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Efficacy and safety study of allogeneic Equine Umbilical Cord derived Mesenchymal Stem Cells (EUC-MSCs) for the treatment of clinical symptomatology associated with mild to moderate degenerative joint disease (osteoarthritis) in horses under field conditions

THESIS

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DEDICATORIA

AGRADECIMIENTOS

INDEX

1. SUMMARY/ RESUMEN

1. SUMMARY

Osteoarthritis (OA) is one of the equine diseases with the greatest economic impact in the industry. Responsible for 60% of lameness, OA is considered one of the main causes of premature abandonment of sports life in horses.

Despite its high economic impact on both direct and indirect costs, few new treatments have been developed for the treatment of equine OA in recent decades.

Non-steroidal anti-inflammatory and / or corticosteroid accompanied by nutritional supplements are the most commonly used conventional treatments in the equine clinic, however, conventional treatments are associated with positive doping in sport horses and can have important medium-long term side effects.

The objective of this work was to demonstrate the safety and efficacy of a new product of advanced therapies based on Equine Umbilical Cord Mesenchymal Stem Cells (EUC-MSC) as treatment of equine OA.

To achieve the objective a superiority, controlled, blind, randomized and multicentre clinical study, has been designed and conduct following the European and Good Clinical Practices guidelines (VICH 9- GCP).

In addition to the main objective, the present work has deep in the knowledge of the EUC-MSC efficacy, for that different epidemiological covariates such as age, chronicity of symptoms, the type of joint affected or the degree of sporting level of the animal has been analysed in order to discover if there were specific equine populations where the EUC-MSC are not effective.

Likewise, a comparative study on the efficacy-safety-price balance of the treatment with EUC-MSC has been made in comparison with conventional treatments in order to discover if EUC-MSC can be considered a real therapeutic alternative.

The present study has demonstrated a robust efficacy of EUC-MSC in the reduction of lameness in horses affected by OA. 72% of the horses treated with EUC-MSC showed a reduction in the level of lameness, presenting non-lameness or an inconsistent lameness, allowing the horses to return to the same level of sport activity than after the injury.

The study of epidemiological covariates found that the efficacy of EUC-MSC is consistent, not being affected by epidemiological factors such as age, chronicity of symptoms or the degree of sports activity.

Finally, it has been established that in a comparative efficacy-safety-price chart, EUC-MSC represents a real alternative to conventional treatments thanks to its long efficacy and few adverse effects.

RESUMEN

La Osteoartrosis (OA) equina es una de las enfermedades equinas de mayor impacto económico en la industria. Responsable del 60% de las cojeras equinas, la OA es considerada una de las principales causas de abandono prematuro de la vida deportiva en équidos.

A pesar de su alto impacto económico tanto en costes directos como indirectos, pocos tratamientos nuevos han sido autorizados para el tratamiento de la OA equina en las últimas décadas.

Los tratamientos farmacológicos especialmente antiinflamatorios no esteroides y/o corticoides acompañados de complementos nutricionales son los tratamientos convencionales más utilizados en la clínica equina, sin embargo, los tratamientos convencionales están asociados a doping en caballos de competición y pueden suponer importantes efectos secundarios a medio-largo plazo.

El objetivo de este trabajo es demostrar la seguridad y eficacia de un nuevo producto de terapias avanzadas basado en células madre mesenquimales de cordón umbilical equino (EUC-MSC) en el tratamiento de la OA equina.

Para conseguir el objetivo un estudio clínico de superioridad, controlado, ciego, aleatorizado y multicéntrico, ha sido diseñado siguiendo las guías y directrices europeas de Buenas Prácticas Clínicas (VICH 9 guidelines).

Adicionalmente al objetivo principal, el presente trabajo ha tratado de estudiar ampliamente la eficacia de las EUC-MSC teniendo en cuenta diferentes covariables epidemiológicas como la edad, la cronicidad de los síntomas, el tipo de articulación afectada o el grado de nivel deportivo del animal; con el fin de descubrir si había poblaciones especificas donde las EUC-MSC no son tan efectivas.

Así mismo, se ha hecho un estudio comparativo sobre el balance eficacia-seguridad-precio del tratamiento con EUC-MSC en comparación con los tratamientos convencionales con el fin de descubrir si efectivamente las EUC-MSC pueden suponer una alternativa terapéutica.

El presente estudio ha demostrado una sólida eficacia de las EUC-MSC en la reducción de la cojera en caballos afectados por OA. El 72% de los caballos tratados con EUC-MSC presentaron una reducción del nivel de cojera, quedando sin cojera o con una cojera inconsistente, permitiendo que los caballos volviesen a su mismo nivel de actividad deportiva posterior a la lesión.

El estudio de covariables epidemiológicas descubrió que la eficacia de las EUC-MSC es consistente no viéndose afectadas por factores epidemiológicos como la edad, la cronicidad de los síntomas o el grado de actividad deportiva.

Por último, se ha establecido que en una comparativa eficacia-seguridad-precio las EUC-MSC representan una alternativa real a los tratamientos convencionales gracias a su larga eficacia y escasos efectos adversos.

2. ABBREVIATIONS

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AAEP: American Association of Equine Practitioners

AEMPS: Agencia Española de Productos Sanitarios

ADAMTS: a disintegrin and metalloproteinase with thrombospondin motifs

AT: Adipose Tissue

BM: Bone Marrow

CD: Cluster of Differentiation

CCL: Chemokine (C-C motif) Ligand

COs: Corticosteroids

COXs: cyclo-oxygenases

CP: Control product

CSP: Chondroitin sulfate

CTX-II: C-telopeptide of type II collagen

EMA: European Medicine Agency

EUC-MSCs: Equine Umbilical Cord derived Mesenchymal stem cells

FAO: Food and Agriculture Organization

FEI: Fédération Equestre Internaciale

GAG: Glicosaminoglycans

HA: Hyaluronic Acid

HLA: Human Leukocyte Antigen

IA: intra-articular

IDO: indoleamine- 2,3-dioxygenase

INF: Interferon

IIAB: Intra-articular Anesthetic Block

IL: Interleukin

ISCT: The International Society for Cellular Therapy

IVP: Investigational Veterinary Product

M: Macrophages

MA: Marketing Authorization

MHC: Mayor Histocompatibility Complex

MLR: Mixed Lymphocytes Reaction

MMP: Metalloproteinases

MoA: Mechanims of Action

MSCs: Mesenchymal stem cells

NO: Nitric Oxygen

NSAIDs : Non-steroidal anti-inflammatory drugs

OA: Osteoarthritis

PBMCs : Peripheral blood mononuclear cells

PGE2: Prostaglandin E2

PGs: Prostaglandins

PHA: Phytohemagglutinin

PP: Population by protocol

PPT: Population to be treated

PSGAG: Polysulphated glycosaminoglycans

SF: Synovial Fluid

Tc: Citotoxic T-cells

TGF: Transforming growth factor

Th: T-helper cells

TIMP: Tissue inhibitor of Metalloproteinases

TNF: Tumor Necrosis Factor

Treg: T-regulatory cell

WJ: Wharton's jelly

3. INTRODUCTION

3. INTRODUCTION

The equine skeletal system is comprised of more than 200 bones that interconnect with the assistance of connective tissues such as tendons, ligaments, and cartilage. Where two or more bones meet it is considered a joint. Joint, is defined in anatomy as a structure that separates two or more adjacent elements of the skeletal system.

There are three basic types of joints in horses: fibrous, cartilaginous and synovial.

a. Fibrous Joints:

In fibrous joints, bones are connected by dense connective tissue fibres (collagen), which pass from one part to the other. Fibrous joints are less common in the equine body; these joints do not allow for movement.

An example of fibrous joints would be those between the bones making up a horse's skull and the articulations between the bodies of the vertebrae that make up the axial skeleton.

Fibrous joints are the least likely to be afflicted with disease because they are more or less immobile.

b. Cartilaginous Joints:

In cartilaginous joints, the interface consists of hyaline or fibrous cartilage. Examples of these joints are the intervertebral disk and the symphysis of the pubic bones in both human and horses.

Cartilaginous joints don't have a high propensity for disease because they have limited movement. These are the joints of the pelvis and vertebrae as well as growth plates, which extend a bone's length during the horse's growing years.

c. Synovial Joints:

The basic structure of all synovial joints is the same, regardless of the type or location. All synovial joints have (Figure 1):

- Two or more bones (ending with a plate of subchondral bone) covered with a thin layer of articular cartilage. The articular cartilage is smooth and resilient and enables frictionless movement of the joint.
- A synovial fluid-filled cavity between the articulating bones, which provides lubrication within the joint itself.
- A synovial membrane.
- A joint capsule that encapsulates the joint and secretes the synovial fluid

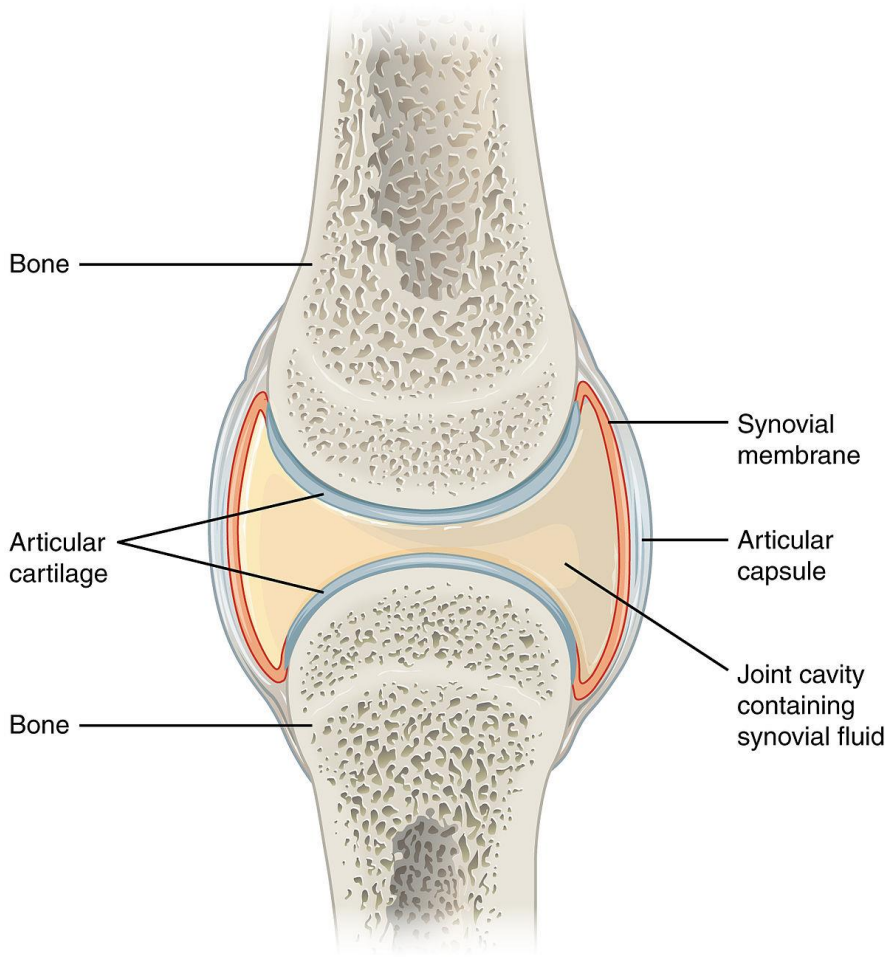

Figure 1: Typical Synovial Joint (Illustration from Anatomy & Physiology, 2013)

Articular cartilage

A synovial joint is more than simply the union of two or more bones; the joint could be considered as an organ.

The highly specialized tissues of the synovial joint come together to perform two main functions: Enable movement and transfer load from one bone to another.

In a normally functioning joint, both of these tasks are achieved in an efficient and pain-free manner. The secret of how this frictionless, painless movement occurs relies on all the joint elements functioning in concert, but requires the involvement of healthy articular cartilage lining bones.

Articular cartilage is an extremely specialised connective tissue capable of withstanding very high loads during physical activity. It is composed largely of water (70- 80%), type II collagen and proteoglycan molecules such as aggrecan and chondrocytes (cartilage cells). On a normal

microscopic section, the articular cartilage appears as a glasslike structure containing cells. The glasslike material outside the cells is called matrix. The matrix is made up of a framework of collagen, and within the framework molecules called proteoglycans are contained, as well as water (Figure 2).

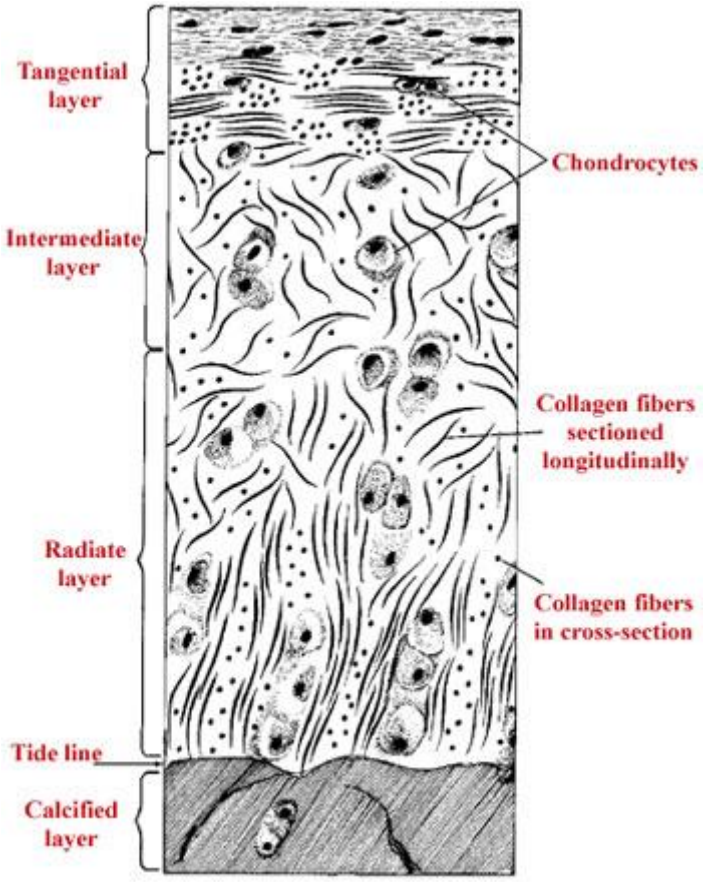

Figure 2: Diagram of adult articular cartilage showing four layers and arrangement of chondrocytes and collagenous fibres (Wayne McIlwraith; Colorado State University)

The chondrocytes are responsible for synthesising, organising, and regulating the extracellular matrix of the articular cartilage. The extracellular matrix is the tissue surrounding the chondrocytes where water, collagen, and proteoglycans are found. The type II collagen forms a fibrillar network within the extracellular matrix, which is responsible for maintaining the shape and strength of the tissue. Also found within the extracellular matrix are large, negatively-charged macromolecules called proteoglycans. These are a mixture of proteins and long chains of sugar that attract large amounts of water, but repel each other. The most common proteoglycan in articular cartilage is the aggrecan—a very large proteoglycan that plays a pivotal role in the function of articular cartilage.

During weight bearing, the aggrecan molecules, which are already very tightly packed together, become further compressed. During this compression, water molecules (that were attracted to the negatively-charged aggrecan molecules) are forced from the extracellular

matrix of the cartilage, and all of the negatively charged branches of the aggrecan molecule repel each other like similar ends of a magnet. That is, the bones are protected by this layer of shock-absorbing articular cartilage, and the load is transmitted from one bone to another.

Synovial Fluid

Joints are lubricated by Synovial Fluid (SF), produced by specialised lining cells, called synoviocytes. The cells produce hyaluronic acid as well as other constituents of synovial fluid, including glycosaminoglycans (GAG). These proteins impart viscosity to the fluid, which is subjectively assessed as part of synovial fluid analysis. A small volume of synovial fluid can normally be extracted from joints in all species (up to 1-2 ml can be extracted from equine joint fluids) and is colourless to light yellow and quite viscous.

In normal, not pathologic, synovial fluid the following characteristic can be found (eClinPath.com):

- Gross appearance: Colourless to light yellow, transparent.
- Nucleated cell counts: This is species-dependent, but counts are usually less than 1,000/uL.
    - Dogs: Counts vary between joints, with higher counts seen in some joints. We generally use 3,000/uL as an upper limit of normal in this species.
    - Cats: One study showed average counts of 161 cells/ul, with a range of 2-1,134/uL in fluids with minimal blood contamination (RBC counts were up to 4,535/uL). We generally use <1,000/uL as the upper limit of normal.
    - Horses: In most horses, counts are <500/uL, but counts up to 1,350/uL have been reported in healthy horses and we commonly see counts of <1,000/uL.
    - Cattle: Similar to horses.
- Red blood cell counts: This should be low (<1,000/uL) unless there is blood contamination or haemorrhage (uncommon) during the SF extraction. In a freshly prepared smear of fluid, erythrophages would support recent haemorrhage into the joint.
- Total protein: This is usually <2.5 g/dL, although fluid from normal horses has a protein as low as 1.5 g/dL.
- Viscosity: A strand of 2 cm should form between two objects. Decreased viscosity is seen with degenerative joint disease, trauma, inflammatory joint disease, hydroarthrosis, hemarthrosis and haemodilution.
- Smear assessment: Normal joint fluid is viscous and of low cellularity. Cells are comprised of 50-90% mononuclear cells, of which 80% or more are macrophages or synovial lining cells with <20% lymphocytes. There are usually <10% neutrophils (non-degenerate). Most macrophages are not “activated” – due to lack of cytoplasmic vacuolation, do not demonstrate phagocytic activity and have eccentric round monocytoid nuclei. Some clinical pathologists use the term “large mononuclear

cells" and "small mononuclear cells" demonstrating the difficulty of distinguishing macrophages from synoviocytes and quiescent (non-activated) synoviocytes or macrophages from small lymphocytes. If there are sufficient cells (rarely in a normal joint fluid) or mild blood contamination, the cells will line up in streams in the smear (called "windrowing"), implying normal or retention of viscosity.

In synovial fluids from horses with Osteoarthrosis (OA), in general, the changes observed in synovial fluid from injured or diseased joints are brought about by alterations in the permeability of the synovial membrane and impairment of its normal secretory functions. Because of the importance of the synovial membrane in maintaining the proper fluid composition, the physiological function of the fluid and membrane are inextricably linked. In fact, synovial fluid analyses have been postulated as an early disease indicator in OA (Ma *et al.,* 2017; de Grauw *et al*., 2006).

Reductions in relative viscosity in the SF can occur by two separate mechanisms; simple effusion or synovitis. Synovial effusion often results from direct trauma to the joint and the resulting reduction in viscosity arises from the dilution of the synovial fluid by the influx of plasma into the joint space. In inflammatory conditions (synovitis), viscosity can be further reduced through decreased synthesis of hyaluronic acid as well as incomplete polymerization (shorter chain lengths). When both simple effusion and synovitis occur simultaneously the viscosity of the synovial fluid may be little more than that of water. Since hyaluronic acid is responsible for the viscosity of synovial fluid, its concentration is similarly reduced by synovitis and effusion.

Normally the protein concentration of synovial fluid is considerably less than that of the serum. Thus, any injury or condition which causes vascular leakage in the synovial membrane will be accompanied by an influx of fluid and protein into the joint space and surrounding tissue producing oedema.

Minor elevations in the leukocyte content occur in many common joint disorders and reflect the degree of the inflammatory response. Marked increase in white cells is highly suggestive of an infectious aetiology. Although cartilage fragments are not truly a property of synovial fluid, their presence in the fluid is indicative of degeneration and erosion of the articular surfaces. Cartilage fragments occur in the synovial fluid as a result of mechanical wear of the articular surfaces and with damage or disease the rate and extent of articular degeneration can dramatically increase. Minor cartilage erosion appears to be a normal consequence of aging and articular fragments are common in the synovial fluid of the elderly. Large amounts of cartilage debris are, however, indicative of significant lesions or deterioration of the articular surfaces. Erosion of the articular cartilage is an important factor in the overall pathogenesis of many degenerative joint conditions and may contribute significantly to mechanical instability of affected joints (Tew *et al.,* 1981).

3.1. Osteoarthritis

3.1.1. Physiopathogenesis

One of the most prominent OA researchers agreed on the following definition of OA: *"Osteoarthritis can be described as the failed repair of damage that has been caused by excessive mechanical stress (defined as force/unit area) on joint tissues*" (Brandt et al., 2009).

Osteoarthritis (OA) is a painful, chronic, debilitating joint disease with no known cure in horses. It is characterized by heat, pain, swelling, crepitus, and a decreased range of motion in affected joints.

This condition can develop suddenly (e.g., secondary to a traumatic injury to the joint), or it can develop slowly over the course of months to years. Trauma to the joint, immobilization of the joint, poor conformation, improper shoeing and age are often preliminary factors that contribute to the onset of OA in the horse (Schlueter & Orth, 2004).

In the horse, synovitis is also regarded as an important primary, or at least concomitant, event. Irrespective of whether there are only single or multiple primary factors, there is general consensus that after this primary event a vicious cycle may ensue that comprises both inflammatory and degradative components. Synovial inflammation is an important component of OA, contributing to the dysregulation of chondrocyte catabolic and anabolic activities (Van Werren & de Grauw, 2010)

OA is characterized by progressive loss of articular cartilage and the existence of an inflammatory environment and the presence of matrix degrading and inflammatory cytokines. It usually has a chronic character and affects all joint structures as bursa, synovial fluid, cartilage and subchondral bone.

It is thought to start as result of damage to the joint tissue by physical forces as a single event of trauma or by repeated microtrauma due to altered mechanical loading of the joint. Synoviocytes and Chondrocytes responds to the physical injury by stopping the production of anabolic factors and by releasing more catabolic enzymes such as metalloproteinases (MMPs), Interleukin 1 (IL-1) and Tumor Necrosis Factor α (TNF-α) which results in further damage to the cartilage (Souza. 2016)

As shown in the Figure 3, during the development of OA the immune response play an important role in the evolution and degeneration of the disease (Haseeb *et al.,* 2013; Lange-Brokaar *et al.,* 2012; Manferdini *et al.,* 2013; Ma *et al.,* 2017):

1. On one hand, the Synovial Fluid and Synovial membrane have an important infiltrate of immune cells including macrophages (65%), T-cells (22%) and B-cells (5%).

