammation due to protein leakage from damaged vessels (Steel, 2008). Our results before treatment confirm the presence of joint injury and showed significant reduction at the end of the study. There were no significant changes in cellularity and neutrophils percentage. The mild increase of TP is not always present in OA joints but TP significant reduction confirmed decreased inflammation after this specific HA treatment.

To our knowledge this is the first time that serum type II collagen and a Fibulin-3 fragment are used to assess IA therapy in OA horses. We found that Fib3-2 presented the highest reduction after treatment, which is different from Henrotin *et al*. (2014) reporting ColI2-1 to be more significantly decreased. Another study reported both type II collagen biomarkers' reduction early after IA HA treatment in humans (Henrotin *et al*., 2013). Biomarkers serum concentration can be affected by the presence of OA elsewhere, even in unassessed joints. That was the reason for treating two joints in one horse. Moreover, we have recently found ColI2-1 and Fib3-2 to be increased in the serum in the presence of OCD. In our study, horses were only excluded if presenting OCD in the treated joint. If OCD was present elsewhere, this could have increased serum biomarkers' values and explain absence of statistical significance. Instead, low number of horses may have precluded significant difference. It was our intention to test the same biomarkers in treated joints but there was not enough SF for all horses before and after treatment. Verwilghen *et al*. (2011) reported a significant correlation between the degenerative score and the synovial levels of ColI2-1. In the future, it would be interesting to study these biomarkers in SF together with serum at additional time points closer to the injection treatment.

In conclusion, our results suggest that high concentration of HMW-HA is safe, induces transient lameness reduction on a straight line but progressive improvement in response to flexion-test either in acute or chronic OA cases. HMW-HA reduces joint inflammation by significant reduction of TP in SF.

A tendency for cartilage biomarker decreased concentrations in serum also reflects improved cartilage breakdown. These results suggest that pain reduction is modest but joint inflammation is reduced in a protracted way. This could represent an alternative for pain relief while preserving joint metabolism to drugs that are banned from competition. A randomized, double blind and controlled study, with a large number of horses should be performed in order to confirm the clinical interest of this therapy.

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GENERAL CONCLUSION

Early recognition of OA is of major importance in both human and in equine medicine, in particular in young athletes. Nevertheless we need to be able to stop and revert the disease progression at any stage. While advances in human and veterinary medicine will progressively increase the longevity of species if we do nothing OA will become one of the most disabling, frequent and important disorders in the future.

Currently, we are still looking for the gold standard therapy for joint disease, which would be able to achieve pain relief and return to function with maximal safety and at the lowest price possible. In this quest, researchers and manufacturers have made available a large arsenal of drugs but ambiguous information is frequently encountered. Therefore, objective evaluation of the effect of various therapies is mandatory, representing the main issue at the origin of our work.

Both in human and equine medicine joint, therapy is difficult due to the limited healing capacity of cartilage and progressive degeneration along side with age and work. As equine surgeons with particular interest on equine orthopaedics we have to deal with challenging cases where cartilage loss is important and diffuse in joints of the distal limb. When we first designed this project we were convinced that objective methodologies were the only way to achieve early recognition of a disease process and would help assessing the effectiveness of a proposed therapy. For the equine species, as for human athletes, effective treatment means returning to the intended function. As veterinarians, it is our responsibility to use the available technology to achieve a specific and correct diagnosis and to recommend the best therapeutical option.

Our goal being to find a medication that could stop the progression of the disease, we have paid attention to the biologic therapies and their promise of true regeneration. We first looked to the human and equine literature on this subject and made a survey amongst equine field practitioners about OA diagnosis and treatment in Portugal. The review article shows that blood and cell derived products induce mild clinical improvement and some cell-therapies are associated with better outcomes in horses, with higher rates of return to work and better histologic repair compared to controls, being described. In spite of the large amount of therapies described, there is still a lack of significant clinical response, which, together whith the high price and associated risk of flare reaction, make veterinarians and owners reluctant to the application of these therapies. Therefore, it was not suprising to find that equine veterinarians, including pratitioners working with sport horses, are still rarely using biologic products on OA patients.

In order to standardize protocols, while achieving good safety and a less expensive therapy, we decided to investigate an allogeneic cell-derived treatment. The adipose tissue stem cells (ASCs) allows a good yield of cells while being easy to obtain from the patient itself or another animal. The novelty of our therapy was the preactivation with IFN-γ. The *in vitro* work allowed testing the effects of the ASCs in equine cartilage explants before their application *in vivo*. Cartilage degradation molecules and GAG release measurements were increased after OA induction and values were reverted by high dose of ASCs. Interestingly, GAG release achieved the lower values after preactivation of ASCs with

IFN-γ, for an extended period of time and, more importantly, when using the lowest dose of cells. The hypothesis that inflammatory conditions further enhance ASCs protective effects were confirmed and then tested in equine experimental disease *in vivo*. The same research group had previously developed the metacarpo-phalangeal groove model of OA in horses specially designed to be closer to the natural occurring disease and allowing to test the efficacy of therapies. Our *in vivo* study confirmed the induction of predictable lameness, radiographic and histological lesions with the groove model. Secondly, the study shows that the ASCs-γ treated joints have significantly reduced PGE2 concentration and radiographic changes 9 weeks after IA treatment. This therapy should be further explored in clinical cases and for an extended period of time before being recommended in practice.

The experimental study performed at the beginning of the research work allowed the PhD student to learn a new methodology for objective lameness detection and has allowed its application on the subsequent clinical part of the work. We performed lameness and radiographic examinations in 51 Lusitano horses in order to correlate objective pain and radiographic signs of OA with cartilage degradation and inflammation biomakers in the blood. Using a heterogenous population, our study confirmed the utility of Coll2-1 as a biomarker of cartilage degradation being more specific in young horses and males. Increased concentration of Fib3-2 was found in the joint-disease group with the advantage of not being influenced by sex or age, which could potentially help to predict disease in certain cases. The Coll2-1NO_2 biomarker was not significantly increased but should probably be part of a biochemical panel to detect more inflammatory than degenerative cases. Instead of an extensive but certainly incomplete radiographic screen, horses can have blood analysis performed in order to assess their osteoarticular status or a response to an OA therapy. With this study we concluded that a biochemical osteoarticular scan both in young males and females is a possibility for early detection of joint disease.

Using the same 51 Lusitano horses we further analysed the relationship between subjective and objective detection of lameness. Straight-line evaluation and flexion-test responses were assessed and correlated with distal limb radiographic findings. Moderate agreement between subjective and objective evaluation for straight-line and low agreement for flexion-test response are described. We confirmed that a subjective assessement of a distal flexion test can be negative in the presence of radiographic changes and positive in the absence of lesions. However, objective lameness evaluation was more likely to detect increased asymmetry when radiographic osteoarticular lesions were present, in particular in hind limbs. These findings support the use of inertial sensor system to objectively assess distal limb flexion-test response evaluation but further analyses are important to better understand these correlations and other inertial sensors' potential. In the future, we would like to explore the inertial sensor data by analysing response of the distal limb flexion not only in the flexed limb but also in the other limbs, in particular the contralateral limb.

In order to use the studied methodologies (inertial sensor device and cartilage biomarkers) in natural occurring OA we have assessed response to HMW-HA therapy. Despite being more accurate to detect radiographic changes in the hind limbs we used the inertial sensors in clinical cases of both the forelimbs and the hind limbs OA. Flexion-test response was significantly decreased using the objective lameness detection in the hind limbs 8 weeks after treatment. Moreover, TP were

significantly decreased and all biomarkers were reduced by the end of the study. Although there were not significant changes, serum Fib3-2 was the biomarker that showed the more pronounced reduction and could be an important tool for therapy response assessment in the future.

We believe that the purpose of our work has been fulfilled. We have explored two objective diagnostic methodologies for equine OA diagnosis and we have used these technologies to assess IA therapies in horses with experimental and naturally occurring disease.

The strategy of our work was to perform all studies in close relationship between human and veterinary medicine, the private industry and field practitioners. Additionally, our association with VetAgro Sup, University of Lyon (France) has allowed us to integrate state of the art technologies to our local resources.

Finally, this doctoral thesis provides a contribution to different aspects of the equine orthopaedic field and has allowed advancing in the student personal skills as well as developing knowledge about specific aspects of distal limb OA diagnosis and treatment in horses. As the objective methodology and therapies were inspired and performed in colaboration with different human and veterinary research centers and laboratories we hope to have contributed to a global medical progress.

Appendix A – Proceedings of the 24th ECVS Annual Scientific Meeting

Effects of pre-activated adipose mesenchymal stem cells on experimental fetlock osteoarthritis model

Effects of pre-activated adipose mesenchymal stem cells on experimental equine fetlock osteoarthritis model

Monteiro S[*1], Maninchedda U[2], Roussignol G[3], Remandet B[3], Segard E[2], Hilairet S[3], Jorgensen C[4], Lepage OM*[*2].*

[1]Departamento de Medicina Veterinária, Escola de Ciências e Tecnologia, Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Instituto de Investigação e Formação Avançada, Universidade de Évora, Évora, Portugal, [2]University of Lyon; VetAgro Sup, Veterinary Campus of Lyon, GREMERES-ICE Lyon Equine Research Center, Marcy l Etoile, France, [3]Sanofi-aventis Recherche, Montpellier, France, [4]CHU Lapeyronie University Hospital, Montpellier, France.

Introduction:

Mesenchymal stem cells (MSCs) have reparative effects due to paracrine actions and need to be activated by inflammatory mediators. Therefore, pre-activation of MSCs could lead to a more efficient therapeutic activity than unstimulated MSCs. Our objective was to study the effects of interferon-γ (INF-γ) pre-activated or "primed" allogeneic MSC in experimental osteoarthritis models, *in vitro* and *in vivo*, in horses.