2. On the other hand, multiple proinflammatory soluble factors such as cytokines ((IL1, IL-6, TNF-α), chemokines (CS846, GAG, HA and CTX-II) and metalloproteinases (MMP-

9, MMP-13, ADAMTS-5,) are released by different cell types not limited to immune cell, but also synoviocytes and chondrocytes. This secretion forms inflamed synovium and develops the degradation of the cartilage matrix that results in further progression of OA symptoms. Among the cytokines, IL-1β, TNF-α and IL-6 are three main pro-inflammatory responsible for the shift of cartilage homeostasis towards more catabolism and degradation of cartilage. In addition, TNF- α, MMP-3 and MMP-2 have been related to be the most important inflammatory reagents related to lameness grade progression in horses (Ma *et al.*, 2017).

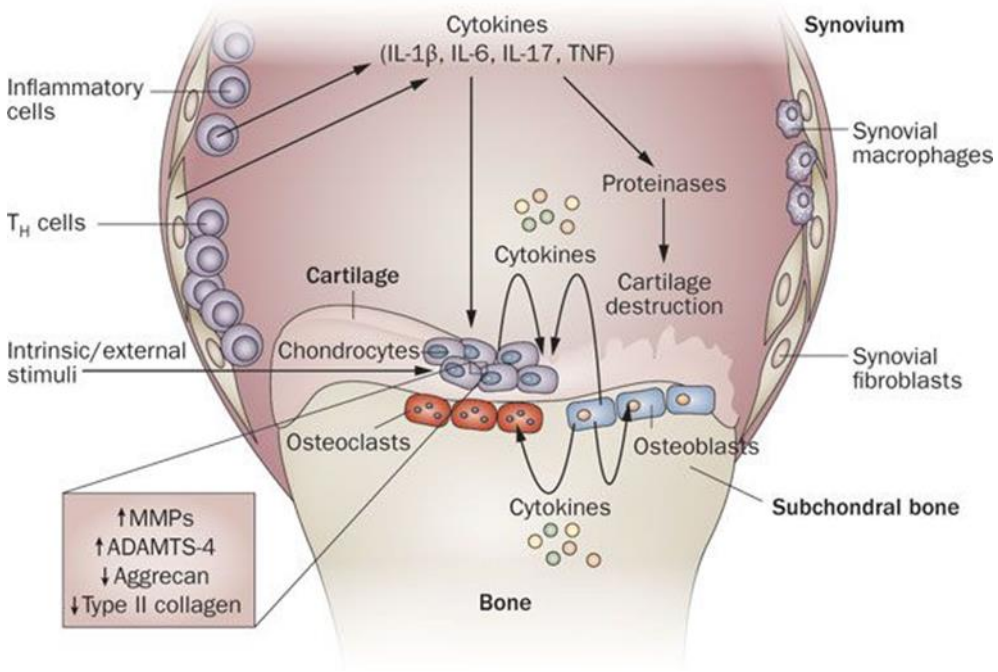

Figure 3: Physiopathogenesis of OA Kapoor *et al.,* 2011

TNF-α is the prime mediator of the acute inflammatory response and promotes the release of IL-1. MMPs are enzymes responsible for the physiological remodeling of the cartilage, but when they are increased, thanks to the upregulated by IL-1 and TNF, they alter the normal regulation of cartilage matrix causing their destruction.

In response to the chronic and progressive loss of cartilage, subchondral bone responds by stimulating the osteosynthesis which causes the formation of new bone and osteophytes (bone spurs) in bone articular surface and general articular incongruity, which triggers chronic joint changes (Figure 4).

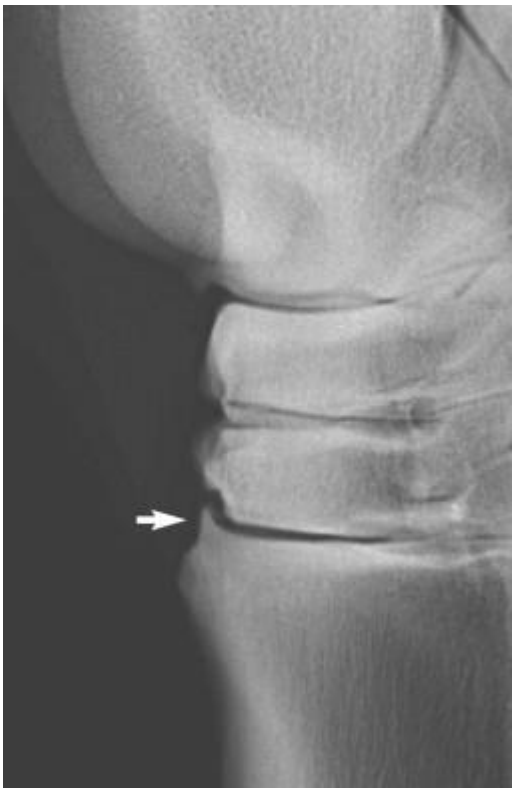

Figure 4: Example of Osteophyte in equine tarsal joint

Because of all these mechanisms, joints affected by OA have a chronic inflammatory environment, chronic cartilage destruction, loss of joint congruence and clinical sings as pain, heat, lameness and loss of sports capabilities.

3.1.2. Economic Impact of OA

OA is a common disorder in horses with a prevalence as high as 80% in both young and old animals (Souza. 2016). OA is considered the most common joint disease in horses, responsible of 60% of lameness in horses (Kim *et al.,* 2003). The most common causes of poor performance and early retirement of equine athletes are joint pain and loss of mobility due to osteoarthritis (Todhunter and Lust, 1992).

Osteoarthritis is expensive to manage per horse to diagnose, treat, and medicate, in addition the value of a horse affected by osteoarthritis also decreases substantially (McIlwraith, 2010).

A review of the direct and indirect cost associated to OA is described in the table below.

Lameness examination	75-500€ (depending on extent of examination, blocking, etc.)
Radiologic (per joint)	120-150€
Conventional treatments (Corticosteroid + hyaluronic acid)	200-300€ (approx. 800€/year considering the time of effectiveness of the treatment)
Physical therapy and related techniques (aqua treadmill, massage, electrical therapies, cold therapies, etc.	100€ (session)
Oral Joint health supplements	3€/day 1095€/year

Table 1: Estimation of direct cost of OA diagnosis and treatment in Europe (based in USA estimations publish by Oke. (2009)

In addition to direct cost, the indirect costs (lost sports days, decrease in the economic value of the horse, expenses associated with the maintenance and care of the injured horse, etc.) of OA are very high in equine practices. It has been estimated that the direct costs of OA in humans only represents the ~30% of the total impact of the OA, so to the direct cost an increase of 250% should be added as indirect costs (Coyte & Chan. 1998).

However, it has been suggest that while indirect costs are rarely considered in veterinary medicine, the estimates of indirect costs associated with equine OA projected from human research are likely substantially higher than they are in reality (Oke. 2009)

Per year, the direct medical costs could amount to approximately 2.500€. If one considers indirect expenses, the cost of this horse could be substantially higher—perhaps as high as 12,000€/year (Oke &Mcllwraith. (2010)).

Bearing in mind that there are 5.7 million horses in Europe (Food and Agriculture Organization (FAO) 2009) and considering that 60% of lameness in horses is caused by OA (Oke & Mcllwraith, 2010) and that 46% of active horses are lame (Greve & Dyson, 2014), it can be estimated that in Europe there are a total of 1.57 million lame horses due to the OA.

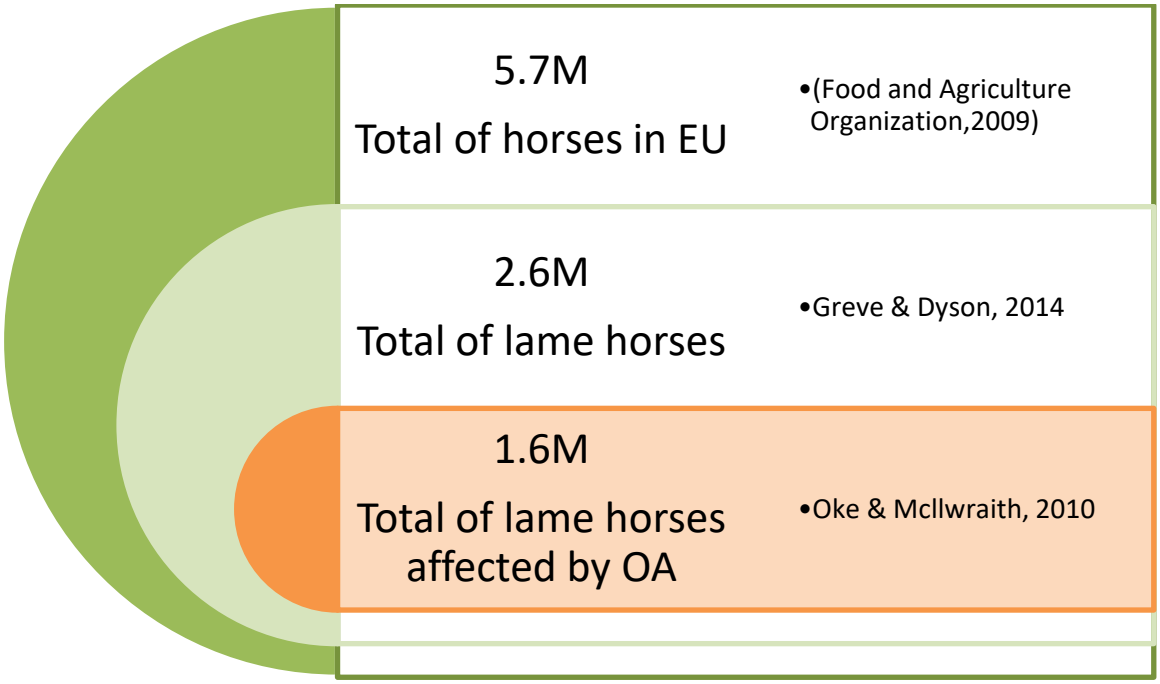

Figure 5: Number of horses lame due OA in Europe

With a total cost of € 12,000 per year and per horse and 1.6 million lame horses due to the OA in Europe, the total economic impact of the OA in the European equine sector can amount to 18,000 million Euros per year.

3.1.3. Treatment

Conventional treatments for OA in horses are mainly focused on relieving this inflammation and controlling pain (Barrachina *et al.,* 2017). The classic treatment of OA in horses has been basically symptomatic by inhibiting the synthesis of eicosanoids through the use of non-steroidal anti-inflammatory drugs (NSAIDs) or intra-articular corticosteroids (COs). Currently, the use of hyaluronic acid (HA), polysulphated glycosaminoglycans (PSGAG) and pentosanpolysulfate have great acceptance in equine clinical practice (Goodrich and Nixon 2006). These substances *have* no anti-inflammatory effect mediated by blocking the eicosanoid cascade. However, they promote the metabolism of articular cartilage and reduce synovial effusion. Generally, these substances are not used as a single treatment. Many clinicians combine them with intra-articular COs. The drugs used in OA can be classified into three general groups:

a) Nonsteroidal anti-inflammatory drugs (NSAIDs) are used in equine OA because they reduce pain and synovial effusion. Its action is symptomatic, although some molecules can be protective of the articular cartilage. NSAIDs belong to different groups that have no chemical relationship with each other, but have a common mechanism of

action, the inhibition of the synthesis of inflammatory prostaglandins (PGs) by blocking the enzymes cyclo-oxygenase (COXs). The most commonly used NSAID in equine practices is phenilbutazone (Mcllwraith,2004). Due to the pharmacokinetic characteristics of the NSAIDs, they must be administered daily to maintain their therapeutic effect (Foreman & Roummler, 2011) so the treatment of equine OA with NSAIDs requires daily treatment of the animal, which is a disadvantage for the owner. Likewise, the use of long-term NSAIDs is associated with adverse effects on the equine digestive system, especially gastric ulcers, and / or kidney problems. It has been suggested that long-term use of nonsteroidal anti-inflammatory drugs might enhance the pathologic process of cartilage degeneration (Van Weeren & de Grauw, 2010). We should not forget that the use of NSAIDs in horses is considered doping, so no horse for sporting purposes could be treated continuously with NSAIDs and continue with their sporting career (Knych, 2017).

b) Corticosteroids (COs) are the most used currently in equine practices for OA treatment. COs act on the metabolism of arachidonic acid by relieving the inflammation that causes swelling, heat and pain (van Weeren and de Grauw 2010, McIIwraith, 2010). The anti-inflammatory effect occurs quickly and effectively, with a consequent decrease in lameness. However, the used of COs is associated with important disadvantages because of their adverse results in the metabolism of chondrocytes by inhibiting the synthesis of proteoglycans and changing the structure of collagen networks (Souza *et al.,* 2016). Moreover, clinical and experimental results are also contradictory, although studies have indicated that these drugs, when administered at low doses are effective and safe, COs should be used carefully with respect to dosage and the frequency and extent of application (Goodrich and Nixon 2006). Because they are immunosuppressive drugs, inhibit proteoglycan synthesis, alter collagen structure, and suppress the biosynthetic activities of many cell types, including chondrocytes (Souza *et al.,* 2016) COs could negatively affect the cartilage homeostasis. In fact, studies have shown that negative impact of COs on the matrix occur if high doses are used, when the treatment is repeated continuously, or when the drugs are used in healthy joints (Souza *et al.*, 2016). On the other hand, Frean *et al.,* (2002) reported adverse responses even when low doses were administered. In addition to the local adverse events described before, the use of COs has been also associated to systemic adverse event as laminitis, endotoxemia and gastrointestinal tract disorders (Johnson PJ, 2012). In addition, COs are also considered doping and in the case that COs are used in sport horses a withdrawal period of about 15 days (Federation Equestre Internationale guidelines) is needed.

c) Modifying drugs for osteoarthritis such as Hyaluronic acid (HA) which is an unsulphated glycosaminoglycan. It is produced by synoviocytes and is responsible for promoting joint lubrication. The HA can modify the course of the OA for two reasons: 1) Production of modulating effects of the biological response mediated by specific

receptors of the cellular membrane of leukocytes and articular cells. 2) Mechanical interference of the interaction between catabolic proteins and their cellular receptors. HA produces inhibition of white cell migration, traps free radicals and possesses chondroprotective actions, since it promotes the synthesis of proteoglycans. Polysulphated glycosaminoglycans (PSGAGs) are sulphate-rich polysaccharides produced by chondrocytes and make up the extracellular matrix of articular cartilage. PSGAGs can be used intramuscularly or intra-articular to treat cases of severe joint injury. The mechanism of action of PSGAGs has not been fully elucidated. *In vitro* studies show that PSGAGs do not affect the production of PGE2, but they can reduce NO production and the expression of inducible NO synthetase. They can also decrease the expression of MMP-1 and promote the synthesis of the central protein of aggrecan and procollagen type II. Chondroitin sulphate (CSP) and glucosamine are two oral glycosaminoglycans used in the treatment of equine OA. CSP is one of the main components of the extra cellular membrane of articular cartilage, on the other hand Glucosamine is a hexosamine precursor of the disaccharide unit of CSP and HA. Exogenous administration of these two molecules can prevent NO production, release proteoglycans and inhibit collagenase and gelatinase activity. Although controlled research has not been conducted on horses with natural disease, well-controlled clinical studies in humans with OA have been conducted.

Considering OA has a multifactorial origin, where certain factors such as age, the conformation and mobility of the joint can influence the onset of the OA, the repercussion of these epidemiological factors can also have a significant effect on the effectiveness of the different products.

Although the impact of epidemiological cofactors on the efficacy of OA treatments seems to be a justified hypothesis, few comparative studies have been conducted with conventional treatments, in order to correlate the efficacy of treatments with equine co-variables (age, lameness grade, joint, etc.)

An ideal therapeutic approach should stop progressive loss of cartilage and stimulate the regeneration of damaged structures without (or with minor) adverse events.

Treatments for equine joint diseases based on the intra-articular (IA) administration of mesenchymal stem cells (MSCs) are gaining importance because of their regenerative role.

MSCs show significant potential for cartilage repair, which is attributed to their trophic and differentiation properties, as well as their immunoregulatory ability (Barrachina *et al.,* 2017).

3.2. Mesenchymal Stem Cells

Mesenchymal stem cells are multipotent stromal cells that can differentiate into a variety of cell types, including osteoblasts (bone cells), chondrocytes (cartilage cells), myocytes (muscle cells) and adipocytes (fat cells).

The International Society for Cellular Therapy (ISCT) proposes a set of standards to define MSC (Dominici *et al.,* 2006)

1. Plastic Adherence: MSCs must be plastic-adherent when maintained in standard culture conditions using tissue culture flasks. (figure 6)

Figure 6: Equine Umbilical Cord Mesenchymal Stem Cell (EUC-MSC)

2. Specific surface antigen (Ag) expression: according ISCT at least 95% of human-MSC population must express CD105, CD73 and CD90, as measured by flow cytometry. Additionally, these cells must lack expression (5/2% positive) of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II. However, for MSCs derived from origins other than human, it is difficult to comply with the standards of the ISCT since some of the antigens cited above do not have equine cross-reactivity. In addition, there is a real lack of reagent specifically designed for equines in the biotechnological industry. For equine MSCs the surface antigen expression usually is limited to: CD45 (negative), MHC-II (negative), 79α (negative), CD90 (positive), CD44 (positive).

3. Multipotent differentiation potential: the cells must be able to differentiate to osteoblasts, adipocytes and chondroblasts under standard *in vitro* differentiating conditions (Figure 5).

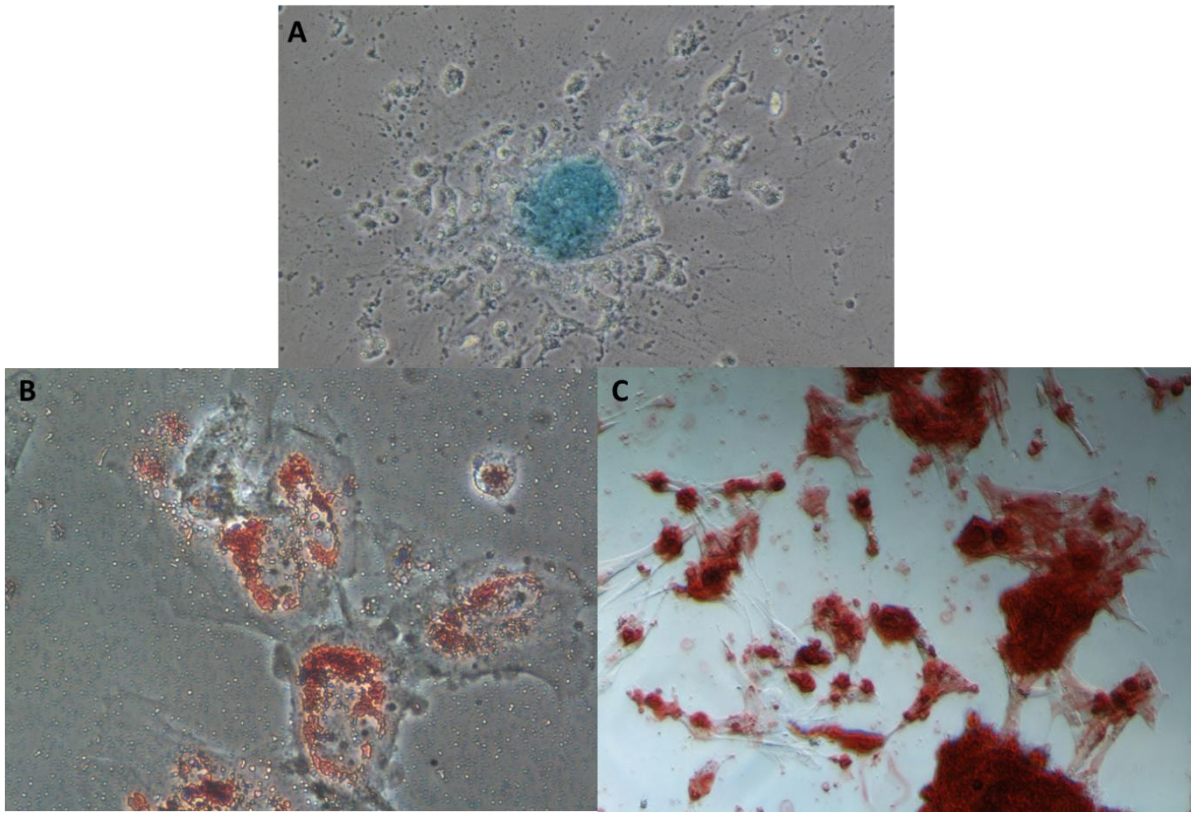

Figure 7: Equine Umbilical Cord Mesenchymal Stem Cells differentiated. A) Chondrogenic differentiation. B) Adipogenic differentiation. C) Osteogenic differentiation.

Mesenchymal stem cells (MSCs) have been a focus in recent research because they are a potential tool in cellular therapies for several clinical applications. These cells can differentiate into mesenchymal lineages and secrete cytokines and growth factors with effects that favour the regeneration of damaged tissues. In addition, MSCs possess an immunoregulatory capacity that allows these cells to be used in the treatment of diseases with an important immune factor (Manferdini *et al.*, 2013, Castro-Manrreza *et al.,* 2015).

MSCs can be isolated from different sources, most common being bone marrow (BM) and adipose tissue (AT). However, in the recent years umbilical sources are growing in popularity.