Materials and Methods:

Equine adipose derived mesenchymal stem cells (ASC) were characterized and activated with IFN-γ. ASC were tested on equine cartilage explants subjected to interleukin-1 (IL-1) and oncostatine-M (n=8). The experimental *in vivo* model of OA was induced by arthroscopy, using an adapted "groove" technique in one distal metacarpal condyle. Group A (n=6) did not receive any treatment; Group B (n=6) received intra-articular injection of 15 million INF-γ activated ASC in a vehicle (3 ml) and Group C (n=4) received the vehicle alone (3ml), 7 days post-surgery. After 15 days stall-rest horses respected a standardized exercise protocol. They were clinically evaluated before surgery (d0), in the middle (d45) and in the end of the study (d75), using an inertial sensor device for objective lameness examinations, synovial fluid analysis and radiography. All articular surfaces were evaluated macroscopically and histologically.

Results:

In the experimental study *in vitro*, IL-1 and oncostatine-M treatment induced a significant ($p<0.001$) increase in glycosaminoglycans (GAG) release to the culture medium. Cartilage explants treated with ASC showed less GAG degradation and IFN-γ pre-activation enhanced the protective effect ($p=0.019$). *In vivo*, there was no significant difference in mean head movement asymmetry and radiographic scores between groups throughout the study ($p>0.05$). Estimated proportions of lame horses at the end of the study was 83%, 33% and 50% and mean radiographic score was 11.8, 7.7 and 9 in group A, B and C, respectively. There was no significant difference in synovial fluid TP and PGE2 mean values, nor in macroscopic and histologic mean scores between groups ($p>0.05$).

Discussion / Conclusion:

IFN-γ primed allogeneic ASC treatment had benefic effects observed *in vitro* by significant reduced GAG production but these effects were less evident clinically. Perhaps there was not enough inflammation by the time of the treatment (d7) or maybe the number of stem cells was not adequate. This could be explained by insufficient number of cells for this type of joint or accelerated clearance of the cells from the joint environment. Using INF-γ primed allogeneic ASC treatment has protective effect in vitro and should be further explored clinically before it can be recommended in acute osteoarthritis in horses.

Appendix B – Review article

Biologic Strategies for intra-articular treatment and cartilage repair

Journal of Equine Veterinary Science 35 (2015) 175–190

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

journal homepage: www.j-evs.com

Review Article

Biologic Strategies for Intra-articular Treatment and Cartilage Repair

Susana O. Monteiro [a,*], Elisa V. Bettencourt [a], Olivier M. Lepage [b]

[a] Departamento de Medicina Veterinária, Escola de Ciências e Tecnologia, Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Instituto de Investigação e Formação Avançada, Universidade de Évora, Évora, Portugal

[b] Equine Department, VetAgro Sup, Veterinary Campus of Lyon, University of Lyon, Marcy l'Etoile, France

ARTICLE INFO

Article history:
Received 20 November 2014
Received in revised form 31 December 2014
Accepted 4 January 2015
Available online 9 January 2015

Keywords:
Equine
Cartilage
Osteoarthritis
Autologous-conditioned serum
Platelet-rich plasma
Mesenchymal stem cell

ABSTRACT

Primary cartilage disease such as osteoarthritis (OA) or secondary degenerative disease to a localized cartilage defect or a soft tissue injury is difficult to treat. Osteoarthritis is associated to important morbidity in human patients and is self-limiting for equine sport activities. Many surgical and medical treatments are described with the common goal of improving joint function and stop the disease progression. Biologic therapeutic strategies are under evaluation and may be promising because they are based on the organism natural capacity to heal and are supposed to have a regenerative potential. In the last decades, these treatments have gained popularity in human and veterinary orthopedics but there is still few clinical prove of efficacy. This review of literature summarizes research and clinical evidence of the so-called blood- and cell-derived therapies, providing scientific evidence and understanding about their mechanisms.

1. Introduction

Compared with other tissues, cartilage composition is quite simple, having only one cell type and no vascularization or innervation, which causes limited healing capacity [1,2]. Osteoarthritis (OA) is a slow progressive and disabling joint disease that most commonly affects old or high-level sportive young people [3]. Cartilage damage can result from joint-level factors such as acute trauma, chronic joint instability, or other joint-tissue diseases. Besides these, individual-level factors contribute to disease progression, and both levels interact in a complex manner making OA treatment difficult [4]. These features are similar both for human and equine patients.

There are two main approaches to cartilage treatment: surgical and medical. The surgical approach is usually used in case of focal cartilage lesions such as osteochondral defects and subchondral bone cysts. The medical approach is used in generalized cartilage disease such as OA [5]. The main goal for both approaches is to alleviate clinical signs of pain allowing humans and horses to continue to work and compete. Moreover, in human medicine, a distinction is made between symptom-modifying drugs and disease-modifying drugs for OA. Drugs of the latter group are expected to improve patient's symptoms and to alter the course of disease however; increased understanding of inflammatory pathways is still needed [6]. In the last decades, a different category of therapies have gained popularity both in human and equine medicine. These are based on biologic and natural organism ability to repair and try to amplify their effects, the so-called regenerative or biologic therapies. These therapies may be classified in two main groups: the blood- and the cell-derived therapies.

There are obvious benefits in the close collaboration with human medicine and on testing advanced therapies in

* Corresponding author at: Susana O. Monteiro, Departamento de Medicina Veterinária, Escola de Ciências e Tecnologia, Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Instituto de Investigação e Formação Avançada, Universidade de Évora, Núcleo da Mitra, 7000 Évora, Portugal.

E-mail address: susana.monteiro.vet@gmail.com (S.O. Monteiro).

0737-0806/$ – see front matter © 2015 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jevs.2015.01.006

horses, but veterinarians and owners should keep a critical mind when extrapolating human treatments for equine patients without further investigation. In this review, we look at several studies that support or bring into question most common biologic therapies for cartilage repair, looking in detail to their clinical findings in horse's joints.

2. Blood-Derived Therapies

Blood-derived therapies are widely available because there are no restrictions and few legislation regulating their use. They are based on autologous products so readily available from the patient and supposed to be safe for the same reason. There are two main products being used in equine patients, autologous-conditioned serum (ACS) and platelet-rich plasma (PRP). The main interest is based on their anticatabolic and anabolic effects. As performance enhancement has not been demonstrated, these therapies are not included in the prohibited list of doping controls, and, consequently, competing horses can benefit from their effects. They have been adapted from human medicine with little investigation but are being widely applied to equine patients. One-step processed products such as bone marrow concentrates and vascular stromal fraction of fat tissue are briefly discussed in the Section 3.1—cell-derived therapies.

2.1. Autologous-Conditioned Serum

Autologous-conditioned serum was one of the first biologic products to be tested in horses. Autologous-conditioned serum is produced from patient's blood and contains, among other biologic products, interleukin (IL)-1 receptor antagonist (IL-1ra) protein [7]. Interleukin 1ra is a natural antagonist of IL-1 cytokine, which is known for its central role in inflammatory responses, binding to the same cell membrane receptor and reducing inflammation [8]. Therefore, ACS would be expected to have an effect on cartilage metabolism and great potential for OA patients.

2.1.1. ACS Technique

Autologous-conditioned serum is a cell-free therapy first collected from patient peripheral blood into syringes containing $CrSO_4$-coated glass spheres followed by whole-blood 24-hour incubation at 37°C, centrifugation, and filtration before serum being injected into joints [9]. Until recently, there was only a commercial available method of producing ACS, Orthokine (Orthogen; Düsseldorf, Germany). There was little research on this product composition except one report from the manufacturer together with an equine research team [10]. Authors report that exposure of blood leukocytes to pyrogen-free surfaces, glass spheres, causes an accumulation of anti-inflammatory cytokines in human blood after 6 hours of incubation compared with baseline. This included 8.5× higher IL-1ra and 4× higher IL-10 for the anti-inflammatory cytokine group compared with 2× higher IL-1β, 2.3× higher IL-6, and 1.5× less tumor necrosis factor α (TNF-α) for the proinflammatory cytokine group. In addition, increased concentration of several growth factors (GFs), modest 1.4× increase in insulinlike growth factor 1 (IGF-1), substantial increase of 190× in platelet-derived growth factor (PDGF), and 84× in transforming growth factor-β1 (TGF-β) were achieved. Analyses were performed in the serum using commercial human enzyme-linked immunosorbent assay kits, but the number of patients was different for each cytokine, from n = 80 for TGF-β to n = 224 for IL-1ra. Authors assume that clinical effects are probably attributed to the synergistic action of all factors and not to IL-1ra by itself. This list of cytokines is probably more extensive, and the high standard deviations indicate great variability between individuals [9].

2.1.2. In Vitro—Laboratory Findings

A recent study [11] reported ACS effects in vitro on cartilage proteoglycan metabolism of autologous-conditioned serum (n = 24) compared with non-conditioned serum (n = 24) as well as cytokine levels in synovial fluid before and after treatment on 14 human patients. They used ACS (Orthokine) with 6 hours of incubation time. In line with other studies, increased levels of anti-inflammatory cytokines IL-1ra, TGF-β, and IL-10 were shown in ACS. However, proinflammatory cytokines IL-1β, IL-6, and TNF-α were also upregulated. There was no apparent in vitro effect of ACS on OA cartilage explants from the six different donors during a 16-day culture. Another interesting finding was that even after six intra-articular in vivo injections of ACS during 21 days, there were no significant changes in cytokines levels. Authors hypothesize that limited effects of this therapy in vivo can be because of fast intra-articular clearance shown in this study.

A newer generation of ACS is now available, and laboratory results published by Hraha et al [12] helped to understand the major differences between the two equine products. Most important conclusion is that the more recent ACS system, IRAP II (Arthrex Inc, Naples, FL) yielded significant increase in IL-1ra compared with the other ACS product, IRAP (Orthokine; Orthogen), and nonconditioned serum. However, IRAP would not cause an increase of IL-1ra significantly higher than 24 hours of nonconditioned serum, which consisted in whole blood incubated 24 hours in borosilicate glass tubes, centrifuged, and filtered before storage. In contrast to the previous study [10], which reported less TNF-α after 6-hour incubation, in this study, this proinflammatory cytokine was significantly increased in IRAP compared with 24-hour controls. Finally, the authors remind that cytokines list present in ACS can be more extensive than tested so far and confirmed great variability in cytokine production among individuals. In conclusion, other factors besides IL-1ra could be involved in the reported clinical effects.

More recently, another research group [13] compared the effects of autologous equine serum (AES) to ACS (IRAPII) on glycosaminoglycan (GAG) synthesis and metabolism of postmortem equine chondrocyte pellets. Treatment with recombinant human IL-1β and 10% of AES decreased the amount of synthesized GAG. Instead, when associated with 20% of AES and 20% of ACS, results showed minimal beneficial effects of ACS treatment. Although both treatments have increased IGF-1 concentrations and 20% of ACS has increased IL-1ra in culture medium, this did not lead to

a different direct effect on in vitro proteoglycan cartilage metabolism compared with AES at same concentrations. Once more, even if in a different study design, results suggest that ACS is not so different in cartilage metabolism in horses than autologous-unconditioned serum.

Gene therapy is beyond the scope of this review, but one study using an osteoarthritic culture model reported results of a combination of proteins IGF-1 and IL-1ra. Transduction with IGF-1 alone promoted cartilage production of proteoglycan and type II collagen, suggesting a beneficial role for healing injured cartilage. Transduction with IL-1ra alone decreased synovial expression of IL-1α, IL-1β, and matrix metalloproteinase (MMP), indicating a mechanism for prevention of matrix degradation. The combination of the two resulted in improved preservation of proteoglycan content of cartilage explants exposed to depleting effects of IL-1. This study sustains a potential benefit of combining anabolic GFs and catabolic blockers [14].

2.1.3. In Vivo—Clinical Studies

In humans, a randomized controlled trial in 376 knees of OA-confirmed patients was performed during 26 weeks and reported for 345 patients at double-blind 6-month follow-up [15]. Three parallel groups were formed and received at least one treatment of ACS (Orthokine), hyaluronic acid (HA), or saline injection. The number and percentage of patients with 50% improvement of symptoms on the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) scores compared with baseline were markedly highest in the ACS group compared with HA and saline patients. There was the same frequency of local adverse reactions in the ACS and placebo groups but significantly more in the HA group. Infection was not reported in any case. An observer-blinded 2-year follow-up in 310 patients still showed improvements for all three groups, and there was still a significant statistical difference between ACS compared with control groups (WOMAC scores). Autologous-conditioned serum reduces pain and increases function and mobility, but authors believe that although ACS can be recommended in low- to medium-grade patients with painful knee OA, this cannot necessarily be generalized to all patients.

Another study [16] reported function and symptoms improvement after six intra-articular injections of ACS (Orthokine) compared with placebo physiological saline. There were 160 patients included in this double-blinded, placebo-controlled, randomized, multicenter, prospective, clinical trial at 3, 6, 9, and 12 months of follow-up. Results showed similar improvements on the WOMAC score between groups but significantly more improvement for Knee injury and Osteoarthritis Outcome Score for ACS group. Orthopedic surgeon and patient evaluations had better but not significantly different results for the ACS group compared with placebo group. The reported adverse effects on ACS group consisted in one case of severe inflammatory reaction and one patient with septic arthritis. In contrast to the previous study, improvement in symptomatology seemed not clinically significant. Additionally, placebo group patients were reluctant to undergo further ACS treatment, and those who accepted it ($n = 20$) did not show clinical improvement [17].

Besides this contradictory results, IL-1ra is considered to be “more than minimally manipulated” by Food and Drug Administration and is therefore subjected to different regulatory laws than PRP and is generally not available for human use in the United States [18].

In horses, ACS is now widely used in equine hospitals and requested by many horse owners, but only anecdotal personal communications report some results and sustain good outcome for mild-to-moderate OA unresponsive to conventional therapies. There are two experimental clinical studies, one testing IL-1ra gene and the other ACS with significant lameness improvement in horses [19,20]. Clinically, a new product, autologous protein solution, seems to be effective in mild cases of OA and safe for intra-articular administration in equine joints. It is a 2-step process, using double centrifugation and filtration through polyacrylamide beads that achieves high concentrations of Il-1ra [21].

In the future, ACS complete cytokine profile and differences from incubated patient’s blood alone need further investigation. The potential for cartilage regeneration also need to be proven in vitro, and large prospective blinded clinical studies are required in natural occurring equine OA.

2.2. Platelet-Rich Plasma

Platelet-rich plasma is another autologous product derived from patient’s blood known for its anabolic effects. By definition, PRP is a volume of plasma fraction of autologous blood having platelet concentration above baseline [22,23]. To expect clinical efficacy, concentrations should reach $1{,}000 \times 10^3$ cells/μL of nonactivated platelets in plasma. This usually corresponds to twofold or more above whole-blood baseline for humans, and in horses, this is also considered the gold standard [24]. The concept that PRP would improve joint disease is based on the physiological role of platelets. Indeed, PRP interest is based on the ability of platelets to release GFs from their α-granules on their activation. Growth factors are cytokine proteins that regulate other cells function and play a role on modulation of the inflammatory response, promoting angiogenesis, attraction of fibroblasts, and local stem cells to the site of injury and induction of autocrine GF production [25].

Platelet-rich plasma has been applied since the 70 seconds in bone, ligament, and tendon injuries and has recently gained special interest for use in improving cartilage repair. Effects of IA PRP injection in injured or degenerated cartilage are promising and interesting results that are being published every year. Ideally, the same PRP product would be consistently used or at least consistently characterized, but many times, this is not the case and that is of major importance when assessing studies results.

2.2.1. PRP Preparation Techniques

Different techniques of processing the blood to separate and concentrate platelets exist (Table 1). Major systems consist in the automated apheresis, only available in human hospitals, and automated centrifugation or “buffy coat”, available for any human doctor or veterinarian. Several companies sell the “buffy coat” technique, which involves a centrifuge machine investment with its own PRP

Table 1
Equine platelet-rich plasma (PRP) preparation techniques [9].

Technique	Preparation	Advantages	Disadvantages
Automated centrifugation: buffy coat method	Single centrifugation; commercial kits	Ease of use; rapid production; good sterility; 10 minutes procedure	Kits price; human PRP protocols; leukocytes and erythrocytes contamination
Manual centrifugation: tube method	Double centrifugation; pure PRP or Leukocyte-poor PRP	Economic; less erythrocytes or WBC contamination	Time consuming; sterility breaking risk
	Double centrifugation; leukocyte PRP or leukocyte-rich PRP	Economic; bigger platelet concentration potential	Less platelet concentration; time consuming; sterility breaking risk; leukocyte and erythrocytes contamination
Filtration: gravity method	Blood collected into a bag and filtered by gravity. Filter retains platelets	No centrifuge needed; good sterility; 10 minutes procedure	Kits price; some erythrocyte contamination

Abbreviation: WBC, white blood cell.

production protocol and separated individual kits. Care should be made to guarantee that protocols for equine patients are available before buying or using these systems. Other options are manual centrifugation or "tube method" and filtration or "gravity method." This last method can be used by equine veterinarians in the field, with no special centrifuge requirements, besides room temperature conditions and the kit cost.

Manual tube centrifugation has the advantage of being economic but requires a centrifuge machine and sterile technique while manipulating samples. Our double centrifugation technique is similar to the one described by Textor [9], which uses a first centrifugation at 200*g* for 15 minutes and second centrifugation at 400*g* for 15 minutes and is described in Fig. 1. Using four acid-citrate-dextrose (ACD) tubes will usually provide 4 mL of PRP at

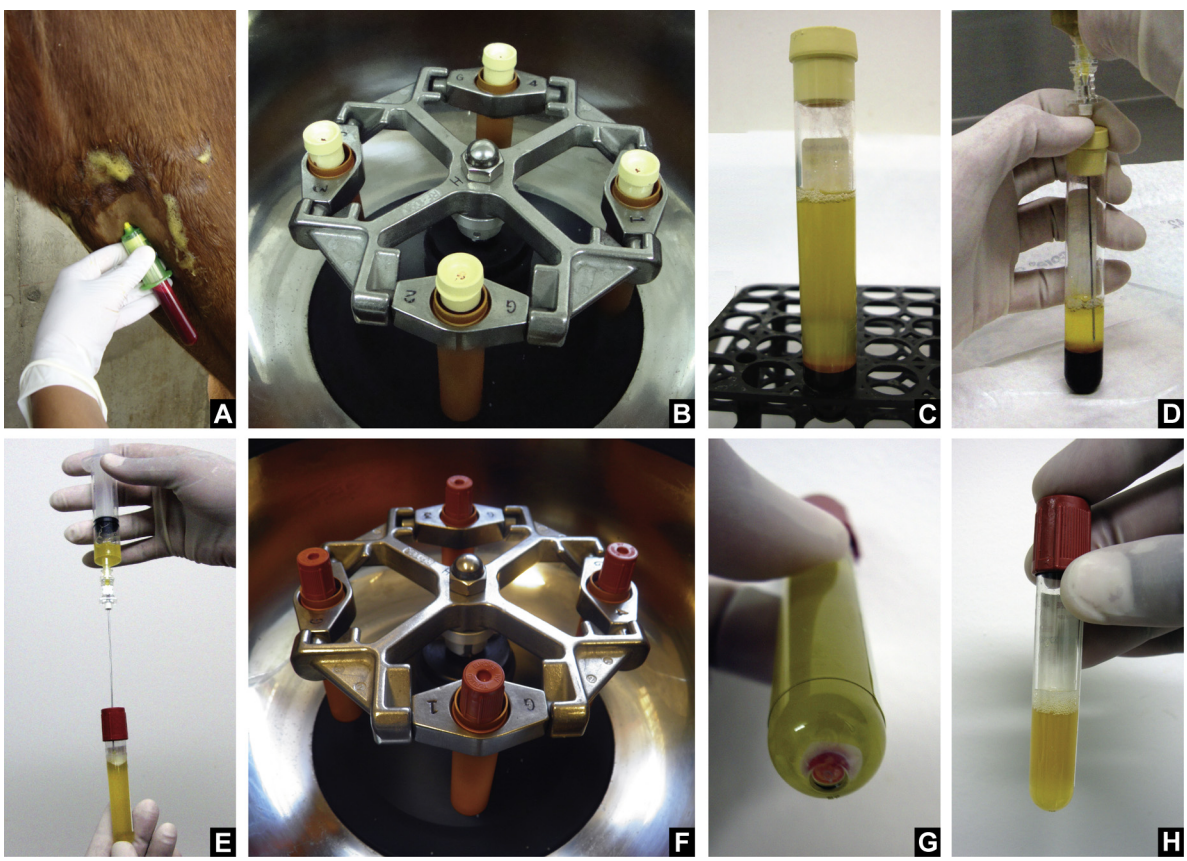

Fig. 1. Platelet-rich plasma preparation using H-19F Regen Centrigel Centrifuge (RegenLab, Mollens, Switzerland)—double centrifugation technique: (A) taking patient's blood into four tubes of 8.5 mL acid-citrate-dextrose (yellow top); (B) centrifuge at 1,000 rpm during 15 minutes (soft spin); (C) three layers: plasma on the top, thin white buffy coat in the middle, and the red blood cells on the bottom; (D) maximum amount of plasma should be collected without disturbing the buffy coat; (E) transferred to 5 mL dry tubes (red top); (F) and centrifuged at 2,000 rpm during 15 minutes (hard spin); (G) a platelet pellet will be created on the bottom of the dry tube; (H) the top plasma (3–4 mL) can be discarded, and the remaining plasma resuspended and mixed with other tubes before treatment.