Umbilical cord (UC) contains two umbilical arteries and one umbilical vein, both embedded within a specific mucous connective tissue, known as Wharton's jelly (WJ), which is covered by amniotic epithelium. The isolation of fibroblast-like cells from WJ of human UC was reported by the first time by McElreavey *et al.,* in 1991. (Nagamura-Inoue *et al.,* 2014)

Umbilical Cord source presents important advantages that made them a very interesting source for advance treatments. The main advantages of umbilical cord are:

i. **Non-invasive extraction:** The extraction of MSCs from the umbilical cord (both tissue and blood) occurs once the animal has been born and without interfering in the birth process, therefore it is considered a non-invasive process. This MSCs source avoids interfering with the animal and subjecting it to a painful procedure. Additionally, it avoids risks associated with the BM or AT extraction processes such as sedation, wound healing, etc. The non-invasive extraction of cells is an advantage in relation to animal welfare. (Nagamura-Inoue *et al.,* 2014)

ii. **Isolation efficiency:** The amount of mesenchymal stem cells, which can be obtained from bone marrow, is very limiting. Only 0.001 to 0.01% of mononuclear cells were reported, while 1 g of adipose tissue yields approximately 5×10^3 stem cells, which is 500-fold greater than in the bone marrow. The isolation efficiency from Wharton's jelly (WJ) is high and ranges from 1 to 5×10^4 cells/cm of umbilical cord. Side-by-side comparison of MSC from bone marrow adipose tissue and Wharton's jelly demonstrated that WJ-MSC have highest proliferative capacity among tested cell types (Kalaszcynska *et al.,* 2015).

iii. **Immunoprivileged status**. The ability to modulate immunological responses ranks umbilical cord MSCs as an important compatible stem cell type for therapeutic applications in allogeneic setting. The mechanisms of immunoprivilege are still investigated; however, low MHC-I level and absence of MHC-II expression protect them from NK-mediated lysis. Despite the fact that they synthesize, though low, amounts of MHC class I, umbilical cord MSCs do not demonstrate immunogenicity. It can be attributed to the lack of co-stimulatory molecules-CD 40, CD80, CD86 expression, and high levels of inhibitors of immune response: indoleamine- 2,3-dioxygenase (IDO) and prostaglandin E2 (PGE2). Of particular importance is the fact that umbilical cord tissue MSCs express high levels of leukocyte antigen G6 (HLA-G6), the same which is produced by trophoblast and protects the embryo from immune-based destruction (Kalaszcynska *et al.,* 2015). In addition, recent studies in horses demonstrate that in MSCs from conventional sources (Bone Marrow) exist a great heterogeneity in the expression of MHC-II surface markers (range 0–98% positive) (Schnabel, 2013). In addition, a study from the same author (Schanabel *et al.,* 2014) showed that the incidence of positiveness of MHC-II in bone marrow samples is about 85%. However in our experience in more than 30 donors of Umbilical Cord MSC that has been characterized, all of them were strongly negative to MHC-II (<1%).

iv. **Low population doubling:** Thanks to working with a tissue as rich in stem cells as the umbilical cord and the large anatomical size of an equine umbilical cord (~1kg), the number of stem cells obtained from a single umbilical cord is extremely greater than the one obtained from other sources. This greater number of cells in pass zero (P0) allows the expansion of the cells efficiently without the need to increase the cellular

duplications in excess. It is well known that as the cells are duplicated in culture, they lose differentiation capacity, immunomodulation and therapeutic actions (Crisostomo et al., 2006).

v. **Safety**: Umbilical Cord MSCs are considered to be safer than other source due different reasons. On the one hand, being a tissue collected at the time of birth, the donor animal has not been exposed to the environment or infectious diseases, so the risk of infectious disease is much lower, being applicable only those of vertical transmission (Nagamura-Inoue *et al.,* 2014). On the other hand, the cells are not exposed to the age or the environment of the donor, so the risk of genetic changes in umbilical cord cells are minor than the one of adult stem cells.

3.2.1. Mesenchymal Stem Cell Mechanism of Action:

After the discovery of the MSCs it was thought that their therapeutic activity was mainly due to their capacity for differentiation and on the functional integration after transplantation (Madrigal *et al.,* 2014). However, in recent years it has been discovered that this capacity is certainly very limited.

Currently it is considered that the main mechanism of action of MSCs is through paracrine actions both cell to cell and through the secretion of cytokines.

MSCs engaged in a pro-inflammatory environment exert an anti-inflammatory and chondroprotective effect. (Manderfini *et al.,* 2013; Manderfini *et al.*, 2015; Saulnier *et al.*, 2004).

Such anti-inflammatory capacity of MSCs is not only depended on the cell-to-cell contact, but also on their secreted paracrine factors (Najar *et al.*, 2010). Many *in vitro* studies have shown their complex and wide range of anti-inflammatory/immunodulatory paracrine effects on adaptive immune system, including T and B-cells, dendritic cells and natural killers (Fontaine *et al.,* 2016, Carrade *et al.,* ,2014).

It has been postulated by the scientific community that the MSCs capacity of PGE2 secretion is a key factor in MSC immunoregulatory function. (Chen *et al.*, 2010; Najar *et al.,* 2010; Solchaga *et al.,* 2012; Carrade *et al.,* 2014; Fontaine *et al.,* 2016). Therefore, the MSCs ability to secrete PGE2 turns into an important cellular aspect for MSC Mechanism of Action MoA.

MSCs are very potent immunomodulatory and anti-inflammatory agents. In fact, many publications have reported the suppression of alloantigen proliferation in Mixed Lymphocytes Reaction (MLR) when MSCs isolated from human and other mammalian species (including baboon, canine, caprine, equine and rodents) are co-cultivated/stimulated with active peripheral blood mononuclear cells (PBMCs), especially T-cells. This suppression is confirmed to be PGE2-mediated since the PBMC proliferation is restored after the PGE2 production

blockage (Nicola *et al.,* 2002; Aggarwal *et al.,* 2005; Chen *et al.,* 2010; Carrade *et al.,* 2012;, Solchaga *et al.*, 2012 Carrade *et al.,* 2014; Auletta *et al.*, 2015; Ayalla-Cuellar *et al.,* 2017).

This inhibition capacity of PBMCs proliferation has been tested in the EUC-MSCs used in the present study.

Although the purpose of this work is not to deepen in the pharmaceutical development that involves transforming cells extracted from umbilical cord in a medicine fulfilling all the European quality requirements (Good Manufacturing Practices, European Pharmacopoeia, European Guidelines, etc.) different *in vitro* studies have been developed by our group, to discover the mechanism of action of EUC-MSCs.

During the pharmaceutical development, special importance was given to the research on the mechanism of action (MoA) of the EUC-MSC.

As part of this investigation, the EUC-MSCs were stimulated with synovial fluid extracted from horses with OA and associated clinical symptoms (lameness). Due to the stimulation with the inflammatory environment of the synovial fluid the secretion capacity of PGE_2 by EUC-MSCs was increased 23-fold compared with the capacity of secretion without stimulation with synovial fluid (Figure 8).

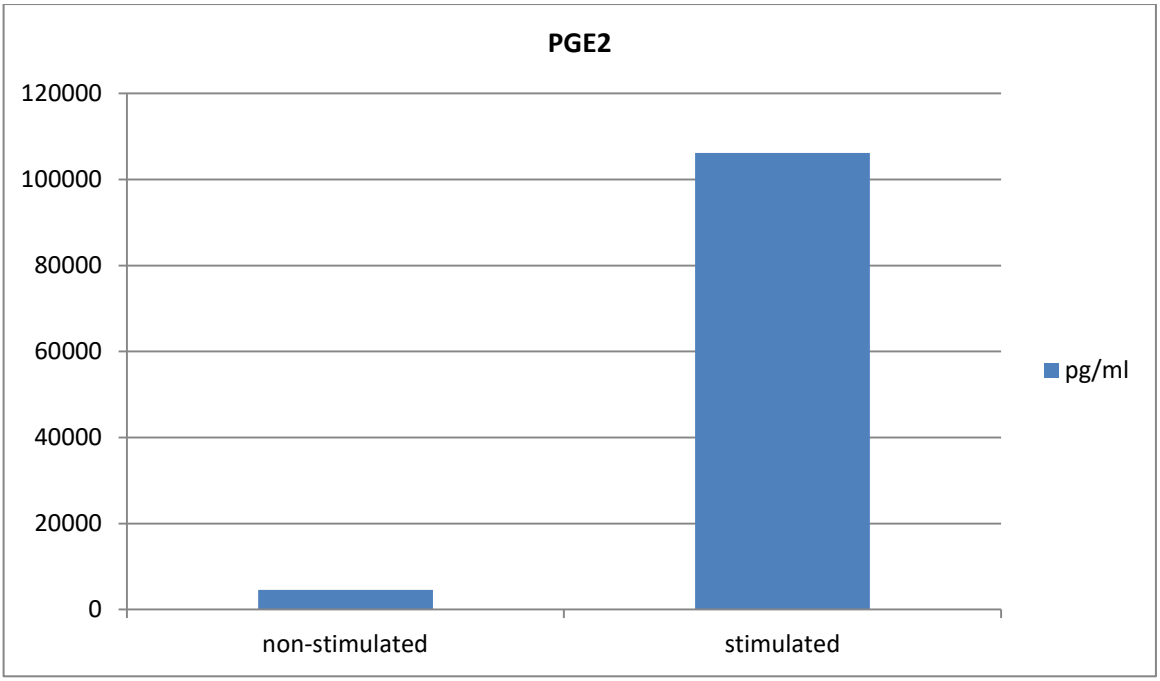

Figure 8: PGE2 secretion by EUC-MSC with or without stimulation with synovial fluid from OA lame horses

In addition Equine Umbilical Cord Mesenchymal Stem Cells used in this work have also demonstrated the PBMCs inhibition in a MLR assay developed where the PGE2 was

demonstrated to be one of the main responsible of this action (Figure 9). In this *in vitro* assay EUC-MSCs were co-cultured with PBMCs previously activated with Phytohemagglutinin (PHA) for 6 days. It was observed that EUC-MSCs were able to inhibit the PBMCs proliferation and that the inhibition was mediated by PGE2, since this could be restored in PGE2 secretion was blocked by indomethacin (INDO).

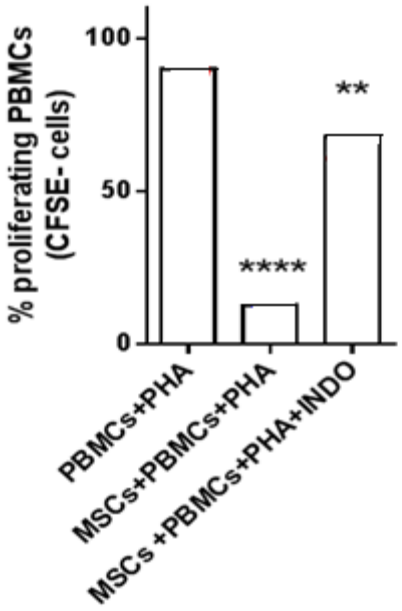

Figure 9: MRL: Activated equine PBMCs are suppressed by EUC-MSC

Therefore PGE_2 has been postulated as one of the main cytokines responsible of the paracrine actions of the EUC-MSC and highlight as an excellent marker of efficacy.

3.2.2. Prostaglandine E2:

Prostaglandins are lipid autacoids derived from arachidonic acid. They both sustain homeostatic functions and mediate pathogenic mechanisms, including the inflammatory response. They are generated from arachidonate by the action of cyclooxygenase (COX) isoenzymes and their biosynthesis is blocked by nonsteroidal anti-inflammatory drugs (NSAIDs), including those selective for inhibition of COX-2.

PGE2 is one of the most abundant PGs produced in the body, is most widely characterized in animal species, and exhibits versatile biological activities. Under physiological conditions, PGE2 is an important mediator of many biological functions, such as regulation of immune responses, blood pressure, gastrointestinal integrity, and fertility. Dysregulated PGE2

synthesis or degradation has been associated with a wide range of pathological conditions. In inflammation, PGE2 is of particular interest because it is involved in all processes leading to the classic signs of inflammation: redness, swelling and pain. Redness and oedema result from increased blood flow into the inflamed tissue through PGE2-mediated augmentation of arterial dilatation and increased microvascular permeability.

Generally, this soluble factor is recognized as a mediator of active inflammation; however, the interest in its immunosuppressive ability is growing since it has been widely demonstrated to suppress both innate and antigen-specific immunity at multiple molecular and cellular levels, *"earning PGE2 the paradoxical status of a pro-inflammatory factor with immunosuppressive activity*" (Kalinski, 2012).

In fact, multiple publications support that PGE2 plays a critical role in the anti-inflammatory effect of MSCs on PBMC modulation (Siegel *et al.*, 2009; Krampera, 2011; Bao *et al.*, 2011; Yanez *et al.,* 2010; Manderfini *et al.,* 2013; Manderfini *et al.*, 2015).

Effect of PGE2 in cells:

PGE2 affects many of the cells present in the development of OA. The effect of PGE2 in the different cell types present in the OA infiltrates have been summarized below:

I. Macrophages (M): these cells are classified in two groups: M1 and M2. M1 are activated by pro-inflammatory Th1 cytokines, as IL-1, IL-12, INF-γ and TNF-α, and M2 produce anti-inflammatory cytokines, such as TGF-β and IL-10. The main form of macrophages in pro-inflammatory environment is the M1 one.

   Numerous studies have demonstrated that MSCs lead the macrophage phenotype towards M2, which involves the decrease of TNF-α, IL-1, IL-6 and IL-12 production and the enhancement of IL-10 (anti-inflammatory). In fact, this ability of MSCs to reprogram the cells has been proven to be mediated not only by high level of PGE2 secreted by active MSCs, but also by constitutive PGE2 level of non-active MSCs. (Kim *et al.*,2009; Maggini *et al.*, 2010 and Fontaine *et al.,* 2015)

II. T-cells:

   - T-helper cells (Th): PGE2 at high doses inhibits IL-2 release (pro-inflammatory) and IL-2 responsiveness in T-cells, non-specifically suppressing T-cell activation and proliferation. In contrast at much lower PGE2 concentrations polarize CD4+ T-cells from aggressive Th1 cells (promoting the inflammatory / cytotoxic form of immunity) towards Th2 and Th17, which are less destructive. This effect is regulated by suppressing production of Th1 cytokine IFN-γ and IL-12 (responsible of the pro-inflammatory M1 macrophages) and by promoting the

production of IL-4 and IL-5 (Aggarwal *et al.,* 2005; Kalinski, 2013; Ayala-Cuellar *et al.*, 2017 and Dutton *et al.,* 2018).

- Citotoxic T-cells (Tc): PGE2 inhibits Tc activity and suppresses their ability to interact with their targets (Kalinski, 2013).
- T-regulatory cell (Treg): multiple studies have demonstrated that PGE2 enhances the differentiation of Treg with suppressive activity, like Foxp3+ Treg. In fact, the content of Foxp3+ Treg was elevated in MLR with a significantly MSC mediated T-cell suppression and an increment of PGE2 level (Auletta *et al.,*, 2015; Ayala-Cuellar *et al.*, 2017; Kalinski, 2013)

III. B-cell: PGE2 interferes with the early phase of B-cell activation regulating the process of Ig class switch in activated B cells and suppressing the cytokine and antibody production (Kalinsky, 2013). In fact, the co-culture of B-cells with MSCs results in a decrease of B-cell proliferation, differentiation to IgM, IgG and IgA-producing cells and expression of CXCR4, CXCR5 and CCR7 (major chemokines involved in the B-cell homing) (Cocione *et al.,* 2006 and Traggiai *et al.,* 2008).

Moreover, apart from immunosuppression of adaptive immune system, MSCs in co-culture with inflamed human synoviocytes and chondrocytes have shown decrease in the expression of IL-1β, IL-6 and IL-8 in both cellular lineages. In addition, it was also observed that significant down-modulation of other chemokines and metalloproteinases promote the progression of the disease (CXCL1, CCL2, CCL3, CCL5 ADAMTS4, ADAMTS5 and MMP13), as well as, the up-regulation of TIMP1, tissue inhibitor of metalloproteinases.

In particular, the role of COX-2 and PGE2 has been demonstrated since the expression of COX-2 (inducible enzyme involved in the PGE2 synthesis) is down-regulated in inflamed chondrocyte and synoviocyte monocultures that produced high levels of pro-inflammatory factors. Likewise, PGE2 released in co-cultures with MSCs have been shown higher than in monocultures.

Once again, these data show the key role of PGE2 in the immunesuppressive properties of MSCs, in particular on inflamed chondrocytes or synoviocytes, since the COX-2 inhibition in them was related to PGE2 increase after MSC co-incubation (Manderfini *et al.*, 2013, Manderfini *et al.*, 2015).

The relation between PGE2 and immune cells, chondrocytes and synoviocytes is summarised in Figure 10.

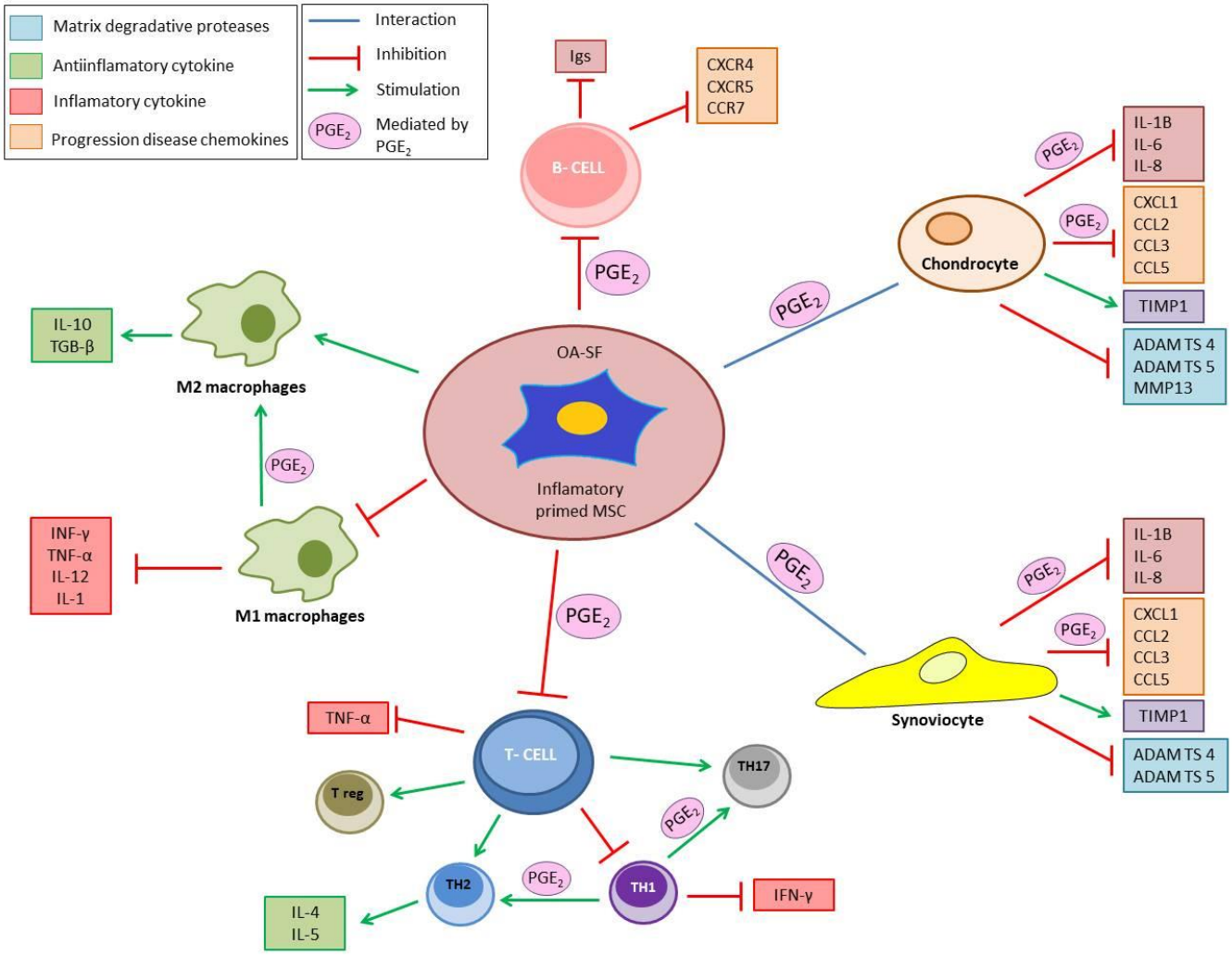

Figure 10: PGE2 Effect of PGE2 secreted by MSC in immune cell, chondrocytes and synoviocytes

3.2.3. Rational use of MSC in equine OA:

OA physiopatogenesis and EUC-MSC MoA has been summarised in Figure 11, that briefly can be resumed as:

Age, genetic factors, or microtraumas cause damage to the equine joints. This repeated damage generates an inflammatory response in the joint and immune cells (specially Tcell and Macrophages) infiltrate the joint. In addition, synovicytes of the synovial membrane are activated as a reaction to the damage and the immune infiltrate.

Both, immune cells and synoviocytes, react secreting inflammatory cytokines such us TNF-α, IL-1β and IL-6.

These inflammatory cytokines (mainly TNF-α and IL-1β) alter the homeostasis of chondrocytes provoking the degradation of the extracellular matrix. This degradation provokes the release

of matrix degradatory proteases, mainly metalloproteases (MMP) that increase the inflammatory environment of the synovial fluid.

Therefore, the OA could be considered as a circle of negative feedback where each one of those involved (immune cells, synoviocytes and chondrocytes) respond to the damage by enhancing the negative effects that favour the evolution of the disease, the inflammatory environment and joint degeneration.

This inflammatory environment leads to clinical sings in the horse, mainly lameness grade, flexion pain, and sometimes joint effusion.

An adequate strategy for OA treatment is to work in the two key points involved in the disease and its progression: the inflammatory infiltrate and the inflammatory cytokines and degradative proteases release to the joint environment. Products intended to treat OA should be related to the intended therapeutic effect: Reduction of infiltrated cells & reduction of inflammatory cytokines/chemokines.

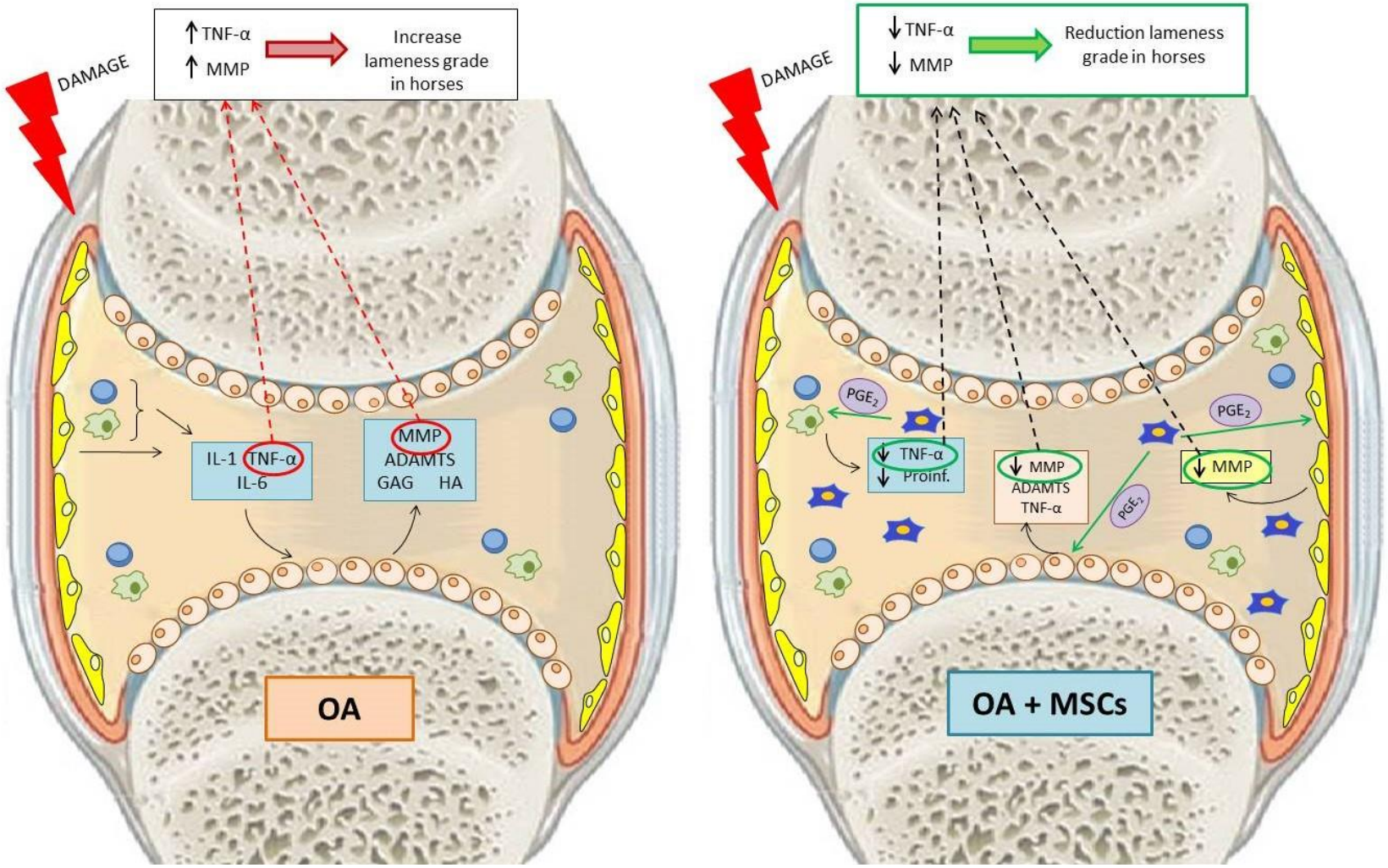

Figure 11: PGE2 relation with EUC-MSC MoA

Equine Umbilical Cord MSC and equine clinical improvement:

As previously explained, equine OA is associated with clinical symptoms in horse, mainly lameness, joint effusion and flexion pain, but also reduced sport performance and abilities. Being the lameness the most limiting symptom of OA, is lameness where products against OA are focused.

It has been established, that among other inflammatory cytokines, the main reason of lameness grade in horses is the increase of TNF-α and MMP (Ma *et al.*, 2017).

In order to scientifically establish a reasonable cause effect between EUC-MSCs´ treatment and equine clinical symptoms improvement (lameness reduction), the effect caused by MSCs on TNF-α and MMP should be probed, since they are linked with the *in vivo* efficacy: Lameness reduction.

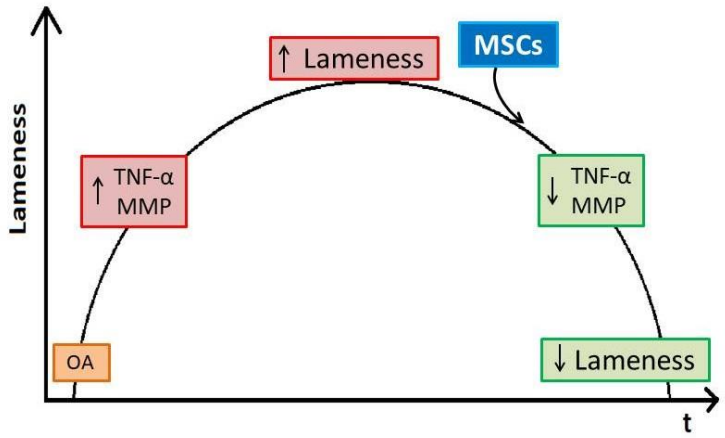

Figure 12: Relation between OA-Clinical Signs-MSC- Efficacy

It has been proven that EUC-MSCs from Wharton´s Jelly in co-culture with activated PBMCs cause PBMCs proliferation inhibition (Figure 9).

Also Carrade *et al.* (2014) demonstrated Tcell proliferation inhibition and a decrease of the secretion of TNF-α and other inflammatory cytokines in a Mixed Lymphocyte Reaction (MLR) model with Equine Umbilical Cord MSC. Carrade work also demonstrated that this anti-inflamatory effect was PGE2 mediated, as when PGE2 secretion was blocked, the Tcell proliferation and TNF-α secretion was restored (Carrade *et al.,* 2014).

On the other hand, Saulnier *et al.* (2014) developed an *in vivo* and an *in vitro* research with Equine Umbilical Cord Mesenchymal Stem Cells. In the *in vitro* model, the effect of MSC in synociocytes was investigated. It was demonstrated that if synoviocytes

were put in direct contact with the cultured medium of EUC-MSCs (that contained all the soluble factors secreted by MSC) synoviocytes expressed significant less MMPs.

The beneficial effect of Equine Umbilical Cord Mesenchymal Stem Cells has been strongly demonstrated in the reduction of cell infiltrated (Tcell), the reduction of TNF-α and the reduction of MMP. In addition, it was demonstrated that the main responsible for this action is mediated by the secretion of PGE2 by EUC-MSC.

Therefore, the lameness grade reduction in horses with OA by the treatment with EUC-MSCs has been established based on the reduction of inflammatory cells infiltrated and the reduction of inflammatory cytokines (TNF-α) and matrix degradation proteases (MMP). In addition, it has been demonstrated that this effect is PGE2 mediated.

3.3. Clinical trials in veterinary medicine

In order to demonstrate the efficacy of veterinary medicines, clinical trials must be conducted in a well manner that allows the scientific community to reach solid conclusions in terms of efficacy and safety of new products.

Clinical trial is defined as a research study in which one or more subjects are prospectively assigned to one or more interventions (which may include placebo or other controls) to evaluate the effects of those interventions on health-related biomedical or behavioural outcomes.

The purpose of clinical trials is to demonstrate or substantiate the effect of the veterinary medicinal product after administration at the proposed dosage regimen via the proposed route of administration and to specify its indications and contraindications according to species, age, breed and sex, its directions for use as well as any adverse reactions which it may have (Directive 2001/82/EC)

Different regulatory bodies work helping researchers on how clinical trials must be designed and conducted if the intention is to submit the results for the approval of a new product as a veterinary medicine. There are public guidelines at national level (Agencia Española de Productos Sanitarios (AEMPS)) or at international level (European Medicine Agency (EMA)). Several guidelines exist to guide researchers in the design of veterinary clinical trials for regulatory purposes of which the below can be highlight:

- VICH GL9 - Good Clinical Practice (June 2000): which provides guidance on the design and conduct of all clinical studies of veterinary medicines in the target species.

- Directive 2001/82/EC on the Community code relating to veterinary medicinal products.
- Guideline on statistical principles for Clinical Trials for veterinary medicinal products (pharmaceuticals)" of 16 January 2012 (EMA / CVMP / EWP / 81976/2010): This document provides guidance on the statistical principles to be considered in the design, conduct, analysis and evaluation of clinical trials to demonstrate efficacy and/or safety of an investigational veterinary pharmaceutical product in animals.

For obtaining a Marketing Authorisation (MA) as veterinary medicine, quality in the manufacturing, safety and efficacy of the product must be demonstrated to the authorities. In order to demonstrate safety and efficacy a well-designed clinical trial must be submitted.

If the intention of the researcher is to use the data to obtain a Marketing Authorisation, regulatory guidelines must comply from the early beginning (design of the trial) to the end of the clinical phase (data analysis).

Different types of clinical trials could be conducted depending on the final objective. One classification for clinical trials could be: observational or interventional.

In observational studies, participants are identified as belonging to study groups and are assessed for biomedical or health outcomes. Participants may receive diagnostic, therapeutic, or other types of interventions, but the investigator does not assign participants to a specific interventions/treatment. This kind of clinical trial provides less compelling evidence than interventional clinical trials. In observational studies, the investigators retrospectively assess associations between the treatments given to participants and their health status, with potential for considerable errors in design and interpretation. This kind of clinical trial is usually used in a post marketing phase or in specific populations groups (pregnant, infants, etc.) but not as a single clinical trial for obtaining a Marketing Authorisation.

In interventional studies, participants are assigned to groups that receive one or more interventions/treatments (or no intervention) so that researchers can evaluate the effects of the interventions on biomedical or health-related outcomes. The assignments are determined by the study's protocol.

Interventional studies are the most common types of studies conducted in the pharmaceutical industry. Different types of interventional studies could be conducted depending on the control, the masking, the place where is conducted, etc.

Well design interventional studies should be:

Controlled clinical trials: Clinical trials which involve one or more test treatments and at least one control treatment. Controlled trials have specified outcome measures for evaluating the studied intervention, and a bias-free method for assigning patients to the test treatment. Depending on the product use in controlled clinical trials different types could be performed:

- Negative Control: This is a trial with the primary objective of showing that the response to the investigational product is superior to a comparative agent: placebo or non-treatment.
- Positive Control: In this trial the primary objective is showing that both products have comparable efficacy (Non-inferiority). In this kind of design usually the comparator used is the conventional treatment and the new product wants to demonstrate that the efficacy and safety is at least the same as the one observed in conventional treatments. The comparator in this kind of clinical trials is always an active molecule.
- Historical Controls: where old data is used to compare with new data from new trials. A historical control group should be chosen so that the trial's endpoints are comparable. If the controls are not carefully chosen so that they are reasonably compatible with the experimental group, this can result in inaccurate results.

Design: depending on how the treatment is allocated into the patients, different kinds of clinical trials can be defined:

- Cross-over assignment: A type of intervention model describing a clinical trial in which groups of participants receive two or more interventions in a specific order. For example, two-by-two cross-over assignment involves two groups of participants. One group receives drug A during the initial phase of the trial, followed by drug B during a later phase. The other group receives drug B during the initial phase, followed by drug A. So, during the trial, participants "cross-over" to the other drug. All participants receive drug A and drug B at some point during the trial but in a different order, depending on the group to which they are assigned.
- Parallel: A type of intervention model describing a clinical trial in which two or more groups of participants receive different interventions. For example, a two-arm parallel assignment involves two groups of participants. One group receives drug A, and the other group receives drug B. So, during the trial, participants in one group receive drug A "in parallel" to participants in the other group, who receive drug B.

Masking: A clinical trial design strategy in which one or more parties involved in the trial, such as the investigator or participants, do not know which participants have been assigned which interventions. Types of masking include: open label, single blind masking, and double-blind masking.

- Open Label: there is no masking. All people involve in the study know the treatment received.
- Single blind: some people involve in the study is blind but other are not blind e.g. the researcher knows the product allocated but the owner is blind.
- Double blind: All people involve in the study is blind (researcher and owner do not know the treatment received).

Site: depending on how different place the trial is conduct:

- Multicentric: the same clinical trial (same protocol of study) is conducted in different places with different researchers and different epidemiologic circumstances. In veterinary medicine for example the design of multicentric clinical trials for antimicrobials or anthelmintic is especially important since the resident microbiota could differ from one location to another. In pathologies where the location, climate or endemic microbiotics have no impact, the need for a multicentric design may not be a priority.
- Single place: only one centre is used. Patients and researchers come from the same place. This type of clinical trials is necessary when, for example, highly specialized machinery is used that is not present in other centres. In non-infectious diseases, where the epidemiology of the disease is comparable everywhere, this type of design does not involve large bias.

In addition to the design, other important aspects of the clinical trial are relevant to the final outcome. One of these key points is the Primary Endpoint Selection.

Primary Endpoint is defined as an event or outcome that can be measured objectively to determine whether the intervention being studied is beneficial or not.

Usually, the primary endpoints are assigned as qualitative classification *"yes"* or *"no"* to identify whether the treatment has been beneficial or not (e.g.: the animal has stopped convulsing yes / no).

At the time of the statistical calculation, the number of patients who have reached the objective established in the primary endpoint in each group is compared to identify the efficacy or lack of.

For primary endpoint selection the intended efficacy of the product must be well known in order to establish an adequate endpoint that shows the benefits of the investigated medicine and gives a clinical relevance result to the animal.

Often preliminary exploratory clinical trials are developed in a small number of animals in order to determinate an adequate primary endpoint in confirmatory clinical trials.

The final objective of this work is to submit the data to the European Medicine Agency (EMA) in order to obtain the Marketing Authorisation of Equine Umbilical Cord Mesenchymal Stem Cells as veterinary medicine.

With the intention to demonstrate the efficacy and safety of Equine Umbilical Cord Mesenchymal Stem Cells in equine OA, a well conducted clinical trial has to be designed and conducted in accordance with regulatory guidelines (VICH GL9).

4. OBJECTIVE AND JUSTIFICATION

4. OBJECTIVE AND JUSTIFICATION

4.1.1. Objective

The objective of this work is:

- To demonstrate the efficacy of Equine Umbilical Cord Mesenchymal Stem Cells (EUC-MSCs) in horses with clinical signs associated with mild to moderate Osteoarthritis (OA).
- To demonstrate the safety of EUC-MSCs in horses with clinical signs associated with mild to moderate Osteoarthritis (OA).
- To investigate the effect of co-variables in the efficacy of EUC-MSC
- To compare the efficacy and safety of EUC-MSC with conventional treatments

4.1.2. Justification of the study

As deeply explain before Osteoarthritis (OA) is a major cause of reduced athletic function and retirement in equine performers. With a prevalence as high as 80%, OA is the most common cause of lameness in horses, being responsible for 60% of total cases, and one of the most frequently responsible for premature abandonment of the sport life (Souza, 2016).

However, despite the high prevalence and the devastating consequences that have for the equine sector with an economic impact valued at 18,000 million € in Europe, the pharmaceutical industry has not provided new solutions or innovative therapeutic alternatives for decades.

Medical treatment of OA may include anti-inflammatory and analgesic drugs to reduce the inflammation and pain, an intra-articular administration of corticosteroids, administration of NSAIDs and nutritional supplements that purportedly improve joint function (Souza, 2016). However symptomatic treatments but are not able to stop the disease and are also associated with long-term side effects.

During this work it will investigate the efficacy and safety of EUC-MSCs, their effectiveness depending on different epidemiological cofactors such as age, physical activity, etc. and a comparison will be made with the efficacy and safety of conventional treatments.

This work has been carried out to investigate EUC-MSCs since they could represent an innovative therapeutic alternative to conventional treatments.

5. MATERIAL AND METHODS

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5.1. Study design

5.1.1. Type of study and overall study design

A comparative superiority study, multicentric, parallel, blinded, randomized and placebo-controlled Clinical Trial was designed. The study was carried out complying with the Good Clinical Practice guidelines (VICH 9 guidelines).

The study was conducted in two groups Group 1 (treated group) and Group 2 (control group).

The owner of all the animals enrolled signed an Informed Consent before the enrolment of the animal and the risk and benefits as well as the objective of the trial were explained to the owner.

Group 1: Treated group; Horses (n=36) were tested with Equine Umbilical Cord Mesenchymal Stem Cells.

Group 2: Control group; Horses (n= 39) were tested with placebo.

The study was designed and conduct according to the schedule that follows:

Activity	Pre treatment (-15 a -7 Day)	Day 0 (treatment day)	Day 1	Day 14 (±2)	Day 35 (±2)	Day 63 (±2)
Checking the inclusion criteria	X	X				
Informed consent		X				
Diagnosis	X					
Blood Analysis		X		X		
Intra-articular treatment		X				
Physical exam	X	X	X	X	X	X
Orthopaedic examinations	X	X		X	X	X
Registration Adverse Events		X	X	X	X	X
Injection site evaluation		X	X	X	X	X
Study completion						X

Table 2: Tabulated summary of the study outline

Before starting the Clinical Trial, a single list of randomisations for the whole trial was developed.

Horses were randomly assigned to Treatment or Placebo Group according to the randomisation list developed.

The randomisation list was developed manually by a non-blinded person by flipping a coin, where head was code A (Treatment) and Tail was code B (Placebo).

None (demographic, symptomatology, etc) criteria were applied for the development of the Randomisation list. The list was developed strictly aleatory and before the clinical trial began.

5.1.2. Blindness

Investigators, study personnel and owners were blinded to Treatment or Placebo.

Both the Treatment and the Placebo were conditioned in identical-looking vials.

Considering Mesenchymal Stem Cells have a particularly cloudy colour, difficult to mask, the syringe used was covered with opaque material before sterilizing, to ensure completely blindness of the researcher (Figure 10).

Figure 13: Syringe with opaque material for ensuring the blindness

In order to ensure full blinding of the investigator, the presence of a dispenser was designed. The dispenser was a person, unknown to the investigator, who was responsible for handling sterile vials and syringes to prevent that the researcher could detect if the product was treatment or placebo during the manipulation of the vials.

The researcher applied the product to the horse, previously prepared by the dispenser.

In addition, different measures were adopted in order to avoid bias:

- In each horse observations were carried out by the same person and instruments.

- Housing conditions were similar for both groups (experimental and control) in each location.
- The researchers did not know the identity of the experimental and control groups (blinded study).

5.2. Treatment and administration

The product used in the trial, is non-toxic and non-harmful for human beings. Nevertheless, farmers, veterinarians and researchers were informed of the characteristics of the products.

Investigational Veterinary Product (IVP)

Name of the IVP: EUC-MSC (HorStem®)

Description: Sterile injectable suspension conditioned in sterile vial, sealed, tight and penetrable for syringes

Composition per one dose (1 ml):

Active ingredients: Equine Umbilical Cord Mesenchymal Stem Cells 15 million (± 20%) with a viability ≥70%

Excipient: Confidential formula 1 mL

Control product Placebo:

Name of the Control Product: Placebo

Description: Sterile injectable suspension conditioned in sterile vial, sealed, tight and penetrable for syringes

Composition per one dose (1 mL):

Active ingredients: None

Excipient: Saline solution 1 mL

Method and route of administration:

Before administration of the VIP or Placebo, the joint was prepared by cleaning the area with an antiseptic soap (alternate washes, betadine or chlorhexidine / alcohol for at least 2 minutes) to reduce the number of bacteria at the injection site. The hair was

not clipped unless the horse was particularly dirty, hairy, or if anatomic landmarks were difficult to identify.

Before aspiration into the syringe the content of the vial was homogenized with gentle movements, this procedure was done by the dispenser in order to maintain blindness.

Next, immediately prior to inserting the needle (20G) into the joint (arthrocentesis), the area was generously cleaned with alcohol until the area was free of soap. At that point, the needle (without the syringe attached) was quickly and easily inserted intra-articularly.

Due to anatomical differences among individual horses' joints, it was allowed that the veterinarian redirected the needle until it was fully inside the joint and synovial fluid was (typically) visible in the hub of the needle. The opaque syringe was subsequently attached to the needle and the drug of choice was delivered directly into the joint space.

After the arthrocentesis and the product administration, a bandage was applied (if applicable) to the area for 24h, with the intention of preventing dirt in the injection site.

The bandage consisted of a softly applied cotton band followed by a bandage (non-compressive) with a cohesive bandage.

5.3. Diagnosis of OA:

The diagnosis of OA was made by each researcher. The final diagnosis is the result of the different diagnosis tools that allow having an overall conclusion to issue a final diagnosis.

Detailed description of the diagnostic procedure:

a) Palpation of the joint

Once the lameness was evaluated, palpation of the lame limb was performed (this is normally performed before trotting the horses). Examination of both soft tissues and joints on the lame limb was performed checking for signs of inflammation, joint distension, pain on deep palpation or increased size of soft tissue structures.

Figure 14: Example of limb palpation

b) Lameness examination

With the objective to detect gait irregularities or lameness in the animal and according to American Association of Equine Practitioners (AAEP) guidelines the horse was trotted and walked in straight line and in circles in both hard and soft ground. The horse was observed from the front, back and both side views.

c) Flexion test

According to the AAEP guideline the veterinarian holds the horse's limbs in a flexed position and then releases the leg. As the horse trots away, the veterinarian watches for signs of pain, weight shifting or irregular movement. Flexing the joints in this manner may reveal problems not otherwise readily apparent (Figure 15).

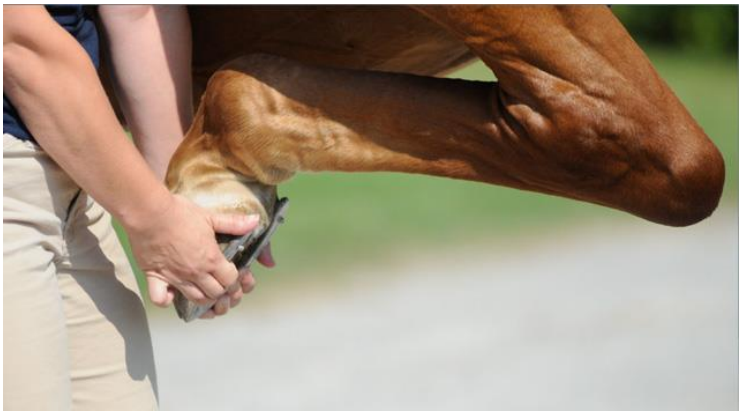

Figure 15: Example of a flexion test

d) Perineural Block

Perineural blocks consisted in the subcutaneous or perineural injection of local anaesthetic (mepivacaine, lidocaine, etc) in the intention to block the pain sensation in the area. Working systematically, the veterinarian temporarily deadens sensation to specific segments of the limb, one region at a time, until the lameness disappears. When the lameness disappears it is considered that the anatomical structure that causes lameness is in the area of action of the blockage (Figure 16).