Table 2
Platelet-rich plasma (PRP)-derived products used in different clinical settings [9,28,31].

Product	Preparation	Advantages	Disadvantages
PRP Pure PRP Leukocyte PRP	Centrifugation	Ready after centrifugation	Cannot be adapted to wounds or cyst cavities
Platelet-rich gel	Platelet activation after centrifugation	Works like a scaffold; possible to apply in wounds and cysts	Time consuming; cannot be used in tendons, ligaments
Platelet-rich clot releasate	Liquid portion retrieved after clotting	No white or red blood cells; good GF content	Time consuming
Platelet concentrate	Small volumes	High concentrations of platelets	Very small volumes
Noncoagulating platelet concentrate	Small volume of plasma replaced by saline	Injectable without clotting	Very small volumes

Abbreviation: GF, growth factor.

least threefold above baseline. As described by Alvarez et al [26], if a sterile technique is used from collection until injection, this PRP product should be safe for intrasynovial injection. Some authors call this technique final product pure PRP or leukocyte-poor PRP because the buffy coat is not disturbed, and slow centrifugation speeds are used. The leukocyte PRP or leukocyte-rich PRP, when the entire buffy coat and some red cells are aspirated, usually results in higher concentrations of platelets and requires faster centrifugation speeds [18,27]. Each clinician should test his technique and centrifuge machine for platelet concentration and sterility before injecting it into patients.

Other platelet products have been used in the clinical setting (Table 2). Platelet-rich plasma can be induced to clot or be injected without activation. To clarify literature terminology, the clot product should be termed of platelet-rich fibrin clot or matrix but is also known as “platelet gel” (Fig. 2). A third product, rich in GFs, refers to the supernatant formed after clotting without the cellular portion and is called platelet-rich clot releasate. The platelet concentrate has an extremely high concentration of platelets in the smallest amount of plasma as possible. Textor et al [28] showed that the liquid clot releasate provided 80% of total available GF after calcium chloride ($CaCl_2$) activation and should be considered a valid method for percutaneous treatment. This activation method takes time to clot (30–60 minutes at 37°C) but has the advantages of being a simple process, safe, and economic. Other studies have studied the PRP releasate (PRPr) potential in vitro and also sustain its use [29,30]. An alternative and optimized way of producing PRP is described by Araki et al [31]. By collecting whole blood into ethylene diamine tetra-acetic acid disodium tubes, these authors show that there is greater platelet aggregation inhibition, obtaining higher PDGF-BB content compared with ACD. Moreover, if plasma of platelet concentrate is replaced by saline, they obtain higher PDGF-BB concentrations after activation but no fibrin gel formation (noncoagulating platelet-derived factor concentrate), which is interesting for injectable purposes such as in OA.

Fig. 2. Platelet-rich gel after injecting 50 IU of human thrombin and 1 mL of 10% $CaCl_2$ into 2 mL of platelet-rich plasma tube and waiting for 30 minutes at room temperature [39].

Few reports describe cellular and cytokine profile present in PRP. Major observations are that red blood cells are not completely eliminated, and white blood cells (WBCs) can even be concentrated depending on the technique being used. One study reported concentrations of cytokines in 11 human patient’s blood using two PRP commercial systems. First, ACP (Arthrex Inc), a plasma-based system, concentrated almost twofold more platelets and decreased leukocytes compared with whole blood. Second, GPSIII (Biomet Biologics Inc, Warsaw, IN), a buffy coat–based system, concentrated more than fourfold both platelets and leukocytes [32]. Hessel et al [33] recently reported the same results in horses ($n = 6$), comparing the Equine Platelet Enhancement Therapy (E-PET) (Pall Corporation, Port Washington, NY), an equine-specific filtration system, and four different centrifugation techniques, including the manual double-centrifugation method as described by Mos et al [34]. All systems were able to concentrate platelets, but only two, GPSIII and E-PET, had significantly different results compared with whole-blood baseline. E-PET had lower platelet (3.8-fold) and WBC (1.8-fold) concentrations compared with GPSIII (5.4-fold and 6.7-fold, respectively), but the highest PDGF-BB and TGF-β1 values of all. Only ACP system reduced the total leukocyte counts but was associated to the lowest platelet enrichment. The manual method showed acceptable cytokine concentrations but high standard deviations. Based on these findings, the E-PET system seems to be an acceptable choice for equine veterinarians in the field.

Table 3
Described platelet's exogenous activation methods.

Activation Methods	Advantages	Disadvantages	Reference
Freeze-thaw cycles	Inexpensive and safe	Additional step and time consuming if not using liquid nitrogen	[28]
10% Calcium chloride ($CaCl_2$)	23 mM comparable with 10 U/mL thrombin and freeze-thaw cycles GF release in vitro	Potentially expensive	[28,29]
Autologous equine thrombin		Not effective in GF release	[28]
Bovine thrombin	Big dose-dependent GF release; fast-inducing clot formation	Reported clinical reactions	[28]
10% Calcium gluconate (CG)	9.3 mg/mL incubated at 37°C for 6 hr. Complete release of GF	Time consuming	[35]
$CaCl_2$ + thrombin	At low concentrations: more protracted GF release (until 6 d)	Potentially expensive	[43]
Shear forces		Do not affect GF release	[44]
Collagen	Inexpensive and safe	Modest dose-dependent increase in GF; not significantly different GF's concentrations in synovial fluid	[44]

Abbreviation: GF, growth factor.

The ideal ratio between platelets and WBC has not been established yet, but it seems that a positive correlation exists between WBCs, especially neutrophils, and both IL-1 and TNF-α concentrations in PRP [7]. Therefore, it is accepted to favorless WBC concentrations, even if this means less platelet numbers.

2.2.2. PRP Activation Methods

Clinicians should be aware of controversial results depending on platelet and GF concentrations. In horses, GF concentrations appear to depend on breed, gender, and age of the patient [35]. Specifically, about GFs, McCarrel and Fortier [36] sustain that GF measurements are the reflex of platelet concentrations in PRP. Controversially, other authors state that GF concentrations in the final product do not always correlate with total platelet number and can be very different between individuals [37,38]. A simple reason for this could be the activation of platelets during PRP production with premature release of GFs into whole plasma fraction (platelet-poor plasma [PPP]), not used in the patient treatment. There are different chemical and physical factors able to activate resting platelets. This can be performed during or before administration, and in equine practice, it has been described the use of thrombin, $CaCl_2$, or both [28,39]. However, because solid scientific facts are lacking, most clinicians use PRP in the resting form. It is believed that platelets would be activated after injection by a collagen-rich lesion environment, but this would vary based on site, type, and duration of the lesion [40]. Resting platelets would have positive clinical effects [36], and in vitro PRP is associated with sustained release of GFs over a minimum of 4 days when relying on endogenous activation only [41]. These findings were confirmed comparing resting and activated PRP injections into normal joints showing no significant difference on both GF contents in synovial fluid [42]. Exogenous activation would only be recommended to ensure a maximal release of GFs or in cases where clot formation is needed. Table 3 allows further elucidation in respect to the use of these activators.

Other implications of the activation method are the possible secondary effects on the tissue to be treated. As reported by Han et al [45], thrombin activation method eliminates the chondroinductive and osteoinductive potential of PRP. In conclusion, if using PRP for cartilage purposes, resting PRP or $CaCl_2$-activated PRP should be considered.

2.2.3. In Vitro—Laboratory Findings

Interest on PRP effects in cartilage environment is increasing, and some studies report surprising and promising results. On healthy cartilage, there are two studies supporting its use. One study showed PRPr effects on stimulation of porcine articular-chondrocyte proliferation and matrix biosynthesis [46]. Treatment with 10% PRP resulted in increased DNA content, proteoglycan, and collagen synthesis compared with 10% PPP and fetal bovine serum (FBS). The effects of PRP were more pronounced on collagen synthesis than on proteoglycan synthesis, especially compared with the FBS group. A more recent work reported biochemical evidences that PRPr supplementation to human chondrocytes could support their chondrogenic properties [47]. At 48 hours of 5% PRPr treatment, there was a 96% increased proliferation of chondrocytes in comparison with 5% PPP releasate treatment.

In fact, anabolic effects of PRP on healthy chondrocytes are well documented, but there is also one study that reports its effects on chondrocytes in the presence of IL-1 to mimic an osteoarthritic environment. Platelet-rich plasma releasate has diminished multiple-inflammatory IL-1β–mediated effects on human osteoarthritic chondrocytes, including inhibition of nuclear factor kappa B activation, a major pathway involved in the pathogenesis of OA [48]. Other interesting feature of PRP potential was reported in a study where synovial cells were isolated from 10 osteoarthritic patients [49]. Synoviocytes cultured in PRP demonstrated increased angiogenesis, cell proliferation, and HA production and secretion, suggesting chondroprotection and joint lubrication after intra-articular application. Biologic effects of PRP-derived preparations on chondrocyte proliferation have been repeatedly demonstrated. These reports suggest that PRP action mainly depends on proliferation and migration, rather than differentiation. Nevertheless, there is one study showing that PRP enhances mesenchymal stem cell (MSC) proliferation, suggesting that PRP could induce chondrogenic differentiation [50].

Another feature that has been proposed is that clinical effects of PRP could also be explained by pain control mechanisms. End-stage arthritic human cartilage was treated in vitro with low WBCs and twofold to threefold platelets PRP. There were decreased markers associated with pain (TNF-α, substance P, and IL-1) which represents possible antinociceptive effects [7]. Martin et al [51] also reports thrombin receptor, possibly triggered after PRP treatments, as an endogenous inhibitor of inflammatory pain by activating opioid pathways.

2.2.4. In Vivo—Clinical Studies

In laboratory animals, three experimental studies were performed in rabbits and support PRP use. In collagenase-induced OA, PRP-treated stifles showed clear macroscopic and histologic improvement compared with controls [52]. In an osteochondral defect experimental study, PRP-treated group demonstrated cartilage regeneration and increased production of GAGs in the extracellular matrix compared with controls [53]. Finally, PRP microspheres injection into OA knees in rabbits stimulated chondrocyte GAG synthesis in vitro. Proteoglycan core protein messenger RNA in the articular cartilage was increased after administration of PRP. Treatment also suppressed progression of OA morphologically and histologically [54].

In humans, there are several clinical studies testing PRP's effects on arthropathy. Autologous platelet gel has been studied on 98 unilateral total knee arthroplasties, 61 had intraoperative platelet gel (PG) treatment, and 37 were used as controls. Patients receiving PG during surgery had less postoperative blood loss and less narcotic medication requirement. PG group also achieved higher range of motion before discharge, which was in average a day earlier than controls [55]. A retrospective cohort study evaluated the effectiveness of intra-articular injections of an autologous preparation rich in growth factors (PRGF) compared with a control group receiving HA injections, each group including 30 human patients with knee OA. Treatments were performed three times, 1 week apart, and WOMAC questionnaires were filled before and 5 weeks after treatment. Pain subscale reached 33.4% in PRGF group compared with only 10% in the HA group but provided useful information about PRGF safety [56].

A group of authors did a series of clinical trials on PRP effect using a double-centrifugation method. This represented a mean increase of fivefold platelet and 1.2-fold WBC concentration compared with whole blood. Platelet-rich plasma was stored at −30°C and given as one injection per week for 3 weeks. The first study analyzed 100 consecutive patients (115 knees) affected by chronic degenerative condition. Evaluations were done before, at the end of treatment, at 6- and 12-month follow-up, through human pain scales. There was significant improvement of all clinical scores at the end of treatment and at 6-month follow-up but became significantly worse at the 12 months. Authors concluded that PRP treatment was safe and had the potential to reduce pain and improve knee function and quality of life in younger patients with low degree of articular degeneration; nevertheless, there was no control group [57]. Ninety-one patients were available for a 24 months of follow-up (114 knees), using International Knee Documentation Committee (IKDC) and Euroqol Visual Analogue Scale (EQ-VAS) scores. Further analysis showed better results in younger patients and lower degrees of cartilage degeneration. The median duration of clinical improvement was 9 months [58]. The same authors also compared a group treated with three injections of PRP (n = 50) with two HA treatment groups: high (n = 50) and low molecular weight (n = 50), again using IKDC and EQ-VAS scores at 2- and 6-month follow-up interval. At the first evaluation, PRP and low-molecular HA had similar and better results than high-molecular HA. In the 6-month follow-up, better results were observed in the PRP-treated group. Moreover, both PRP and low-molecular HA had similar results in patients more than 50 years, and PRP showed better performance in younger patients affected by cartilage lesions or early OA [59]. These clinical results have not been confirmed by a randomized controlled clinical trial. Once more, PRP-treated group (n = 55) was compared with a HA control group (n = 54) and patients were evaluated by OA human pain scales at 2, 6, and 12 months after a three-dose injection treatment. There were some cases of mild pain and effusion after injections, in particular in PRP group. Both groups presented a clinical improvement, but there was no statistical difference. A trend favorable to PRP group was only found in low-grade articular degeneration [60]. On contrary, a prospective cohort study with a control group analyzed 120 patients with grade 1, 2, or 3 OA using the Kellgren and Lawrence radiographic grading scale. One group was treated with three PRP and the other three HA injections. There was a fourfold to fivefold platelet concentration in PRP, and no adverse effects were observed. Three-month and 6-month follow-up had significantly better results in the WOMAC and pain numeric rating scale in the PRP-treated group [61]. More recently, Patel et al [62] compared one high-concentrated PRP, 10-fold above baseline, injection to one saline injection, and two low-concentrated PRP, fourfold above baseline, injections in humans (n = 150). Patients had significant improvement of all subscales of the WOMAC scores, especially at the 3-month follow-up and decreased slightly after 6 months. The saline group worsened over the 6 months.

In conclusion, in humans, PRP have beneficial clinical effects on pain reduction and function improvement on a short-term period and especially in young and low-grade severity OA cases. Compared with other therapies, PRP can have the additional advantage of being economic. Further consensus is still lacking on preparation, activation methods, and treatment protocols. However, a single injection of high-concentrated PRP, prepared using centrifugation techniques and activation by $CaCl_2$ or freeze-thaw cycles, seems to be valid for cartilage treatment at this time point.

In horses, the first studies in cartilage focused on IGF-1, and they have proven that this GF had anabolic effect on chondrocytes. An experimental study with extensive loss of cartilage showed, 8 months after treatment, that IGF-1 group had enhanced incorporation into surrounding cartilage, improved gross filling and higher proportion of cells producing type II collagen [63]. Using the same model, chondrocytes genetically modified encoding IGF-1 also

showed beneficial effect on cartilage healing [64]. On equine natural occurring OA, there are few communications reporting clinical outcome after intra-articular PRP treatment. The first clinical report describes a decrease of lameness and synovial effusion scores after three treatments, 2-week apart, but only evident from 2 to 8 months after the last injection, over a 1-year period [65]. This is in agreement with clinical human studies. The second communication reports no side effects in any of the 30 patients after intra-articular PRP treatment; however, there is the evident bias of a retrospective study such as big differences in number and clinical presentation of OA between groups [66]. Both studies were performed using double-centrifugation technique and 50 μL of $CaCl_2$ per mL of PRP for activation [26]. This is not sufficient to support PRP treatment but clearly suggests that this is a safe, economic, and beneficial treatment in acute cases of OA in horses. More recently, intra-articular platelet concentrate has been applied to chronic cases of refractory OA with 80% of the horses returning to work, and no adverse reactions reported [67].

Platelet-rich plasma injection into normal joints was performed with the PRP gravity filtration system (E-PET). Authors obtained 3.2-fold and 1.9-fold increases in platelets and WBCs, respectively, which are similar to the concentrations described in vitro before. Thrombin-activated PRP increased proinflammatory cytokines (IL-6 and TNF-α) and caused periarticular heat and swelling, effusion, and increased flexion scores. These clinical findings were not observed in the resting or $CaCl_2$-activated PRP groups. Growth factors were globally low, so they have suggested that PRP benefits are not coming from PDGF or TGF-β1 concentrations [68].

The platelet gel has gained surgeons interest for local cartilage lesions, used alone or combined with other therapies such as stem cells, as a GF scaffold as discussed in Section 3.1. A recent review was done in different species about PRP application on abnormal cartilage. Eight studies in vivo showed PRP can be used as adjunct in microfracture surgery and implants scaffold and graft insertion besides intra-articular injection, but not all studies concluded that PRP has a positive effect on cartilage repair [69]. Some authors state negative effects of PRP could only be exceeded by using specific anti-GF antibodies [70].

In conclusion, platelet-derived products seem promising as an intra-articular therapy, but consistent nomenclature and standardized protocols would be highly beneficial for future randomized controlled trials application. Moreover, negative effects of some of its GFs should be further investigated and modulated in the future.

3. Cell-Derived Therapies

Stem cells are unspecialized cells, which have the ability to renew themselves indefinitely, and under appropriate conditions give rise to a variety of mature cell types [71]. They have gained increased interest in human and veterinary medicine because of their therapeutic potential in many fields. Intensive research has been performed to achieve true regeneration, but in an equine clinical perspective, functional recovery would be the primary goal. There are different sources of mesenchymal stem cells (MSCs), and a hierarchy in stem cell plasticity exists such that totipotent cells can differentiate into all cell types and are available from the 1- to 3-day oocyte (morula) [72]. Pluripotent cells are able to differentiate into tissues from the three germ layers but not to placenta and are available from later embryonic stages or can be reprogrammed from an adult differentiated tissue—induced pluripotent stem cells [72,73]. Multipotency means they can differentiate into two or more cell lines, and unipotent cells can only produce their own cell type [74].

In practice, autologous multipotent cells are normally used because they have good differentiation capacity and require simple procedures to be collected from the patient. Mesenchymal line, containing undifferentiated cells from bone marrow and adipose adult tissues are the two populations most frequently used in cartilage repair [75]. Some studies report a significant higher chondrogenic potential of bone marrow–derived stem cells, synovial membrane, and intra-articular fat [76–78]. However, adipose subcutaneous tissue has gained popularity because it is more readily available and allows obtaining a high number of cells in the initial yield which means little need of expansion and culture time [77,79].

Allogeneic administration of stem cells is also possible because they are able to escape immune recognition [80]. Mesenchymal stromal cells are characterized by their ability to adhere to plastic and by their capacity for multipotent differentiation, such as into osteogenic, chondrogenic, and adipogenic lineages. Furthermore, they must express a panel of mesenchymal stromal cell markers such as CD29, CD44, CD73, CD90, and CD105 despite lacking distinctive hematopoietic antigens such as CD34, CD45, CD14, CD79α, and MHC [81]. In equine bone marrow and adipose tissue MSCs, gene expression of membrane surface markers has been reported by Ranera et al [82].