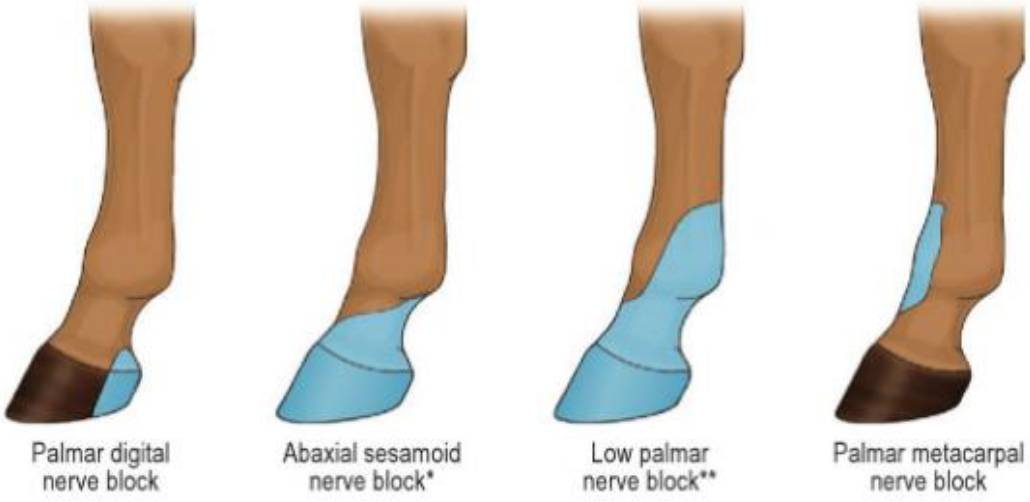

Figure 16: Example of perineural block. In blue it is represented the pain area of each block

e) Radiology

Once the region of pain was localised with palpation, flexion pain test and perineural blocks, radiographs of the area were taken making as many projections as necessary to make a diagnosis.

f) Intra-articular anaesthetic block (IAAB)

Once these diagnosis tools were done, usually a joint was selected as the cause of lameness. At this point an intra-articular anaesthetic block (IAAB) was performed (note that the IAAB was always done in a different day than perineural blocks) in order to localise the source of pain to the joint. For IAA blocks an anaesthetic (mepivacaine, lidocaine, etc) is introduce inside the joint. After ~5 minutes if the pain is located inside the joint the horses should stop limping.

If the IAAB showed a clear improvement of the pain and radiography signs of OA were detected with no other changes compatible with soft tissue pathology a diagnosis of OA in that joint was made.

The compliance of all the detail tools listed below is the gold standard for diagnosis in equine practice, and has been widely reported in clinical trials. (Lynn *et al.,* 2004; Cayzer *et al.*, 2011; Tnibar *et al.,* 2016.).

In the figure below is represented, as an example, how to perform a diagnosis of distal phalangeal joints, and how each diagnostic measure helps us, guiding us to the final diagnosis.

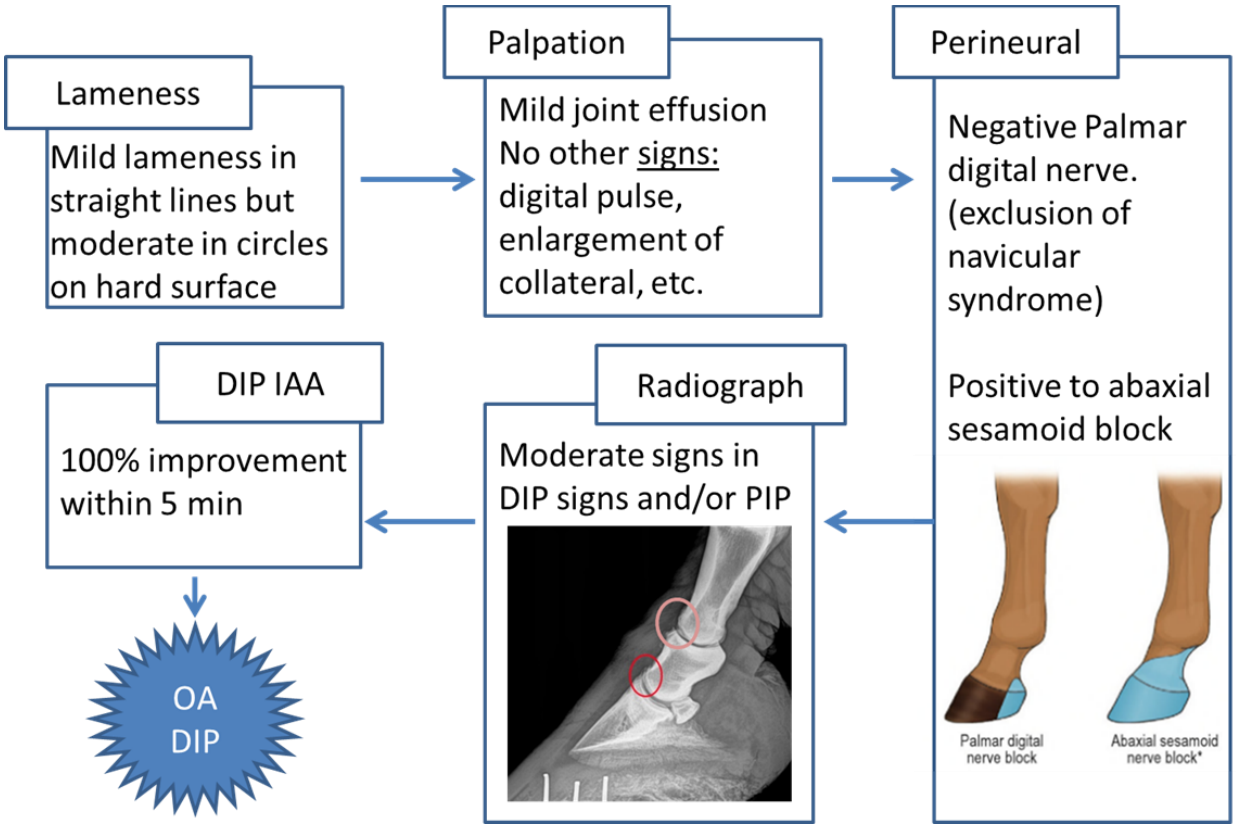

Figure 17: Image of a typical diagnosis during the clinical trial for distal joints

Only horses with clinical sings in a single joint per leg were included. Horses with clinical signs in more than one joint per leg (e.g. proximal and distal joint in the right forelimb), which present improvement after anaesthetic block of one joint (e.g.: distal joint), but still maintain a lameness degree corresponding to the other affected joint (e.g.: proximal joint), have not been included in the study.

Horses with bilateral diagnosis and clinical symptoms (in contralateral limbs) could be included in the present study, however only the joint which manifests a greater lameness degree was medicated. Bilateral horses showing no clear limp lameness presenting bilateral shortening stride have not been included in this study.

The diagnosis had to ensure the following information:

- The horse had radiological signs of mild to moderate according to the scale of Cornelissen Radiographic Scale (adapted to other joints) (Table 2)
- The clinical symptoms (lameness, flexion pain and effusion) met the inclusion criteria of the protocol.
- The clinical symptoms corresponded only to the affected joint by OA.
- The horses had no other pathologies underlying that may alter the results.

Radiographic Scale	
Score	Radiographic findings
0	Rounded joint margins. No subchondral bone sclerosis. No signs of anomalies
1	Pointed joint margins of the joint or minimal localized subchondal bone sclerosis
2	Small spur(s) on joint margins or mild localized subchondal bone sclerosis
3	Moderate spur(s) on joint margins or mild localized subchondal bone sclerosis
4	Large spur(s), severe subchondal bone sclerosis, cyst, osteochondral fragments or evidence of joint space narrowing.

Table 3: Cornelissen Radiographic Scale adapted to all joints (Cornelissen 1996)

5.4. Orthopaedic examinations:

Once between day -15 and -7, days 0, 14 (±2), 35 (±2) and 63 (±2), the enrolled animals were Orthopaedic examined of the next parameters:

- Assessment of lameness according to the American Association of Equine Practitioners (AAEP) guideline. The use of half points was allowed when the lameness grade of the horse was between two integer values. The use of half points in the lameness scale increases its precision, making it a more suitable scale for clinical studies where lameness is the primary efficacy endpoint, and

therefore it is necessary to determine the improvement in the most reliable and precise way possible (Keegan *et al.,* 2009, Back *et al.,* 2007, Hu *et al.,*, 2009, Erket *et al.*, 2004, Schneider, 2013)

AAEP Lameness Scale	
0	Lameness not perceptible under any circumstances.
1	Lameness is difficult to observe and is not consistently apparent, regardless of circumstances (e.g. under saddle, circling, inclines, hard surface, etc.).
2	Lameness is difficult to observe when trotting in a straight line but consistently apparent under certain circumstances (e.g. weight-carrying, circling, inclines, hard surface, etc.).
3	Lameness is consistently observable at a trot under all circumstances.
4	Lameness is obvious at a walk.
5	Lameness produces minimal weight bearing in motion and/or at rest or a complete inability to move.

Table 4: AAEP Lameness score (Scale of the American Association of Equine Practitioners AAEP). The use of half points was allowed when the lameness grade of the horse was between two integer values.

- Assessment of joint effusion according the following table:

Joint Effusion Scale	
0	No Swelling
1	Mild Swelling
2	Severe Swelling

Table 5: Joint Effusion Score

- Assessment of flexion pain according to the following table:

Flexion Test Scale	
0	No flexion response
1	Mild flexion response
2	Moderate Flexion response
3	Severe flexion response

Table 6: Flexion Pain Score

5.5. Physical examinations:

Physical exploration was always performed by the veterinarian and consisted of a general medical examination, where the following actions should be performed:

- Cardiac and pulmonary auscultation
- Abdominal auscultation (peristalsis)
- Lymph nodes palpation
- Revision of the general state (hydration, corporal condition, lachrymation, nasal discharge)
- Inspection of the skin and the injection point
- Rectal body temperature

The physical examination was performed once between day -15 and -7, days 0, 1, 14 (±2), 35 (±2) and 63 (±2).

The results of the physical examinations were described as normal (physiological parameters e.g. rectal temperature between 37ºC and 38ºC) or abnormal. Those abnormal or non-physiological values were investigated case by case and included as an adverse event if applicable.

5.6. Laboratory examinations:

The laboratory examinations were outsourced in a specialist veterinary clinical analysis laboratory.

Before inclusion (Once between day -15 and day 0) and at day 62 (±2) blood samples were collected for haematology and serum chemistry.

Haematology: Haematies, Haemoglobin, Haematocrit, Medium Corpuscular Volume, Leucocytes, Platelets, Eosinophil, Basophils, Lymphocytes, Monocytes, and cytology of the blood cells.

The serum chemistry: Urea, Creatinine, AST, ALT, Total Protein, Alkaline phosphatase, Gamma Glutamyl Transpeptidase, total bilirubin, Albumin, LDH and Creatine phosphokinase.

5.7. Inclusion, exclusion and post-inclusion removal

5.7.1. Inclusion criteria

Horses diagnosed with OA and with clinical symptoms associated as lameness and flexion pain were included.

Animals that met the criteria of the list were included:

- Healthy (except of OA) mature horses.
- Animals with radiological signs of mild to moderate degenerative joint disease. 1, 2 and 3 degrees on the scale of Cornelissen 1996 adapted to all joints (Table 3).
- Animals with signs of lameness Grades 1, 2 and 3 (Table 4)
- Animals with joint effusion Grades 0, 1 and 2 (Table 5)
- Animals with Flexion Pain Grades 1, 2 and 3 (Table 6)
- The sum of the three clinical signs (lameness, joint effusion and flexion pain) between 4 and 7 points, inclusive.
- Animals clearly may respond, at the field investigator criteria, to the intra-articular anaesthetic joint block
- Animals whose lameness has remained stable for at least 4 weeks prior to the inclusion of animal
- Animals who have not received intra-articular medication in the last three months
- Animals who have not received systemic medication in the last 15 days
- Patients without intra-articular free fragment (joint chip)

5.7.2. Exclusion criteria

- Animals with signs of joint infection
- Bilateral horses showing no clear limp lameness presenting bilateral shortening step.

- Horses with lameness produced by more than one joint per limb.
- Animals whose indication is surgical rather than medical
- Animals with any other disease (e.g. concomitant injuries (e.g. Laminitis, lymphangitis, associated ligament injuries, etc ...) that could require the administration of anti-inflammatory drugs or other medications not permitted during the study
- Animals with presence of a free fragment articular
- Horses who have been introduced to any change in routine ride, rider, horseshoes, and type of training that can help or hinder its clinical course or mask test results
- Animals with open wounds in the joint to be treated
- Pregnant or lactating mares
- Animals destined to enter the food chain
- Animals that have received intra-articular medication in the last three months
- Animals that have received systemic medication in the last 15 days

5.7.3. Post-inclusion removal criteria

Criteria for removal

- The inclusion in this study was voluntary, and therefore an animal may be withdrawn if its owner wishes at any time during the test
- Animals in whom a serious adverse event, such as colic, accident, requiring treatment not allowed which makes it incompatible to continue in the study
- Animals where the evolution of degenerative joint disease worsens markedly and it is necessary to administer additional rescue medication
- Animals unable to properly fulfil the plan set out in the protocol plans
- Animals who needed to implement any changes in their routine that can mask or alter the results of this study

- Females that remain pregnant during the study
- Other causes (e.g. pre-existing higher protocol deviation, undetected disease on day 0, etc ...)
- For animals receiving non-authorised treatments, the permanence or withdrawal of concomitant treatments in the study was evaluated in each case depending on the treatment used, and the time lapsed to the next clinical evaluation. As a general rule the guidelines proposed by the Fédération Equestre Internaciale (FEI) were used on drugs withdrawal time before a competition, since these drugs can have an effect on clinical or sports performance of animals. Depending on the date of application of concomitant therapy, and analysing treatment case by case, it was determined whether the animal should be removed of the trial, or not.
- Pregnant or lactating mares during the study

5.8. Efficacy Assessment

5.8.1. Lameness Improvement

Equine lameness is one of the clinical symptoms with the most consequences in their sporting life and in their quality of life.

Likewise, for the competition, the horses must be free of consistent lameness at trot (<1 grade AAEP) in order to participate in international competitions (Fédération Equestre Internationale (FEI)).

For this reason, to evaluate the efficacy of a medication for the equine locomotor system, the evaluation of lameness is essential.

In order to evaluate the effectiveness of the EUC-MSC the reduction of equine lameness was elected as endpoint. However in this work we wanted also to confirm that the efficacy was sufficient to significantly improve the quality of life and sports capacities of horses.

The primary variable of this study aimed to establish a cut off where the animal did not present a clear or consistent limp and that allowed it to develop a normal sporting life after the treatment.

Also, it is well known that the mechanism of action of MSCs is a complex mechanism based on joint immunomodulation, so the time from the administration of the product until the animal improves significantly is rather weeks than days.

For all these reasons the primary endpoint was establishes as: improvement in the lameness grade to a non-lame or an inconsistent lameness (≤ grade 1 AAEP scale) 63 days post treatment.

Horses that reach the primary endpoint were classified as: Therapeutic Success.

In addition to the primary variable defined above, we also evaluated: the average decrease in lameness at day 63, the decrease or not in lameness at day 63 and the therapeutic success at day 14 and 35. All these variables were assessed as secondary endpoint.

5.8.2. Effusion

Effusion is an indirect measure of the degree of joint involvement and the degree of joint inflammation. Horses affected by OA may have increased joint effusion, have it normal or have it decreased.

In the present study all degrees of joint effusion were allowed.

In order to evaluate the capacity of EUC-MSC in the improvement of joint effusion, the effect on this clinical symptom was evaluated as a secondary variable. The response to effusion was evaluated as secondary endpoint.

5.8.3. Flexion Pain

The pain and increased lameness after active (or passive) flexion test of the affected joint is a very frequent sign in equine OA.

For participation in the present study all horses should show increased pain after active flexion.

Being flexion pain a characteristic sign of OA, it was interesting to know the effect that EUC-MSC has on flexion pain in treated horses. The response to flexion pain was evaluated as secondary endpoint.

5.8.4. Overall Improvement

The overall improvement was defined as the sum of the lameness (punctuated according to the AAEP from 0-5), the flexion pain (from 0-3) and the effusion (from 0-2) summation. The sum of the three variables gives a total of 10 points. The overall improvement as defined above was evaluated as secondary endpoint.

5.8.5. Subjective improvement

With the objective to find an added value, the researcher and the owner (or rider) was asked upon completion of the Clinical Trial about their opinion in the results of the treatments, unbeknownst to them if placebo or IVP was used (blind).

The subjectively opinion of the degree of improvement of the animals was taking into account the following parameters:

- The improvement, in cold, of the horse immediately outside the box
- Degree of improvement of the horse during the exercise practice
- Degree of improvement in sports skills
- Degree of improvement in pain sensation

To each animal, both the veterinarian and owner (or rider), assigned a punctuation of improvement from 0 to 10, where 0 was no improvement and 10 complete improvement.

5.9. Safety Assessment

For the safety assessment a detailed analysis of all adverse events suffered by the participating horses were evaluated.

On a case-by-case basis, each adverse event was evaluated in order to determine its relationship or not with the drug under study and / or the route of administration.

The safety assessment has been carried out based on the assessment of the severity and prevalence of the adverse events occurred.

5.10. Statistical Methodologies

This study was a superiority comparative, parallel, controlled, multicentric, blinded Clinical Trial.

Statistical analyses were carried out based on the guidance document "Guideline on statistical principles for Clinical Trials for veterinary medicinal products (pharmaceuticals)" of 16 January 2012 (EMA / CVMP / EWP / 81976/2010).

Statistical analysis was conducted in the population by protocol (PP) and for population to be treated (PTT).

Basal Homogeneity: for qualitative explanatory variables the appropriate test for comparing treatment groups will be used (Chi-Square test, Fischer's exact test or LR Chi-Square test). For quantitative explanatory variables the appropriate test for comparing treatment groups will be used (t-test, Mann-Whitney's test).

Qualitative Response Variables (reduction > value): the appropriate test for comparing qualitative variables between treatment groups will be used (Chi-Square test, Fischer's exact test or LR Chi-Square test).

Quantitative Response Variables (Longitudinal Analysis): A linear mixed model will be used considering the animal as a random Factor and Time, Treatment and their interaction as fixed factors. The compliance of application conditions will be performed qualitatively through residuals plots. Alternatively, methods considering the appropriate generalised linear mixed models will be applied

In order to account for the basal severity of the animals, all analyses use basal evaluations as reference values.

5.11. Co-variables

OA has a multifactorial origin where certain factors could influence both the evolution of the disease and the effectiveness of treatments against it.

According to clinical experience and bibliography, there are some variables that could influence in the efficacy outcome of a product.

In order to elucidate if some equine population could have better or worse response to EUC-MSCs than others depending of different variables (age, affected joint, activity level…) an efficacy co-variable study was made.

The effect of many different co-variables was investigated:

- Lameness grade before treatment
- Radiological Image
- Horse weight
- Sex
- Age
- Affected Limb (frontlimb or hindlimb)
- Life habits (box, semi-liberty or life in meadow)
- Joint (fetlock, proximal, interphalangeal, etc)
- Chronicity
- Activity level

The election of the co-variables was based in veterinary parameters: clinical experience of the author and bibliographic research, considering that those variables could have some kind of influence in the efficacy of the product.

Based on veterinary parameters and bibliographic references it could be hypothesised that: the greater the lameness, the less effective; the greater the radiological signs, the less efficacy; the greater the age, the lower the efficacy and the greater the chronicity, the less effective.

On the other hand, no relationship is expected between sex, weight, affected limb, joint and activity level on the efficacy outcome, neither in the bibliography nor in the clinical experience of the author.

5.12. Comparison with conventional treatments

In the equine sector, there are different treatments available for equine OA, however almost all of them are symptomatic treatments that are incapable of slowing down the evolution of the disease and are associated with long-term side effects.

Of all the treatments available, the use of intra-articular corticoids + hyaluronic acid (Cos+HA) can be considered the most commonly used conventional treatment in the treatment of equine OA.

MSCs has being postulated as a therapeutic alternative for the treatment of OA, however, to be able to effectively consider them as a realistic alternative, a comparison of both products must be carried out objectively.

The information of conventional treatments has been provided by bibliographyc references: Harkins et al., 1993; Van Weeren & de Grauw, 2010; de Grauw et al., 2015; Souza, 2016.

The information of EUC-MSC has been obtained by the present work and the author's knowledge.

For this comparison, different aspects will be taken into account: cost, effectiveness, adverse events and indirect cost.

Both products will be compared by representing them in a radial chart. The chart will be divided into 5 radius or sections. The larger area occupied the better result for product.

Cost: The cost have been estimated considering the final price that the owner of the horse will pay. The graphic is divided into 5 radius: between 801-1000 €, 601-800 €,

401-600€, 201-400 € and 0-200€. Price data has been established asking to different veterinarians in Spain.

Effectiveness: Considering the effect of the product in lameness reduction. For evaluate the efficacy of CO+ HA the publication of de Grauw, 2015 was used. The efficacy is divided into 5 sections: between 0-20%, 20-40%, 40-60%, 60-80%, 80-100%.

Adverse Event: Considering the safety of the product in general terms. For evaluate the safety/adverse event of COs+HA publications of Harkins et al., 1993, Van Weeren & de Grauw, 2010; de Grauw et al., 2015 and Souza, 2016 has been used. To classify safety, different adverse effects have been scored according to their frequency and severity as follows:

Temporal- Local adverse effects

Frequency	Description	Score
Rare (<10%)	Mild local sing such us local effusion or subcutaneous sweelling	1
Common (>10%)	Mild local sing such us local effusion or subcutaneous sweelling	2
Rare (<10%)	Joint Flare: acute lameness and swelling	1
Common (>10%)	Joint Flare: acute lameness and swelling	2

Temporal or Permanent Systemic events adverse effects

Frequency	Description	Score
Very Rare (<1%)	Sings of systemic illness such us Laminitis, adrenal insufficiency, hyperadrenocorticism, tumors, etc.	2
Rare (<10%)	Sings of systemic illness such us Laminitis, adrenal insufficiency, hyperadrenocorticism, tumors, etc.	4

Permanent Local adverse effects

Description	Score
Negatively affect the cartilage homeostasis and biosynthetic after repeated and/or high dose	3

The safety is divided into 5 sections: ≥9 points, 7-8 points, 5-6 points, 3-4 points and ≤2 points.