3.1. In Vitro—Laboratory Findings

Ability of equine multipotent adult MSCs to differentiate into cells of chondrogenic lineage [83] have led to experimental strategies to investigate whether they can be used for regeneration. This includes research on cartilage implants or vehicle solutions. Injecting MSC suspensions would be responsible for initiating endogenous regenerative activities in the OA joint by means of bioactive factors production [84]. Some studies have shown the capacity of MSCs to adhere to articular cartilage [85], but after intra-articular injection, cell-labeling techniques demonstrated that MSCs localized preferably in soft-tissue structures with little or no retention within cartilage defects [86]. According to these studies, beneficial effects of intra-articular administered MSCs on articular cartilage are likely mediated through soluble factors primarily on other articular tissues and by changing tissue microenvironment.

When determining the optimal source of cells for cartilage repair, two main criteria are considered: cells performance and easy availability [87]. Intrinsic and clinical superiority of purified MSCs populations over more heterogeneous cell isolates is questionable [88]. In fact, after harvesting tissues, samples can be processed by simple

techniques, such as centrifugation, or be cultured during specific periods. The major differences are the total number and purity of stem cells injected into the patient and the processing time, which can take from several minutes for centrifugation to several weeks if culture is performed [89]. Some authors defend that more heterogeneous products can enhance MSCs proliferation and variably induced MSCs migration, mediator secretion, and gene expression. Recently, Kol et al [90] found that soluble factors from adipose-derived stromal vascular fraction (ADSVF) induced significantly more MSCs proliferation and migration in vitro compared with bone marrow mononuclear cells, cord blood mononuclear cells, and platelet lysate. The use of bone marrow aspirate concentrates (BMAC) has been applied effectively to improve cartilage resurfacing in horses [91].

Mesenchymal stem cells are commonly applied together through vehicles and scaffolds containing other molecules. It is assumed that for chondrogenic differentiation cells need low oxygen environment and GFs [92]. A recent study showed PRP to be a three-dimensional scaffold with a mesh-like microstructure with capability of endogenous GF release and ready cell incorporation in vitro [93]. After implantation in PRP, bone marrow MSCs had higher proliferation rate and higher expression of cartilage-specific genes and proteins than adipose-derived stem cells. Using the same constructs in osteochondral defects in rabbits, bone marrow also exhibited better gross appearance and histologic and immunohistochemical characteristics, higher cartilage-specific gene, and protein expression as well as subchondral bone regeneration. However, when associating GFs to MSCs' suspensions, there are possible secondary effects as induced osteophyte formation [94]. Surprisingly, in the same study, osteophytes were not found when GFs were injected without the stem cells.

In inflammatory arthritic conditions, MSCs inhibit activation of T lymphocytes and secretion of inflammatory cytokines while concurrently stimulating secretion of anti-inflammatory ILs and reducing cartilage damage [95]. These MSC-mediated activities could be clinically beneficial in OA joints and support chondrocyte metabolism in the face of arthritic disease. Lozito and Tuan [96] support that MSCs are able to secrete active forms of MMPs, but their activity is not detected probably because of tissue inhibitors of metalloproteinase (TIMPs) inhibition. These authors showed that MSCs of the perivascular niche inhibited high levels of exogenous MMP-2 and MMP-9 through TIMP-2 and TIMP-1, respectively. Stem cells remained matrix protective when exposed to proinflammatory cytokines and hypoxia, responding with increased TIMP-1 expression and augmented MMP inhibition. In another study, using synovial explants subjected to TNF-α and interferon gamma (IFN-γ) to create short-term osteoarthritic conditions, the group receiving MSCs had decreased gene expression of IL-1β, MMP-1, MMP-13, and increased cytokine signaling suppressor (SOCS) 1. In cartilage explants subjected to the same circumstances, IL-1ra was upregulated, whereas disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) 5 and collagen type II alpha 1 were downregulated. These findings are quite interesting and support that MSCs inhibit inflammatory processes in short-term osteoarthritic synovium [97]. Other in vitro study compared equine MSCs derived from bone marrow, adipose tissue, and umbilical cord blood and tissue. All tissue types decreased lymphocyte proliferation, increased prostaglandin (PGE2) and IL-6 secretion, and decreased production of TNF-α and IFN-γ. The only parameter by which MSCs differed was that bone marrow and cord blood MSCs produced nitric oxide, whereas adipose and cord tissue did not [98]. Despite these findings, selecting the source of MSCs will depend on differentiation potential but especially on the practicality of obtaining, culturing, and banking each tissue.

3.2. In Vivo—Clinical Studies

In laboratory animals, several experimental studies have been developed using stem cell implants. Yan and Yu [99] demonstrated that lesions in the femorotibial joint in rabbits treated with chondrocytes resulted in hyaline-cartilage production. Comparatively, lesions treated with MSCs had better cellular organization and integration into surrounding cartilage. In another experimental osteochondral study, defects were created on the patellar groove of the right distal femur of 12 rabbits, divided into four groups ($n = 3$) and managed by scaffold only, scaffold seeded with adipose stem cells (ASCs), scaffold with TGF-β2 and bone morphogeneic protein 7 (BMP-7), or scaffold with TGF-β2 and BMP-7 seeded with ASC. After 9 weeks, scaffolds were filled with dense tissue and had distinct margins with adjacent normal cartilage and GF groups had more foreign-body giant cells and blood vessels. In practice, the use of GF-immobilized scaffolds did not lead to better histologic scores, because they were not significantly different between groups, but increased International Cartilage Repair Society macroscopic scores. This improvement was not observed with ASC implants [100]. More recently, equine meniscal sections were harvested from animals euthanized for unrelated reasons, treated with fibrin glue or fibrin glue and equine bone marrow MSCs, and then implanted subcutaneously in nude mice. At 4 weeks of evaluation, BMSCs-containing constructs had significantly increased vascularization, and histology showed subjectively decreased thickness of repair tissue and increased total bonding compared with fibrin alone constructs [101].

Compared with cartilage implants, stem cell suspensions are practical and increasingly studied. One report used a caprine knee model of OA by complete excision of the medial meniscus and resection of the anterior cruciate ligament [102]. Adult stem cells were isolated from caprine bone marrow, expanded in culture, and transduced to express green fluorescent protein. After 6 weeks, a unique dose of 10 million autologous cells suspended in a dilute solution of sodium hyaluronan was injected by intra-articular administration, whereas controls received sodium hyaluronan alone. There was a marked regeneration of the medial meniscus, and implanted cells were detected in the newly formed tissue. Degeneration of the articular cartilage, osteophytic remodeling, and subchondral sclerosis were reduced in cell-treated joints compared with joints treated with vehicle alone. There was no evidence of repair of the ligament of the joints. Using a rabbit anterior

cruciate ligament transection model, one million allogeneic MSCs from infrapatellar fat pad were injected 12 weeks after destabilization. There was reduced cartilage degeneration, osteophyte formation, and subchondral bone sclerosis [103]. In contrast, in a monoiodoacetate model in rats, MSCs reduced pain scores but not degenerative changes [104]. In this study, bone marrow MSCs were compared with bone marrow mononuclear cells and saline. There was significantly pain reduction in MSC-treated joints, compared with other groups, but no differences were observed regarding cartilage damage, subchondral bone alterations, and synovial inflammation.

There are limited human in vivo studies. Kuroda et al [105] treated a large full thickness articular cartilage defect in the femoral condyle of an athlete with autologous bone marrow stromal cells, cultured and embedded within a collagen gel, transferred to the articular cartilage defect, and covered with an autologous periosteal flap. Seven-month arthroscopy showed the defect to have smooth tissue, histologically being hyaline-like type cartilage, and 12-month follow-up showed clinical improvement and returned to previous activity level. Another single human case study also suggests that intra-articular administered MSCs can restore articular cartilage volume, as assessed by magnetic resonance imaging and improved clinical signs of arthritic disease for several months [106]. Globally, bone marrow stromal cell results have been similar to those yielded using other cell-matrix systems; the repair tissue formed being principally fibrocartilaginous in nature, but of variable quality and durability. Although many teams have investigated stem cell use in orthopedic tissue regeneration, limited evidence is currently available to support its routine use; therefore, large-scale clinical trials are lacking [107].

In horses, there is a study comparing single intra-articular injection of autologous, related and nonrelated, allogeneic placental-derived MSCs in 16 healthy horses [108]. All groups had similar systemic and local response with minimal joint swelling and lameness but marked inflammation in synovial fluid during the first 72 hours after injection. Despite the short period of evaluation, it is important to clarify in vivo safety of allogeneic treatments, although flares can appear several days after injection or after second treatment [109]. Most clinical studies are experimental, and the main reason for this is the lack of scientific support together with elevated price of stem cell therapy. The first experimental study in horses used bone marrow MSCs–enriched fibrin or fibrin alone scaffolds. Improvement in early cartilage healing was very variable and not persistent at 8 months [110]. Using fibrin scaffold to retain cells within cartilage defects requires cells to be prepared before surgery which is not always possible in clinical cases, or a two-step surgery is needed which is expensive and time consuming. The other strategy involves intra-articular injection of stem cell suspension, avoiding collection and preparation before ascertaining the need of the treatment [5]. However, treating extensive lesions is more challenging than the repair of focal cartilage defects. Two studies, using a carpal osteochondral fragment model of OA and focused on clinical assessment of pain beside radiographic, synovial fluid and histologic evaluation, did not found significant improvement in pain score [111,112]. These studies in experimental OA models give little support to the use of MSCs in the acute phase. However, a prospective multicenter study was designed in naturally occurring OA. Treated joints were mostly stifles with advanced fibrillation and loss of soft-tissue structures and received bone marrow MSCs and HA. In a mean of 21-month follow-up, authors reported 76% return to work, from which, 38% of horses returned to their intended use. Authors also found a superior long-term outcome when treatment was performed at least 1 month after diagnosis [73,113]. More recently, this has been published in 33 clinical cases of stifle lesions using autologous BMSCs and allogeneic peripheral blood MSCs plus PRP in 91 clinical cases of OA in different joints [114,115]. Both studies reported high rates of returning to work, 75% at 24 months [114] and 78% at 4.5 months of follow-up [115]. Based on these reports, intra-articular administration of stem cells seems an interesting and straightforward therapy, but it would still be necessary to develop more clinically controlled studies to support these findings.