Indirect Cost: : For the analysis of indirect costs, it has been taken into account: the days of sports loss by dopping, the days for sports loss from the application of the product until its effectiveness and duration of the clinical effect. For evaluate the indirect cost of COs+HA publications of Harkins et al., 1993, de Grauw et al., 2015 and Souza, 2016 has been used.

To classified indirect cost the following points have been scored:

Description	Point
Lose of sport days due dopping	1

Description	Point
Lose of sport days until the treatment is efficacy	2

Description	Point
Duration of the effect ≤ 1 month	4
Duration of the effect 1-3 months	3
Duration of the effect 3-6 months	2
Duration of the effect 6-12 months	1
Duration of the effect ≥12 months	0

The indirect cost is divided into 5 sections: ≥5 points, 4 points, 3 points, 2 points and ≤1 points.

When both products have been scored according to the guidelines describe bellow, both products will be represented in a radius chart. The product with more area will be considered better than the other.

5.13. Long Term Follow up

With the intention of knowing the long-term effect of EUC-MSCs, efficacy and safety data were collected 2 years post product administration.

It is important to note that this study of long term follow-up was not done according to Good Clinical Practice. Possible adverse effects, concomitant treatments, deviations etc. were not recorded following the VICH GL9 regulations. On the other hand, both investigator and owner of the animal were not blind in this follow-up period.

Veterinarians were asked two years after product administration about the moment of relapse and about the occurrence of adverse event during the period.

6. RESULTS

6. RESULTS

6.1. Study Design

The study was designed and conducted according EU guidelines and regulatory requirements. The study was conducted in Spain from November 2014 to September 2017 with two separate enrolment periods.

In this study there were three well-defined figures:

Researcher: in this trial the researcher was the clinical vet. Veterinarians were in charge of: animal selection, product administration, efficacy and safety reviews, adverse event registration, etc. More than 20 different veterinarians from all parts of Spain (Table 7) with an average of 20 years of equine practice experience participated in the clinical trial as researchers. Each horse was always evaluated by the same veterinary in order to avoid inter-observer variability.

Clinical Trial Monitor: An individual responsible of overseeing a clinical study and ensuring that it is conducted, recorded and reported in accordance with the study protocol and good clinical practice. The tasks of the monitor were to ensure compliance with the protocol, provide support and training to veterinarians regarding the clinical trial, review the data collection notebooks, as well as any aspect related to the execution of the trial (days of visits, concomitant treatments, adverse effects, etc.).

Sponsor: An individual, company, institution or organization which takes responsibility for the initiation, management and financing of a clinical study for the veterinary product under investigation.

Name	Credentials	County / Country
W.M	Vet Graduated 1972. Equine Specialist Practitioner since 1973.	Madrid
G.C	Vet Graduated 2004. Equine Specialist Practitioner since 2005	Alicante
M.P	Vet Graduated 1990. Equine Specialist Practitioner since 1992	Valladolid
J.A	Vet Graduated 2003. Equine Specialist Practitioner since 2003	Ciudad Real
F.G	Vet Graduated 1995. Equine Specialist Practitioner since 1998	Madrid
D.J	Vet Graduated 2009. Equine Specialist Practitioner since 2009	Madrid

F.R	Vet Graduated 1988. Equine Specialist Practitioner since 1992	Cantabria/ Asturias
J.G	Vet Graduated 1990. Equine Specialist Practitioner since 1992	Cataluña
P.S	Vet Graduated 1992. Equine Specialist Practitioner since 1995	Cataluña
G.G	Vet Graduated 1991. Equine Specialist Practitioner since 1995	Cataluña
I.M	Vet Graduated 2008. Equine Specialist Practitioner since 2008	Madrid
M. V	Vet Graduated 1992. Equine Specialist Practitioner since 1995	Asturias
I. G	Vet Graduated 1990. Equine Specialist Practitioner since 1993	Valladolid
J.V	Vet Graduated 1997. Equine Specialist Practitioner since 2000	Andalucía
JM. R	Vet Graduated 2011. Equine Specialist Practitioner since 2012	Andalucía
JM. M	Vet Graduated 2000. Equine Specialist Practitioner since 2001	Madrid
J. R.	Vet Graduated 1998. Equine Specialist Practitioner since 1999	Andalucía
V. O.	Vet Graduated 1990. Equine Specialist Practitioner since 1991	Valencia
H. R	Vet Graduated 2007. Equine Specialist Practitioner since 2007	Barcelona
M.V	Vet Graduated 2000. Equine Specialist Practitioner since 2001	Gerona
P.A	Vet Graduated 1988. Equine Specialist Practitioner since 1990	Madrid
S.G	Vet Graduated 2011. Equine Specialist Practitioner since 2012	Valencia
R. H	Vet Graduated 1987. Equine Specialist Practitioner since 1987	Madrid

Table 7: Researchers credentials

The study has a fixed visit schedule that was met by all the vets and horses with the exception of rare minor deviations.

6.2. Enrolled Animal and withdrawals

In the present study a total of 76 horses were enrolled (36 received treatment and 40 placebo).

A total of seven horses were withdrawn before completion the clinical trial due the following reasons:

Lack of adherence to the protocol:

It had been impossible for the monitor to make the proper monitoring of the animal. The investigator did not answer the requests on the monitoring plan established. The monitor was unable to maintain a fluid communication with the investigator that allows the monitor to know the appearance of adverse events or concomitant therapy.

It had been impossible to recover the Data Collection Notebook. Therefore, this horse had not been included in the data analysis.

Changes in the sport and life habits:

During the clinical trial, as reflected in the protocol of study all the horses must have the same activity and life habits during the clinical trial and at least 4 weeks before the stating of the trial, in order to prevent possible bias of the results. For example, if a horse reduces or increases the activity level during the evaluation period, the animal was withdrawn.

Four horses suffered changes in their routine habits and/or changes in the activity level during the trial (or 4 weeks previous the enrolment). The changes suffered were: climatological reasons that meant that 2 horses were not able to perform normal activity level during 15 days (from day -15 to day 0); horse owner was not able to ride the horse as it was used to (2 horses from day -15 to day 0 in one horse and from day 0 to day 14 in other horse).

Non-related adverse event:

One horse was withdrawn because due to the trial suffering a non-related adverse event. The horse suffered a Superficial Flexor Tendon (SDFT) injury that requires a change in the habits (rehabilitation plan) and change in the shoeing, as an anti-inflammatory treatment.

According to the protocol of the study, in the case an AE is detected all the possible measures would be taken in order to discover the origin of the AE. In order to diagnose the horse an ultrasound was made and an image compatible with Superficial Flexor Tendon injury was observed.

Horse sold outside Spain:

After product administration (day 45) one horse was sold outside of Spain. It was impossible for the researcher to perform the last revision visit and therefore the horse was withdrawn.

The horse was not included in the statistical data at any time-point since the data collection notebook was not available.

6.3. Basal Homogeneity

Breed

Any breed was accepted. Pure race horses as Arabian, Spanish Pure Race (PRE) and sport horses (Spanish sport horse, Westfalian, sille francais, etc) but also crossbreed horses were enrolled. The different breeds in the trial are represented bellow:

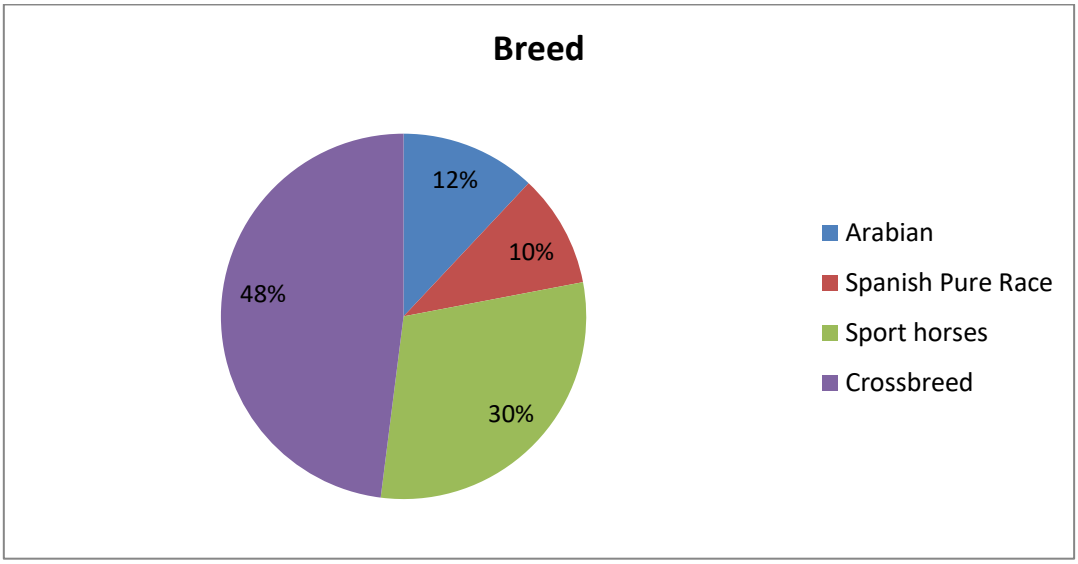

Figure 18: Breed enrolled in the clinical trial

Age

Mature horses were eligible for the study. The main age in the clinical trial was 11.8 years. The homogeneity in term of age within the groups has been demonstrated as represented bellow:

	Nobs	Nmiss	Mean	Median	Std	Max	Min	Lower CL 95%	Upper CL 95%
Total	69	0	72.7	18.0	70.9	216	4	55.6	89.7
Placebo	37	0	70.4	18.0	71.2	216	4	46.6	94.1
Treatment	32	0	75.3	34.5	71.7	204	5	49.5	101.2

	P-Value
Kruskal-Wallis Test	0.7678

Table 8: Basal Homogeneity- Age

Sex

Female, Male and castrated male were accepted (pregnant mares were excluded). The homogeneity in terms of sex within the groups is represented bellow:

SEX	TX			
	Placebo		Treatment	
	N	%	N	%
Male	4	11.1%	2	6.3%
Castrated Male	24	63.9%	19	59.4%
Female	9	25.0%	11	34.4%
TOTAL	37	100.0%	32	100.0%

	P-Value
LR - Chi-Square Test	0.5987

Table 9: Basal Homogeneity- Sex

Weight

The main body weight during the trial was 465 kg. The homogeneity in terms of body weight within the groups is represented bellow:

	Nobs	Nmiss	Mean	Median	Std	Max	Min	Lower CL 95%	Upper CL 95%
Total	68	1	464.7	450.0	66.8	650	300	448.5	480.9
Placebo	37	0	455.9	450.0	76.3	650	300	430.5	481.3
Treatment	32	1	475.2	480.0	52.7	600	400	455.9	494.5

	P-Value
Kruskal-Wallis Test	0.1521

Table 10: Basal Homogeneity- weight

Life Habits

All kind of life habits were accepted in the study, from horses that live in complete freedom in a meadow to horses that live in a box.

The homogeneity in terms of life habits within the groups has been evaluated.

LIFE HABITS	TX			
	Placebo		Treatment	
	N	%	N	%
Box	16	43.2%	12	37.5%
Meadow	6	16.2%	4	12.5%
Semi-Liberty	15	40.5%	16	50.0%
TOTAL	37	100.0%	32	100.0%

	P-Value
Chi-Square Test	0.7244

Table 11: Basal Homogeneity- Life Habits

Horse use

Different types of horses were accepted from high level sport horses to leisure horses. Approximate half of the horses were dedicated to sport activities and the other half were leisure horses.

The types of horses used are detailed in the following figure:

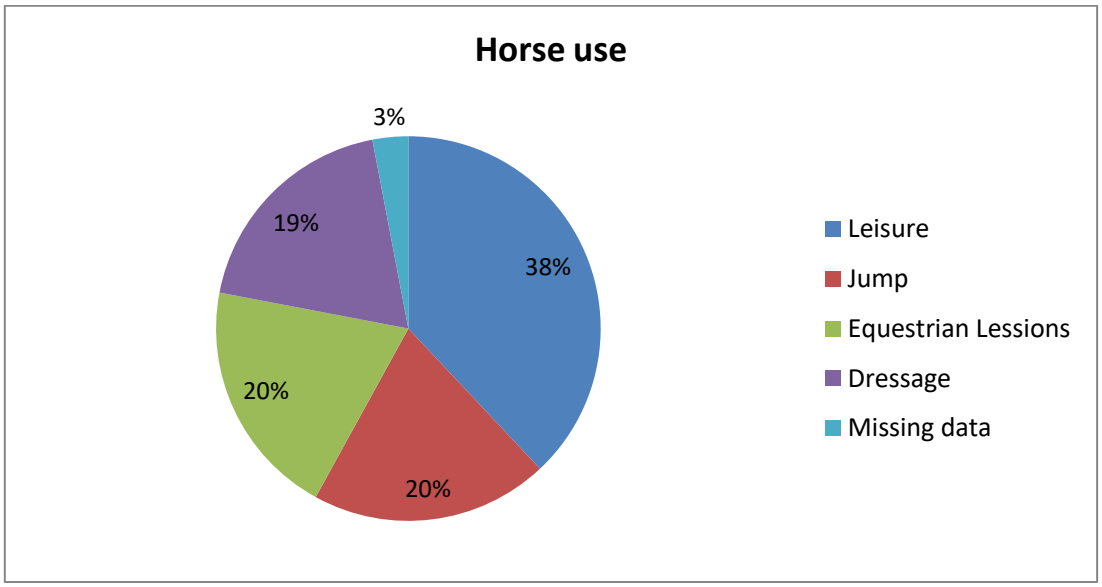

Figure 19: Used of the enrolled horses in the clinical trial

The homogeneity within groups in terms of use of the enrolled horses has been investigated and demonstrated:

PRINCIPAL ACTIVITY IN THE LAST 12 MONTHS	TX			
	Placebo		Treatment	
	N	%	N	%
Jump	7	18.9%	5	16.7%
Dressage	7	18.9%	8	26.7%
Leisure	14	37.8%	11	36.7%
Equestrian Lessons	9	24.3%	6	20.0%
TOTAL	37	100.0%	30	100.0%

	P-Value
Chi-Square Test	0.8882

Table 12: Basal Homogeneity-source

Activity level of horses during the clinical trial:

During the present clinical trial all kind of activity level were allowed. The activity level has been defined as: low (box rest or hand walk), moderate (ridden occasionally 2 days per week), high (ridden at all gaits often), very high (sport competitions or hard work). The activity level of the horses during the clinical trial is described in the following figure:

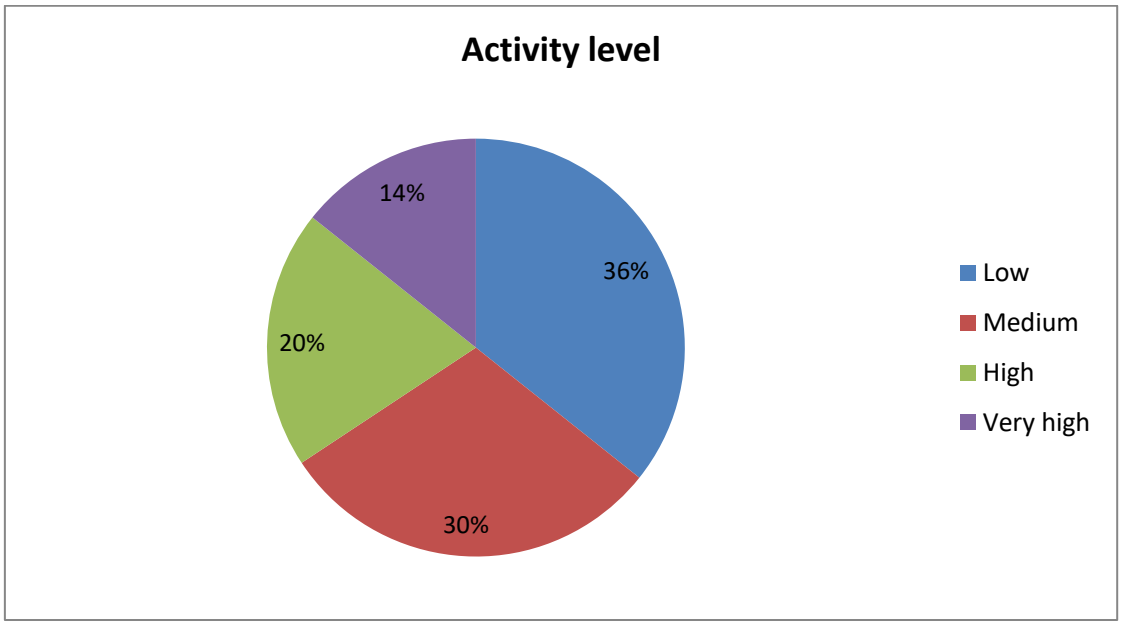

Figure 20 : Activity level of horses during the clinical trial

Both groups were homogenous with respect to the activity level during the present study as represent bellow:

ACTIVITY	TX			
	Placebo		Treatment	
	N	%	N	%
Low	11	29.7%	14	43.7%
Medium	13	35.1%	7	21.9%
High	9	24.3%	5	15.6%
Very high	4	10.8%	6	18.8%
TOTAL	37	100.0%	32	100.0%

	P-Value
LR - Chi-Square Test	0.3580

Table 13: Basal Homogeneity- Activity Level

6.4. Diagnosis

The diagnosis was made as previously described without deviations. During the diagnosis some horses were not enrolled in the study due to different reasons such as: severe OA, presence of joint chips, no clear response to the intra-articular block, etc.

The radiological degree (According Cornelissen Radiographic Scale adapted to all joints) of the enrolled horses is described in the table below:

Radiological degree	Treatment	Placebo
Grade 1	9	7
Grade 2	13	16
Grade 3	10	14

Table 14: Radiological degree according Cornelissen Radiological Scale

The homogeneity within the groups in the radiological degree has been demonstrated as described below:

Radiological degree	TX			
	Placebo		Treatment	
	N	%	N	%
1	7	18.9%	9	28.1%
2	16	43.2%	13	40.6%
3	14	37.8%	10	31.3%
TOTAL	37	100.0%	32	100.0%

	P-Value
LR - Chi-Square Test	0.5545

Table 15: Basal Homogeneity- Radiological degree

With respect to the enrolled joints, several joints were enrolled, though mainly interphalangeal joints were included. The list of the enrolment joints and its distribution within groups is detailed in the table below:

Joint	Treatment	Placebo
Distal interphalangeal	14	12
Proximal interphalangeal	3	12
Metacarpal-phalangeal	8	11
Radiocarpal	1	0
Tarsal	6	1
Stifle	0	1

Table 16: Joint distribution

Interestingly, the joint distribution was not homogeneous within groups (p=0.0426). The impact of the joint in the treatment effectiveness will be evaluated further.

	P-Value	
LR - Chi-Square Test	0.0426	*

Table 17: P-value joint distribution

Considering the lameness evaluation, groups were also balanced before treatment administration as reflected in the tables below:

	TX			
Lameness D0	Placebo		Treatment	
	N	%	N	%
1	5	13.5%	2	6.3%
1.5	2	5.4%	1	3.1%
2	17	45.9%	16	50.0%
2.5	4	10.8%	3	9.4%
3	9	24.3%	10	31.3%
TOTAL	37	100.0%	32	100.0%

	P-Value
LR - Chi-Square Test	0.8210

	Nobs	Nmiss	Mean	Median	Std	Max	Min	Lower CL 95%	Upper CL 95%
Total	69	0	2.2	2.0	0.6	3	1	2.1	2.4
Placebo	37	0	2.1	2.0	0.6	3	1	1.9	2.3
Treatment	32	0	2.3	2.0	0.6	3	1	2.1	2.5

	P-Value
Kruskal-Wallis Test	0.3659

Table 18: Basal Homogeneity- Lameness degree

The balance between groups in terms of joint effusion was also tested and confirmed.

EFFUSION D0	TX			
	Placebo		Treatment	
	N	%	N	%
0	13	35.1%	13	40.6%
1	18	48.6%	14	43.8%
1.5	1	2.7%	1	3.1%
2	5	13.5%	4	12.5%
TOTAL	37	100.0%	32	100.0%

	P-Value
LR - Chi-Square Test	0.9691

	Nobs	Nmiss	Mean	Median	Std	Max	Min	Lower CL 95%	Upper CL 95%
Total	69	0	0.8	1.0	0.7	2	0	0.6	0.9
Placebo	37	0	0.8	1.0	0.7	2	0	0.6	1.0
Treatment	32	0	0.7	1.0	0.7	2	0	0.5	1.0

	P-Value
Kruskal-Wallis Test	0.6945

Table 19: Basal Homogeneity-joint effusion

By last, groups were also balance in terms of flexion pain.

	TX			
Flexion D0	**Placebo**		**Treatment**	
	N	**%**	**N**	**%**
1	7	18.9%	6	18.8%
1.5	1	2.7%	0.	.
2	18	48.6%	18	56.3%
2.5	2	5.4%	0.	.
3	9	24.3%	8	25.0%
TOTAL	37	100.0%	32	100.0%

	P-Value
LR - Chi-Square Test	0.4153

	Nobs	Nmiss	Mean	Median	Std	Max	Min	Lower CL 95%	Upper CL 95%
Total	69	0	2.1	2.0	0.7	3	1	1.9	2.2
Placebo	37	0	2.1	2.0	0.7	3	1	1.8	2.3
Treatment	32	0	2.1	2.0	0.7	3	1	1.8	2.3

	P-Value
Kruskal-Wallis Test	0.9476

Table 20: Basal Homogeneity- Flexion Pain

In addition, the basal homogeneity in terms of the chronicity of the symptoms (expressed in months) has been evaluated, and both groups were comparable:

	Nobs	Nmiss	Mean	Median	Std	Max	Min	Lower CL 95%	Upper CL 95%
Total	69	0	7.5	4.0	7.9	30	1	5.6	9.4
Placebo	37	0	6.6	4.0	6.5	30	1	4.4	8.7
Treatment	32	0	8.5	4.0	9.2	30	1	5.2	11.8

	P-Value
Kruskal-Wallis Test	0.6232

Table 21: Basal Homogeneity- Chronicity

6.5. Efficacy Assessment

6.5.1. Lameness

This study has been designed to demonstrate the efficacy of the intra-articular administration of 15 million Equine Umbilical Cord Mesenchymal Stem Cells (EUC-MSCs) in the treatment of symptoms associated in mild to moderate osteoarthritis in horses under field conditions.