Similar to other biologic treatments, there are still practical questions about stem cell therapy that need to be answered, for example, to know the optimal cell source for each type of tissue or lesion, if there is a dose-dependent response or if a high number of cells do not necessarily improve clinical symptoms. Should cells be administered locally or can be delivered systemically, and if so, can they be injected alone or which type of scaffolds or vehicles should be used? Furthermore, as for other biologic products, there is still no well-defined window for treatment. It is usually avoided to inject MSCs into acutely traumatized inflamed tissues, but it is also possible that anti-inflammatory and neoangiogenic activities of MSCs might have positive effects in early phases of repair and affect long-term results. Some authors believe MSCs would beneficiate from an inflammatory joint environment for their activation to occur. In practice, there is still the possibility to activate these cells and use them as allogeneic products.

The sooner standardized protocols are available the sooner will be possible to have more conclusive clinical answers. It is also important to realize that positive or negative results in experimental settings are not always repeatable in other species or in the naturally occurring disease. Moreover, equine veterinarians need to be aware of each drug legislation and doping rules, if applicable.

4. Discussion

Different biologic strategies are available for treating osteoarthritic joints, but until now, there is little evidence of significantly clinical improvements. Table 4 shows 11 in vivo studies using intra-articular biologic products in horses: six experimental clinical studies and five studies in naturally occurring OA. Not surprisingly, most therapies are targeting a single intra-articular injection. This is mainly because of the fact that there is a decreased risk of infection and inflammatory reaction for the patient, fewer expenses for the owner, and it is easier to standardize protocols for equine veterinarians. In experimental clinical studies, most

Table 4
Effects of intra-articular biologic therapies on equine joint disease.

Product	Preparation Method	Study Design	Treatment	Follow-up	Findings
Autologous-conditioned serum (ACS) versus placebo [20]	- Autologous (Orthokine; Orthogen) Platelet: WBC: NA IL-1ra (mouse antibody): 5-, 2-fold increase	n = 16 Experimental Comparative mechanical carpal fragment model Strenuous exercise: 5 d/wk	14-d post-OA 4 ACS injections, 1 wk apart (days 14, 21, 28, 35) 6 mL saline (n = 8) 6 mL ACS (n = 8)	- Every week: synovial fluid analysis (total protein cytological and biochemical analysis) - Every 2 wk: clinical and lameness evaluation (AAEP scale) - Before, after OA, and in the end: X-rays - After euthanasia (70 d): gross and histologic evaluation	Compared with placebo, ACS group had: - Significant lameness degree improvement on ACS group on 70 d - On OA joints, there was significantly decrease of synovial membrane hyperplasia - Significant increased IL1-ra after 35 d in synovial fluid
Autologous protein solution (APS) versus placebo [21]	Autologous Double centrifugation (Nstride; Biomet) Platelet: 1-, 6-fold increase WBC: 12-, 1-fold increase Il-1ra: 5-, 8-fold increase	n = 40 Clinical cases - Predominantly 1 limb with 1 high motion OA joint - Exercised 2× per week on treadmill (−1, 4, 7, 10, and 13 d)	Single dose 5 mL saline (n = 20) 5 mL APS (n = 20)	- Before, 4, 7, 10, and 14 d: assessment of joint pain and swelling - Before, 7, and 14 d: lameness (AAEP scores and kinetic gait analysis) - Before and 14 d: synovial fluid, blood, X-rays - Before, 12, and 52 wk: client questionnaire	- APS significantly improved lameness by 14 d compared with baseline and controls - No adverse effects - Clinical improvement at 12 and 52 wk by clients - Better results if mild lameness and X-ray scores
Platelet-rich plasma (PRP) versus corticosteroids [65,66]	Autologous Manual double centrifugation Reported concentrations per mL of PRP [65] WBC: $8.68 \pm 3.78 \times 10^6$ cells Platelet: $250 \pm 71.8 \times 10^6$ cells TGF-β1: 12,515 ± 2.443 pg	n = 42 Clinical cases Group 1 (n = 12 chronic OA) Group 2 (n = 10 acute OA and n = 20 chronic OA)	Group 1: rest and IA corticosteroids Group 2: 1–3 PRP injections, 2 wk apart Reported doses*: 10–20 mL	Evaluations before, 45 d, 2 m, and 4 m after treatment: - Lameness (AAEP scale) - X-rays - Joint effusionFollow-up available from 1 to 3 y and 5 y: - Owner's questionnaire	Group 1—75% went back to previous work with 33% relapse Group 2—70% went back to previous work with 9.5% relapse Worst prognosis if chronic or more radiographic changes Better outcome if more than 1 injection
Platelet concentrate (PC) [67]	Autologous Manual double centrifugation PC concentrations: Platelet: $560 (\pm 62) \times 10^3$ cells/μL PDGF-BB: 1,280 (±70.9) pg/mL	n = 20 Clinical cases Chronic OA refractory to rest or corticosteroids Not blinded nor controlled study	- 3 PC injections; 2 wk apart - PC activated with 3 mL saline with 23 mM $CaCl_2$ 1 hr before injection	15 d, 30 d, 1 y: - Lameness examination - Owner's questionnaire using modified Western Ontario and McMaster Universities Osteoarthritis index for pain, stiffness, and physical function - 1-y follow-up examination	- Significant lameness degree and owner's index improvement between 1st, 2nd, and 3rd injections. - Significant correlation between clinician's and owner's index - 80% of horses returning to work after 1-y follow-up. - No adverse reactions
Bone marrow aspirate (BMA) + microfracture versus microfracture alone [91]	Autologous bone marrow aspirated from sternum (60 mL) and centrifugation (SmartPreP2., Harvest Technologies) 6 mL of BMA; 1 mL for cytologic and flow cytometry: MCS: 13.4% in gate 5	n = 12 Experimental One-step surgery/treatment 15-mm cartilage defects on both lateral trochlear ridge	- Microfracture +5 mL BMA (n = 12) - Microfracture alone (n = 12)	- 3 mo–2nd-look arthroscopy: International Cartilage Repair Society (ICRS) macroscopic scoring system	- Significant improvement in BMA group in gross and histologic evaluations - Increased defect fill and improved integration of repair tissue at MRI

(*continued on next page*)

Table 4 (*continued*)

Product	Preparation Method	Study Design	Treatment	Follow-up	Findings
	Platelets: 8-, 7-fold increase WBC: 7-, 4-fold increase	– 2-wk stall rest – 10-min walking exercise/d increasing each week until 3 mo		8 mo euthanasia: gross, histologic (ICRS), and MRI evaluation	- Greater type II collagen content and improved orientation in BMA group - More GAG in BMA group - No adverse reactions
Bone marrow-derived stem cells (BMSC) versus autogenous fibrin [110]	• Sternum BMSC • Self-polymerizing autogenously fibrin vehicle (calcium-activated thrombin, 500 U)	n = 6 Experimental 15-mm cartilage defects on both lateral trochlear ridge – 5-wk stall rest – 7-wk 5-min walk - Pasture exercise	Single implant – 1 mL autogenous fibrin vehicle + 12×10^6 MSCs (n = 6) – 1 mL autogenous fibrin vehicle alone (n = 6)	– 0, 4, 7, 14, 21, 30 d: synovial fluid WBC and TP – 1 mo–2nd-look arthroscopy: macro and biopsy – 8 mo euthanasia: gross, histologic, histochemical, and matrix biochemical evaluation	- No significant differences in synovial fluid between groups - 1-m assessment: MSC-implants treated defects had significantly improved scores - 8-m assessment - No significant difference between groups
BMSC versus adipose-derived stromal vascular fraction (AD-SVF) [111]	Autologous BMSC from sternum AD-SVF (Vet-Stem) from tail head–nucleated cells	n = 24 Experimental Comparative mechanical carpal fragment model Strenuous exercise: 5 d/wk	Injection 14 d post-OA BMSC (n = 12): 10.5×10^6 cells per joint AD-SVF (n = 12): 16.3×10^6 cells per joint	- Every week: synovial fluid analysis (TP, cytologic, and biochemical analysis) - Every 2 wk: clinical and lameness (AAEP scale) - Before, after OA, and in the before 70 d: X-rays - After euthanasia (70 d): gross, histologic, and histochemical evaluation	- No lameness improvement - AD-SVF increased flexion scores and synovial fluid TNF-α - BMSC-reduced synovial fluid PGE2
BMSC + hyaluronic acid (HA) versus HA [112]	Autologous BMSC	n = 10 Experimental 10 × 10-mm cartilage defects on both medial femoral condyles + microfracture - 2-wk rest - 4-m hand walking - Treadmill exercise	Single injection 1 mo after surgery: - 20×10^6 cells + 22 mg HA (n = 10) - 22 mg HA (n = 10)	- Lameness evaluation and X-rays every 2 mo - 6 mo–2nd-look arthroscopy - 12 mo–euthanasia: gross, histologic, histomorphometric, immunohistochemical, and biochemical evaluations	- No significant clinical improvement in BMSC group - Significant increase in repair firmness at arthroscopic and gross evaluation - Significantly increased aggrecan content in repair tissue of BMSC group
Arthroscopy + BMSC ± HA [114]	Autologous BMSC	n = 33 Clinical cases Multicenter prospective study: meniscal, cartilage, and ligamentous stifle lesions treated by arthroscopy, BMSC and HA	Single BMSC injection 3–4 wk after surgery (n = 30) or the day of surgery (n = 3): mean $13.2 \times 10^6 \pm 5.6$ cells per stifle ± 20 mg HA	24 mo: clinic records, evolution, and return to work	43% returned to previous level of work 33% returned to work 24% failed return to work - Meniscal injuries had significant higher return to some level of work (75%) compared with previous studies (60%–63%) with arthroscopy alone. - Postinjection flare in 3 horses receiving BMSC + HA (9%)
PRP + peripheral blood native MSC (PBMSC) versus PRP + chondrogenic-induced PBMSCs [116]* [115].	Allogeneic PBMSC and PRP Reported technique* Platelets: 200×10^6 cells/mL PRP MSC: NA	n = 165 Clinical cases Number of OA joints: stifle, n = 30; fetlock, n = 58; pastern, n = 34; coffin, n = 43	Single IA injection 24 hr after local anesthesia PRP with native PBMSC or induced PBMSC	6 wk (n = 165) and 18 wk (n = 91): 0—failure return to work 1—rehabilitation 2—return to work 3—return to previous level	- Induced PBMSC had higher average scores than native PBMSC but not significantly different.

					- 6-wk follow-up: 45% of native and 60% of induced MCS had score 2 or 3 - 18-wk follow-up: 78% native and 86% of induced MCS had score 2 or 3 - 1-wk postinjection flare in 3 horses (1.8%)
BMSC versus hyaluronic acid (HA) [117]	Autologous Bone marrow recovered from lateral side of humerus bone BMSC green fluorescent protein (GFP) labeled	n = 27 donkeys Experimental 2 mL amphotericin-B 50 mg in both carpal joints Exercise protocol: NA	Single injection at 3 wk (n = 9); 6 wk (n = 9); or 9 wk (n = 9) post-OA Right joint: 5.4–6.9 × 10^6 cells suspended in 3 mL HA (1.8–2.3 × 10^6 cells/mL) Left joint: 3 mL HA	Different euthanasia times for 3 horses in each group (1, 2, and 6 mo): lameness (AAEP score); X-rays (Crawford score)	- GFP-labeled MSCs seen in the newly formed articular surface but also soft tissues - Reduced X-ray and histologic scores for all different degrees of MSCs-treated OA joints - Lameness improvement at 2 and 6 mo of follow-up, better for less severe OA grades

Abbreviations: AAEP, American Association of Equine Practitioners; GAG, glycosaminoglycan; IA, intra-articular; IL1-ra, interleukin 1 receptor antagonist; MRI, magnetic resonance imaging; MSCs, mesenchymal stem cells; NA, not assessed; OA, osteoarthritis; PDGF-BB, platelet-derived growth factor-isoform BB; PGE2, prostaglandin; TGF-β1, transforming growth factor β1; TP, total proteins; WBC, white blood cells.

authors decided to inject the treatments between 2 and 4 weeks after disease induction. However, despite some authors [5] believe regeneration should be attempted after inflammation resolution, others [6] state that inflammatory mediators are the key of therapies success, and IA therapies should be administered as soon as possible. Product concentration and volume are variable. Authors using blood-derived therapies try to concentrate GFs and anti-inflammatory cytokines, such as IL-1ra, fivefold above baseline. Authors using cell-derived therapies injected between 10 and 20 million cells per joint. Frequently stem cells are diluted in a 2- to 5-mL vehicle, saline, or HA, or in association with a PRP product. Most often, biologic products have autologous origin, and as ADSVF has shown increased flexion scores and TNF-α concentration in synovial fluid, most of the equine research has been done with bone marrow–derived stem cells. The exception is the comparison of allogeneic PRP together with allogeneic native or chondrogenic-induced peripheral blood-derived stem cells. A large population of horses was used, but the results have not shown significant difference between groups even if induced MSC group presented higher percentage of horses returning to work [115].

In Table 4 review, equine lameness improvement after intra-articular biologic products injection was reported in refractory chronic OA [67] but must often related to less severe clinical OA cases [19,20,66,117]. However, there were studies where lameness improvement was not observed, and in two stem cells studies, one associated to HA and the other to PRP, there was postinjection flare in 9% and 1.8% of the cases, respectively [114,115]. This should always be considered when doing intra-articular injections, despite this could be solved as it usually responds to conservative treatment.

Biochemical pathways are still only partially understood, but histologic scores were always improved in the five experimental studies using autologous bone marrow stem cells and one using bone marrow aspirate. Cartilage assessment time varied between 2.5 and 12 months and showed better repair tissue quality and integration. One study showed labeled MSCs are present in the newly formed articular surface beside soft tissues. Soft tissue affinity can explain why stifle meniscal injuries had higher return to work compared with previously published reports [117].

5. Conclusions

In the future, maybe there will be no need for exogenous MSCs administration by recruiting clinically effective numbers of host MSCs to injury sites. The perivascular location of most MSC niches facilitates their mobilization. Delivery of key chemokine and/or GFs to sites of injury could be used to attract and retain sufficient MSCs for effective repair. Ideally, readily available allogeneic biologic products or inexpensive autologous products such as PRP, ACS, BMAC, and ADSVF could preclude the need of stem cell processing and be sufficient to improve repair. With more biochemical understanding and molecules combination, biologic strategies could also be used in tissue engineering to achieve true regeneration. Beside treatment products,

researchers should control several factors such as differentiation, cartilage matrix synthesis, and collagen type II production.

More than standardized products and treatment protocols, it would be extremely important to define objective methods to evaluate clinical outcomes. In cartilage assessment and orthopedic pain detection, it would be important to use advanced imaging modalities, biochemical analysis, and objective lameness detection. Creating specific disease diagnostic scales would also help to standardize practitioner's evaluations. Finally, multicenter standardized protocols and randomized controlled trials are probably the only way to achieve sufficient patient numbers for significant answers from ACS, PRP, and stem cell intra-articular therapies.

Acknowledgments

The authors thank António Bettencourt for providing language assistance.

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Appendix C – Questionnaire

Survey to Portuguese equine veterinarians

Questionário

Osteoartrite em Cavalos

O estudo da osteoartrite em cavalos insere-se num programa de doutoramento em Ciências Veterinárias da Universidade de Évora. Neste inquérito considera-se a osteoartrite (OA) como a doença primária na sua fase aguda ou crónica (osteoartrose). Quando não souber ou não tiver a informação escolha a hipótese N/A (não se aplica).

1 **Qual é a percentagem da actividade que exerce em clínica de equinos?**

.....

2 **Há quantos anos exerce clínica de equinos?**

.....

3 **Em que disciplina equestre diagnostica mais OA em cavalos?**

.....

4 **Nessa disciplina, a que nível de trabalho diagnostica mais OA?**

- ☐ Baixo (0-5h/semana)
- ☐ Moderado (6-10h/semana)
- ☐ Elevado (11-15h/semana)
- ☐ Não se aplica

5 **Nessa disciplina, em que faixa etária diagnostica mais casos de OA?**

- ☐ menos de 5 anos
- ☐ 5-9 anos
- ☐ 10-14 anos
- ☐ 15-19 anos
- ☐ mais de 19 anos
- ☐ não se aplica

6 **Encontra sinais clínicos de OA sem presença de lesões radiográficas? Se sim, em que articulação/situação?**

.....

7 **Encontra lesões radiográficos de OA sem presença de sinais clínicos? Se sim, em que articulação/situação?**

.....

8 **Que opções recomenda para o tratamento de OA primária?**
Ordene do mais ao menos frequente | *Marcar apenas uma oval por linha.*

	1º	2º	3º	4º	5º	6º	7º	8º	N/A
Ferração ortopédica	◯	◯	◯	◯	◯	◯	◯	◯	◯
Adaptar maneio alimentar e de trabalho	◯	◯	◯	◯	◯	◯	◯	◯	◯
Anti-inflamatórios não esteróides sistémicos	◯	◯	◯	◯	◯	◯	◯	◯	◯
Ácido Hialurónico sistémico (endovenoso)	◯	◯	◯	◯	◯	◯	◯	◯	◯
Polisulfato glicosaminoglicanos sistémicos (intra-muscular)	◯	◯	◯	◯	◯	◯	◯	◯	◯
Suplementos alimentares	◯	◯	◯	◯	◯	◯	◯	◯	◯
Tetraciclinas	◯	◯	◯	◯	◯	◯	◯	◯	◯
Tratamento intra-articular	◯	◯	◯	◯	◯	◯	◯	◯	◯

9 **Que tratamento intra-articular recomenda em articulações muito móveis (interfalângica distal, metacarpo/tarso falângicas e tíbio-társicas?**
Ordene do mais ao menos frequente | *Marcar apenas uma oval por linha.*

	1º	2º	3º	4º	5º	6º	7º	8º	9º	N/A
Acetato de triamcinolona	◯	◯	◯	◯	◯	◯	◯	◯	◯	◯
Acetato de metilprednisolona	◯	◯	◯	◯	◯	◯	◯	◯	◯	◯
Sulfato de betametasona	◯	◯	◯	◯	◯	◯	◯	◯	◯	◯
Ácido Hialurónico	◯	◯	◯	◯	◯	◯	◯	◯	◯	◯
Polisulfato-glicosaminoglicanos (PSGAG)	◯	◯	◯	◯	◯	◯	◯	◯	◯	◯
Antagonista do receptor da interleuquina I (IRAP)	◯	◯	◯	◯	◯	◯	◯	◯	◯	◯
Plasma Rico em Plaquetas (PRP)	◯	◯	◯	◯	◯	◯	◯	◯	◯	◯
Células estaminais	◯	◯	◯	◯	◯	◯	◯	◯	◯	◯
Antibiótico (ex: amikacina, gentamicina)	◯	◯	◯	◯	◯	◯	◯	◯	◯	◯

10 **Que protocolo (ex: dose e n.º de tratamentos) utiliza na sua primeira opção de tratamento intra-articular?**

.....

UNIVERSIDADE DE ÉVORA
INSTITUTO DE INVESTIGAÇÃO
E FORMAÇÃO AVANÇADA

Contactos:
Universidade de Évora
Instituto de Investigação e Formação Avançada - IIFA
Palácio do Vimioso | Largo Marquês de Marialva, Apart. 94
7002-554 Évora | Portugal
Tel: (+351) 266 706 581
Fax: (+351) 266 744 677
email: iifa@uevora.pt