Due to the importance of Lameness grade in the ability of horses to develop normal life, the lameness improvement 63 days after product administration has been established as primary endpoint.

In the treatment group 84.4% of the horses presented some kind of improvement in the lameness grade compared with 48.6% in the placebo group, being this difference statistically significant (p=0.0019 Chi-Square Test).

However, the objective of this study was not only to demonstrate the improvement in the lameness grade of horses treated with EUC-MSCs, on the other hand, the objective of the present research was to investigate if EUC-MSCs were able to produce an improvement in the lameness grade that was substantial enough to allow the horses to perform normal life or sport abilities.

In this respect the primary designed endpoint was a more restricted endpoint, in order to not only ensure the improvement in the lameness grade but also ensure a clinically relevant improvement.

Primary endpoint:

The primary efficacy endpoint was based in the comparison of the percentage of animals classified as Therapeutic Successes at day 63 (± 2) in the treatment group versus the control group inoculated with placebo.

The percentage of animals in the treatment group vs. placebo with an improvement in the lameness grade to a non-lame or an inconsistent lameness (≤ grade 1 AAEP scale) 63 days post treatment was compared.

Represented below are the overall results (in percentage) separated by groups (treatment group and placebo group) in tabular form.

THERAPEUTIC SUCCESS D63	TX			
	Placebo		Treatment	
	N	%	N	%
No	29	78.4%	9	28.1%
Yes	8	21.6%	23	71.9%
TOTAL	37	100.0%	32	100.0%

	P-Value	
Chi-Square Test	<.0001	*

Table 22: Percentage of therapeutic success by groups at da 63

This difference was strongly statically significant (p<0.0001) by Chi-Square test.

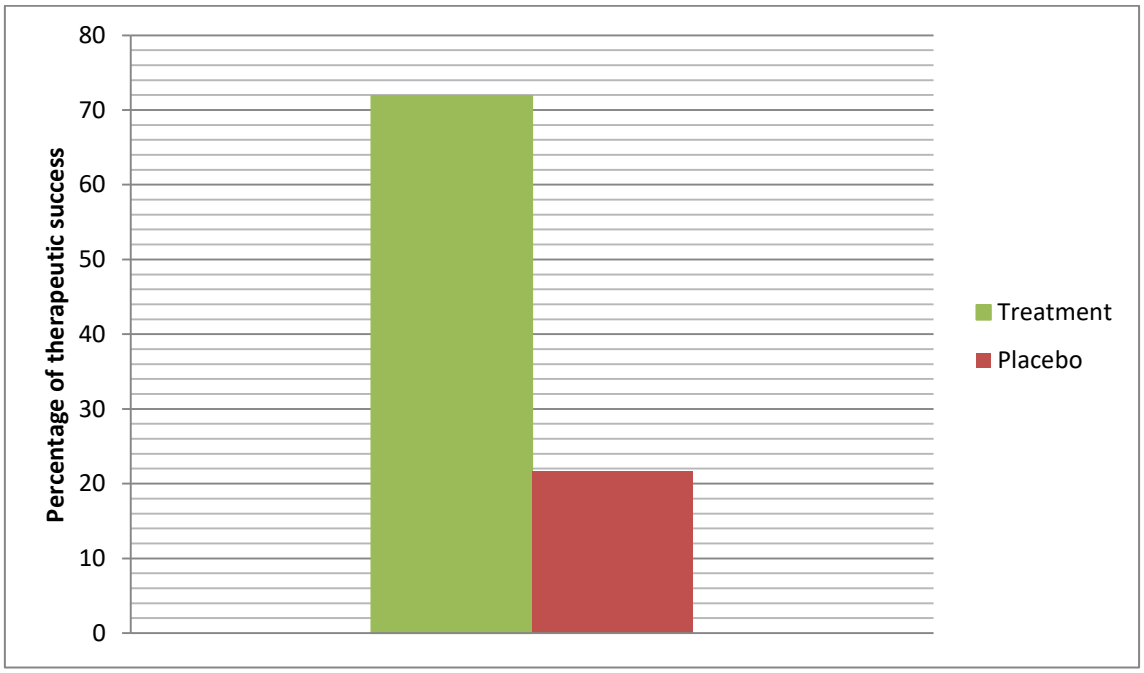

Figure 21: Primary endpoint

Secondary endpoints:

In addition, the same endpoint (improvement in the lameness grade to a non-lame or an inconsistent lameness (≤ grade 1 AAEP scale)) was investigated at day 35 and 14 post treatment.

At day 35 post treatment the efficacy of the treatment group was statistically significantly superior compared to the placebo group (p=0.0066). However, at day 14 both groups had similar efficacy not finding statistical significance (p=0.2369).

THERAPEUTIC SUCCESS D35	TX			
	Placebo		Treatment	
	N	%	N	%
No	29	78.4%	15	46.9%
Yes	8	21.6%	17	53.1%
TOTAL	37	100.0%	32	100.0%

	P-Value	
Chi-Square Test	0.0066	*

Table 23: Percentage of therapeutic success by groups at day 35

THERAPEUTIC SUCCESS D14	TX			
	Placebo		Treatment	
	N	%	N	%
No	29	78.4%	21	65.6%
Yes	8	21.6%	11	34.4%
TOTAL	37	100.0%	32	100.0%

	P-Value
Chi-Square Test	0.2369

Table 24: Percentage of therapeutic success by groups at day 14

In terms of main improvement of the lameness grade, the treatment group had more than 2-fold lameness improvement (according to AAEP lameness scale) compared to a main improvement of the placebo group (1.78 points vs 0.88 points) being this difference statistically significant (p<0.0001).

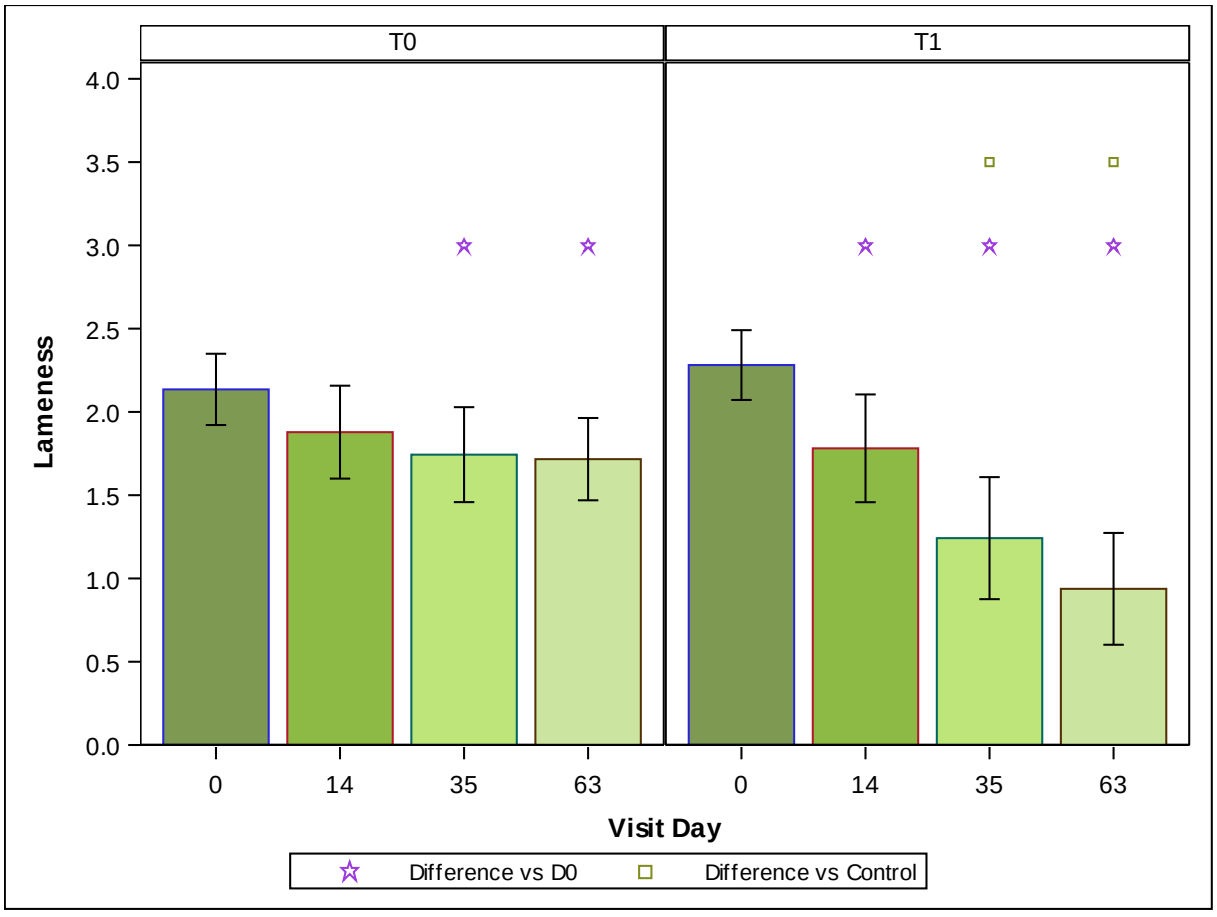

Figure 22 : Lameness longitudinal Analysis (T0 represents placebo horses; T1 represents treatment horses)

Simple Effect Comparisons of t*TX Least Squares Means By t							
Simple Effect Level	TX	_TX	Estimate	Standard Error	DF	t Value	Pr > |t|
t 0	T0	T1	-0.03084	0.1656	207	-0.19	0.8524
t 14	T0	T1	0.2124	0.1656	207	1.28	0.2010
t 35	T0	T1	0.6136	0.1663	207	3.69	0.0003
t 63	T0	T1	0.8940	0.1656	207	5.40	<.0001

Table 25: Logitudinal Lameness statistical significance

6.5.2. Effusion

Here, the average improvement in swelling or effusion in the treatment group, compared with the improvement average in the placebo group was measured.

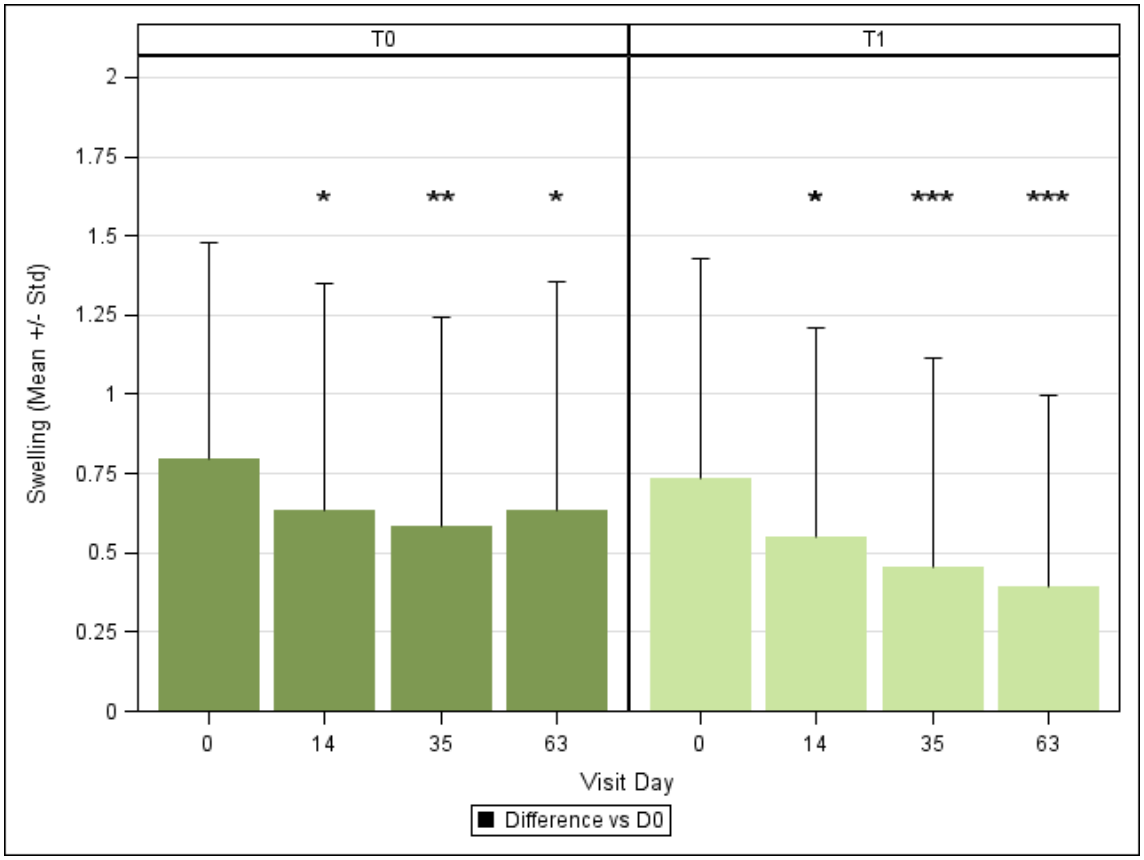

Figure 23: Swelling (effusion) longitudinal Analysis (T0 represents placebo horses; T1 represents treatment horses)

The mean improvement in the effusion or swelling degree in the treatment group was 0.34 points and the main improvement in the placebo group was 0.16 points. Despite having double improvement in effusion degree in the treatment group compared to placebo group, no statistically significant differences were found at any time points between groups.

Simple Effect Comparisons of t*TX Least Squares Means By t							
Simple Effect Level	TX	_TX	Estimate	Standard Error	DF	t Value	Pr > |t|
t 0	T0	T1	-0.1509	0.09913	207	-1.52	0.1295
t 14	T0	T1	-0.1256	0.09913	207	-1.27	0.2067
t 35	T0	T1	-0.08098	0.09944	207	-0.81	0.4164
t 63	T0	T1	0.03069	0.09913	207	0.31	0.7572

Table 26: Swelling statistical significance

6.5.3. Flexion pain

At this point, the average improvement in flexion pain in the treatment group, compared with the average improve in the placebo group was measured.

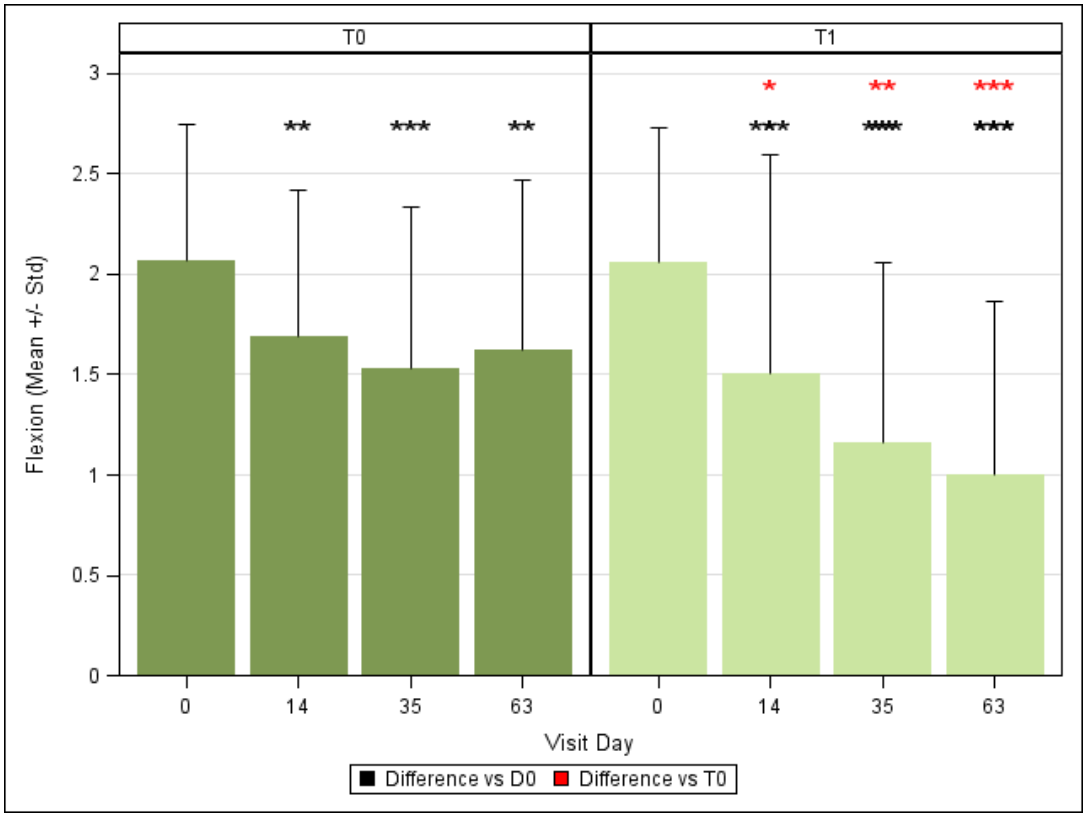

Figure 24: Flexion Pain longitudinal Analysis (T0 represents placebo horses; T1 represents treatment horses)

Differences were observed between the two groups in reference to the flexion pain at day 63 (±2) with a Statistical significance of p< 0.0001. Statistical significance p= 0.0012 was also observed at day 35(±2), and at day 14(±2) (p=0.0224).

Simple Effect Comparisons of t*TX Least Squares Means By t							
Simple Effect Level	TX	_TX	Estimate	Standard Error	DF	t Value	Pr > |t|
t 0	T0	T1	0.2244	0.1775	207	1.26	0.2077
t 14	T0	T1	0.4085	0.1775	207	2.30	0.0224
t 35	T0	T1	0.5868	0.1784	207	3.29	0.0012
t 63	T0	T1	0.8409	0.1775	207	4.74	<.0001

Table 27: Flexion Pain statistical significance

6.5.4. Overall improvement

As seen in previous points, lameness and flexion pain were significantly reduced in all study time points compared with placebo, although this effect was not observed in joint effusion.

It can be seen that the overall score reduction in the summation of lameness + effusion + flexion pain was 2.8 points in the treatment group compared to 1 point in the placebo group, being this difference statistically significant.

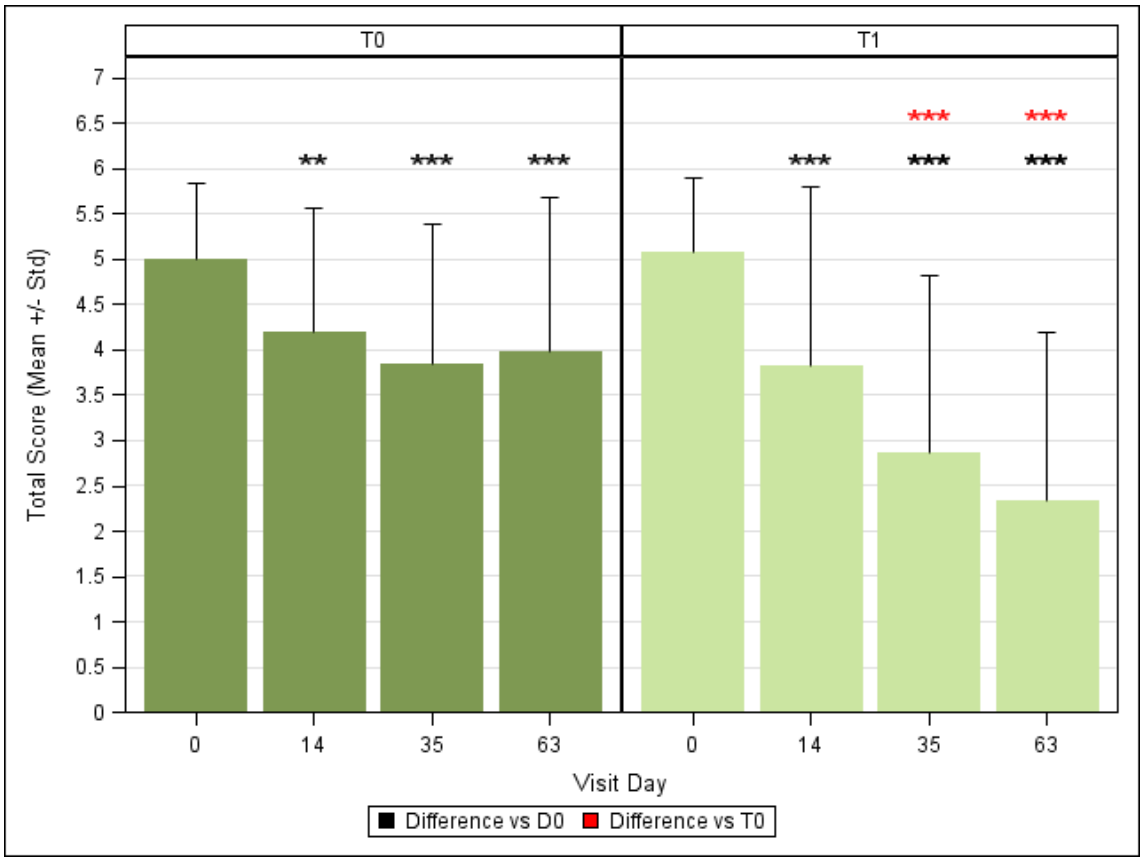

Figure 25: Total Score longitudinal Analysis (T0 represents placebo horses; T1 represents treatment horses)

Simple Effect Comparisons of t*TX Least Squares Means By t							
Simple Effect Level	TX	_TX	Estimate	Standard Error	DF	t Value	Pr > |t|
t 0	T0	T1	0.1209	0.3340	207	0.36	0.7176
t 14	T0	T1	0.5736	0.3340	207	1.72	0.0873
t 35	T0	T1	1.1973	0.3355	207	3.57	0.0004
t 63	T0	T1	1.8439	0.3340	207	5.52	<.0001

Table 28: Overall score statistical significance

6.5.5. Subjective improvement opinion

Owners (rides) and veterinarians were asked about the subjective improvement of the animal 63 days after product administration. The improvement was scored from 0 to 10, being 0 no improvement and 10 complete improvement

The average results obtained (expressed in percentage) in both groups are shown below:

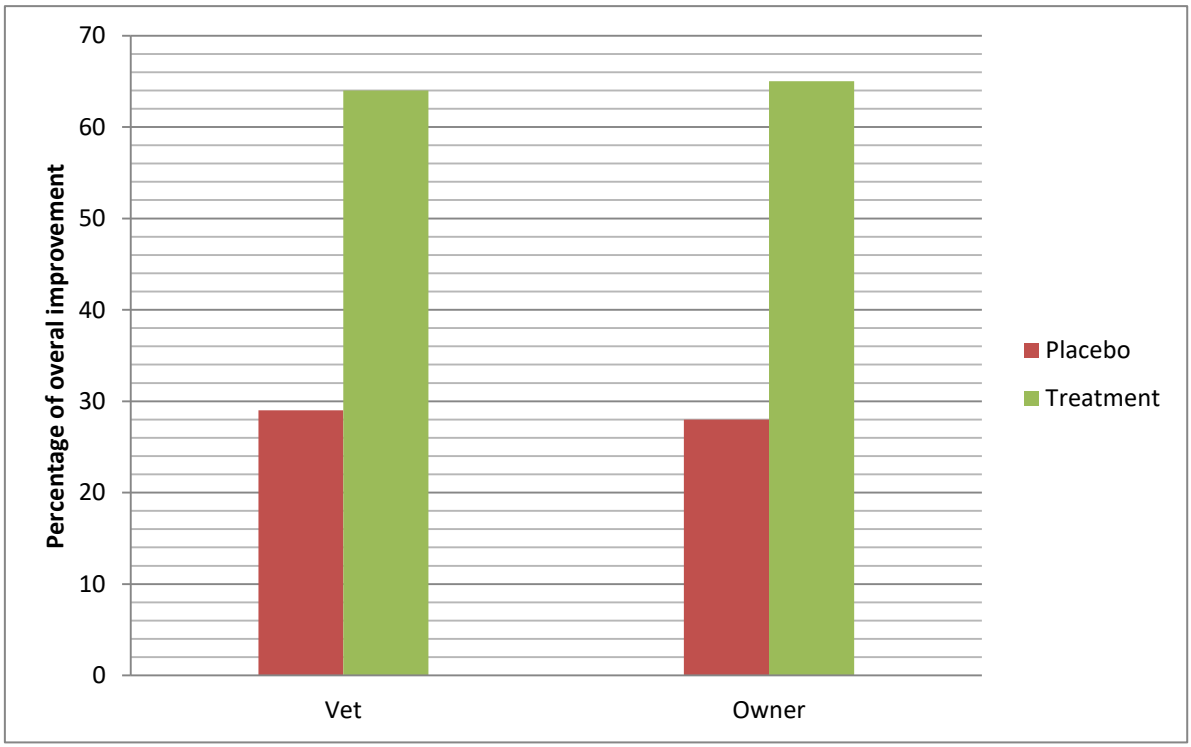

Figure 26: Subjective improvement by owner and vet

As shown in the graph, the improvement in the treatment group was more than 2-fold than in the placebo group.

6.6. Co-variables study

The potentially influence of co-variables or epidemiologic circumstances in the horses has been evaluated in order to elucidate if the efficacy of EUC-MSC could be dependent on certain epidemiological factors.

To assess the impact of the co-variables, the efficacy (defined as lameness reduction to non-lame or inconsistent lameness-primary endpoint) of each co-variable has been evaluated and a statistical analysis has been performed.

Multiple co-variables have been analysed (Table 29) showing that none of the mentioned have influence in the efficacy outcome.

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Lameness	1	48	2.63	0.1113
RX image	1	48	3.37	0.0728
Weight	1	48	2.79	0.1012
Sex	2	48	1.00	0.3756
Age	1	48	0.38	0.5431
Limb	1	48	1.71	0.1972
Life Habits	2	48	1.08	0.3466
Joint	3	48	0.81	0.4944
Chronicity	1	48	0.00	0.9746
Activity level	1	48	0.04	0.8388

Table 29: Gross co-variable study

Those variables that according to the bibliography or clinical criteria may have more influence on efficacy have been evaluated in more detail

Lameness grade: the influence of the lameness grade previous to product administration has been evaluated in the efficacy outcome. It could be expected that the greater the degree of lameness, the less effective the product, however, as seen in the table below the efficacy of EUC-MSC was comparable regardless of the lameness of the horse prior to administration.

Efficacy dependant on lameness grade before product administration	
Lameness grade ≤2 before product administration	78%
Lameness grade ≤3 before product administration	66%

Table 30: Lameness grade and efficacy

Radiologic degree: the influence of the radiologic degree in the efficacy outcome has been evaluated. It could be expected that the higher the degree of radiological signs, the less effective the product, but as can be seen in the table 30, the efficacy rate of MSCs seems to be reduced as the radiological signs increase, however these differences were not statistically significant (p=0.0728).

Efficacy dependant on radiologic degree before product administration	
Rx grade <1	100%
Rx grade ≤2	76%
Rx grade ≤3	60%

Table 31: Radiological Degree and efficacy

Age: the effect that age could have in the efficacy of EUC-MSC is described in the table below. It might be expected that the older the horse is, the lower efficacy of the product. As seen the efficacy of EUC-MSC is very similar in young horses (<10 years) than in mature horses (>10 years). (Table 32)

Efficacy dependant on horse´s age	
≤10 years	70%
>10 years	67.5%

Table 32: Age and efficacy

Chronicity: it could be expected that the more chronic the symptoms are the poor prognostic the treatment has. However as seen bellow the efficacy of EUC-MSC is even better in horses with more than 3 months of symptoms evolution.

Efficacy dependant on horse´s chronicity	
≤3 months	80%
>3 months	60%

Table 33: Chronicity and efficacy

Activity level: the impact of the activity level of the horse after product administration has been evaluated since could be expected that horses with high activity level could have worse efficacy after treatment administration. However, in light of the results, the exercise after treatment does not negatively affect to the efficacy. However, it is important to keep in mind that, in the present study, each horse adapted its level of sport activity based on its clinical condition, so it is possible that horses with less sports activity also had a greater clinical symptomatology.

Efficacy dependant on horse activity level before and after product administration	
Low	58%
Medium	83%
High	80%
Very high	100%

Table 34: Activity level and efficacy

6.7. Comparison of conventional treatments

A comparative assay between conventional treatments Costicosteroids + Hyaluronic Acid (COs+ HA) and EUC-MSC has been done. The information of conventional treatments has been provided by bibliographic references. The information of EUC-MSC has been obtained by the present work and the author's knowledge.

Cost: Considering the high manufacture price of Advance Therapies, the cost of this kind of medicines for the final owner is about 800€, on the other hand, conventional treatments (CO+HA) are quite cheap products with an estimate cost around 200€. Therefore EUC-MSCs receives a score of 2 and CO + HA a score of 5 in the radius graph.

Effectiveness: Considering the effect of the product in lameness reduction. For evaluate the efficacy of CO+ HA the publication of de Grauw, 2015 was used. In this work de Grauw evaluated the intraarticular efficacy of CO+HA in 39 horses. The efficacy in lameness reduction 3 weeks after CO+HA injection was 64%. As seen above the efficacy of EUC-MSC is 72%. The efficacy of both products is between 60-80% therefore 4 points in the graph were assigned for both products.

Adverse Event: Considering the safety of the product in general terms. Temporal- Local adverse effects describe in COs are: 2% risk of joint flare (Harkins & Tobin, 1993) and ˜3% of mild local swelling (de Grauw et al., 2014), on the other hand the risk of joint flare in EUC-MSC is 11% and the incidence of local swelling ~8% (see point 5.11 Adverse Events). In addition the use of COs have been associated with systemic adverse event such us laminitis, adrenal insufficiency, hyperadrenocorticism, despite the incidence of this kind of systemic adverse event is very low could not be ignored.

Moreover, the use of COs has been related with severe deleterious effect in the cartilage homeostasis that potentially accelerates joint degradation or even leading to a devastating disease known as Steroid Arthropathy ((Harkins & Tobin, 1993). Although the negative effects of corticosteroids, they are usually associated with repeated administrations or high doses (Van Weeren & de Grauw, 2010) considering that OA is a chronic disease and that the therapeutic action of corticosteroids is limited, repeated use of corticosteroids is practically inevitable.

Therefore, with respect to adverse event, the total score of EUC-MSCs is 3 points (1 point rare mild effusion + 2 points common joint flare) while the total score of COs + A is 7 (1 point rare mild effusion + 1 point rare joint flare + 2 points very rare systemic adverse event + 3 points deleterious cartilage effect in repeated or high doses). These scores are transferred to the graph by assigning a punctuation of 4 to EUC-MSC and 2 to CO + HA.

Indirect Cost: For the analysis of indirect costs, it has been taken into account: the days of sports loss by doping, the days for sports loss from the application of the product to its effectiveness and relapses from the disease. For evaluate the indirect cost of COs+HA, publications of Harkins et al., 1993, de Grauw et al., 2015 and Souza, 2016 have been used.

COs+HA have lost of sport days due doping that depending on the product could be 15 days; on the other hand, as seen before, the time until efficacy of EUC-MSC is about 30 days; therefore both products are considered to lose useful days until the horses can enter into competition, either by doping or by time until the product is effective.

An important point to assess in the calculation of the indirect cost is the duration effect of the product. It is considered the less duration effect of a product, the more indirect cost associated, since when the horses relapse, the horse needs to be removed from the competition, the veterinarian must be called and treated horse again and a new doping period begins.

The duration of corticosteroids depends on the type of corticosteroid used, the type of injury, the times corticosteroids have been administered before, etc. The most used COs in the equine clinic is triamcinolone with a medium duration (Van Weeren & de Grauw, 2010). In de Grauw (2014) study it was seen that 3 months after treatment with COs+HA less than 50% of the horses were working at previous level. On the other hand the efficacy of EUC-MSC is long term, with more than 70% of the treated horses without relapse 12 months after treatment.

Has been considered that CO+HA have indirect cost due doping period and medium effect duration (2 points score in the radius graph), on the other hand EUC-MSCs have lost of working days due the time until efficacy, but have long-term efficacy, so a score of 4 in the radius graph has been assigned.

Therefore, by representing both products in a radius chart (Figure 27), the product that occupies more area is considered better.

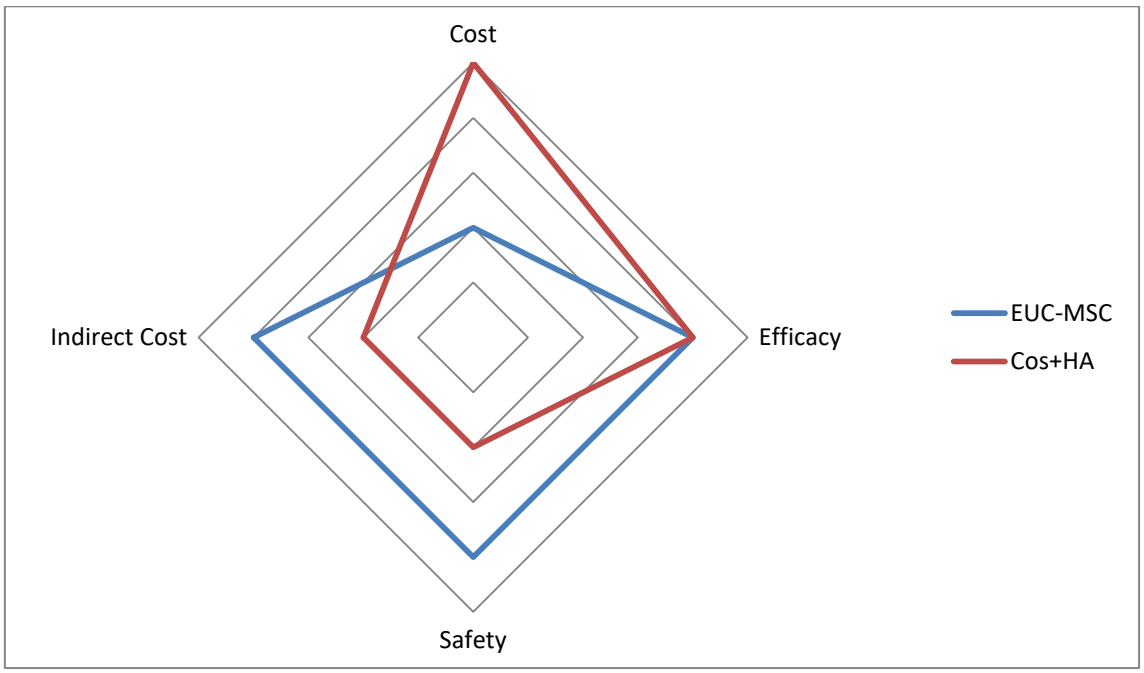

Figure 27: Comparative between EUC-MSCs and COs+HA

Graphically, it can be seen that EUC-MSC is a competitive treatment compared to conventional treatments, despite having a sale price substantially higher than COs + HA, its high safety profile and its lower indirect cost thanks to its long therapeutic effect, generates that EUC-MSCs are not only a real alternative to conventional treatments but also EUC-MSC have fundamental advantages for the equine sector, especially in sports animals.

6.8. Physical Examination

Horses were reviewed daily by the stud staff for general health condition and as per protocol on day -15 to -7, day 0, 1, 14(±2), 35(±2) and 63(±2) by the researcher.

The physical exam included: Attitude, corporal condition, hydration, mucous, denture, lymph nodes, respiratory auscultation, nasal discharge, pulse, quality pulse, cardiac auscultation, peristalsis, injection point and digital pulse.

The physical exam only detected a couple of minor alterations limited to digital pulse (detected in 7 horses), or mild distension (detected in 3 horses) all them related to Adverse Event (see point 4.8).

Eventually non-related product alterations were detected, such as eye discharge (1 horse), Auricular Block Grade II (1 horse) or alterations in the denture (three horses).

6.9. Laboratory Examinations

No clinically relevant data was obtained in the laboratory examinations.

Minor findings detected were previous to the product administration and considered without pathological relevance.

6.10. Safety Assessment

Horses were reviewed daily by the stud staff for general health condition as per protocol on day -15 to -7, day 0,1, 14(±2), 35(±2) and 63(±2) by the researcher.

According to Good Clinical Practices, adverse events are defined as any "untoward medical occurrence" in a patient who receives a drug while participating in a clinical study with or without relation to the study product.

Adverse events (AE) observed in each treatment group during the study have been listed, and their incidences were compared. The relationships between AE and treatment have been classified in probable, possible, and unlikely by the researcher.

Probable:

- Joint Flare:

EUC-MSCs treatments have been related to acute inflammation 24h after its application (Ferris *et al.,* 2013). This described adverse reaction is usually called joint flare, the incidence, description, and resolution of this adverse event is detailed in the table below.

Incidence	Appearance	Description	AE-drug relation	AE resolution
4 horses from 36 treated (11%)	The AE appeared after 24 hours of treatment application	Acute inflammation. Synovitis. Pain. Acute lameness (4-4.5/5 AAEP scale)	Probable	Complete resolution

Table 35: Joint Flare adverse event

Following the World Health Organization criteria a rate classified as "Very Common" with a frequency ≥ 1/10 incidence. Therefore, the incidence of this adverse event has been classified as: very common.

- Local inflammation without lameness associated:

Local inflammations as joint swelling or effusion, and/or mild local oedema but without an acute increase of lameness associated were reported. The incidence, description and resolution of this adverse event are detailed in the table below.

Incidence	Appearance	Description	AE-drug relation	AE resolution
3 horses from 36 treated (8.3%)	The AE appeared after 24 hours of treatment application	Moderate Joint Effusion. Harm at palpation, Subcutaneous Edema, digital pulse. Mild lameness increase	Probable	Complete resolution

Table 36: Mild inflammation adverse event

Following the World Health Organization criteria, a rate classified as "Very Common" with a frequency ≥ 1/10 incidence. Therefore, the incidence of this adverse event has been classified as: very common

Unlikely related:

During this clinical trial, some adverse events occurred without treatment product relation. These unrelated adverse events are detailed bellow:

Incidence	Appearance	Description	AE-drug relation	AE resolution
1 horse from 36 treated (2.7%)	The AE appeared after 14 days of treatment application	Surface wound in both hindlimbs cause by a fall	Unlikely	Complete resolution
1 horse from 36 treated (2.7%)	The AE appeared after 14 days of treatment application	Mild lung mucus	Unlikely	Complete resolution
1 horse from 36 treated (2.7%)	The AE appeared after 14 days of treatment application	Relapse of SDFT lesion	Unlikely	Anticipated Clinical Trial Withdrawal
1 horse from 36 treated (2.7%)	The AE appeared after 25 days of treatment application	Mild Lameness in left hindlimb	Unlikely	Continuous

Incidence	Appearance	Description	AE-drug relation	AE resolution
1 horse from 36 treated (2.7%)	The AE appeared after 19 days of treatment application	Mild Lameness in the right forelimb (contralateral treatment limb)	Unlikely	Continuous

Table 37: unlike related adverse event

6.11. Long Term Follow up

A follow up 2 years after product administration was made in order to know the efficacy of the long-term of the product and evaluate the occurrence of adverse effects not detected in the initial evaluation period. Data from some horses were lost during the 2 years follow up.

The following results were found:

Safety: No horse recorded any adverse events related to the product during the follow-up time. Special attention was paid to the possibility of tumour occurrence, although stem cells manufactured according Good Manufacturing Practice have demonstrated their wide assurance in regards to tumour formation (European Veterinary Guideline: Questions and answers on allogenic mesenchymal stem cell-based products for veterinary use: specific questions on tumorigenicity) the relationship of tumours and MSC is an unfairly frequently performed association. After this study, it can be confirmed that there is no relationship between the administration of EUC-MSCs and the formation of tumours in horses.

Efficacy: The incidence of relapse in the clinical sings (lameness) and the need of re-treating have been evaluated. Veterinarians were asked when the horse needs to be re-treated due relapse in the lameness (Table 38). As can be observed only ~16% of the animals were re-treated before 12 months after EUC-MSC treatment, in addition more than 40% of the animals still working at normal level 2 years after product administration.

Relapse before 6 months	Relapse 6-12 months	Relapse 12-24 months	No relapse in 2 years follow up	Lost data
8.3%	8.3%	29.2%	42%	12%

Table 38: Relapse after EUC-MSC treatment

7. DISCUSSION

7. DISCUSSION

Equine OA is a chronic and devastating disease for equine sports medicine that presents itself with local inflammation, pain, lameness and loss of joint congruence.

In this study all horses enrolled presented the typical clinical signs of OA as: lameness, flexion pain and joint effusion.

It is a double-blind design, common in the design of studies for scientific and regulatory purposes (Denoix *et al.,* 2003; Lynn *et al.*, 2004; Back *et al.*, 2009; Gough *et al.,* 2010 and Koene *et al.,* 2010) that allowed to evaluate the efficacy of the product by clinical veterinarians in an impartial and objective manner.

Regarding the multicentric design, the equine bibliography highlights two types of multicentric designs: multicentric with two or three reference centres where the veterinarians derive their cases (Denoix *et al.*, 2003; Back *et al.*, 2009 and Koene *et al.*, 2010) or studies where multiple researchers are recruited throughout the country's geography and follow the protocol of the study without deriving the animals to a reference hospital (Lynn *et al.,* 2004 and Gough *et al.,* 2010).

For the present design, the methodology study was chosen with multiple researchers for two reasons:

1) It was considered that the inclusion of many researchers better represented the conditions of field use, the final objective that every clinical study should have, by increasing the variability of enrolled animals.

2) Allowed a greater visibility of the study and availability for enrolment.

With respect to the duration of the study, when designing a clinical study, it is essential to know the mechanism of action (MoA) of the product. As earlier described, the MoA of the MSCs is through a complex paracrine mechanism mediated mainly by the secretion of cytokines (PGE2) that exert an anti-inflammatory and immunomodulatory function in the joint.

This complex MoA causes the therapeutic effect of MSCs to be attained after a few weeks after administration, as previously reported by other research groups. Broeckx *et al.,* (2013) reported that the improvement after the application of MSCs in horses with naturally occurred OA is time dependant, increasing the improvement over the weeks. The delayed effect after the use of MSCs was also reported by other authors like Saulnier *et al.,* (2014) and Mokbel *et al.,* (2011) where improvement in the animals treated with MSCs increased from two months post-treatment.

Considering the bibliography, the primary efficacy endpoint was established 2 months after treatment administration. In line with the literature, the effectiveness of EUC-MSCs has been progressive over time, on day 14 post-administration the efficacy rate was placed in the 34% while two months after the administration, the efficacy was 75%.

Another of the fundamental points of the design of this study (and in general of any study) is the establishment of the primary efficacy endpoint.

The primary endpoint was selected because an improvement to a non-lame or inconsistent lameness (1 point or less according to the AAEP scale) represents a clinically relevant improvement that allows a horse to re-enter normal training and sport life. In addition, this is the criteria of the Fédération Equestre Internationale (FEI) for participating in international competitions. (FEI 2018 Veterinary Regulations)

In many other equine OA-studies, a parameter of 1 grade reduction in lameness (AAEP scale) has been defined as therapeutic success (Koene *et al.,* 2010). This means that a horse would be classified as therapeutic success if its lameness grade was reduced from 3 points to 2 points. Despite one point lameness grade reduction was occurred the horse still present a consistent lameness grade that prevents the horse to perform normal working.

Therefore the primary efficacy endpoint in this trial was a more restrictive and clinically relevant endpoint, and it was selected to ensure that horses had a real improvement that allows them to recover normal life, not only a partial improvement.

A total of 76 horses were enrolled from different breeds, life habits and horse use. Considering that veterinarians from all parts of Spain (central, north, east and south) and the diversity of veterinarians participating (more than 20 different vets) in the present study, it can be considered that the sample of enrolled horses represents the Spanish equine population.

As previously reported by Todhunter and Lust, (1992) due to the clinical symptoms, in the present work many of the horses had to reduce their level of sport activity once the symptoms appeared, one of the main non-clinical symptoms associated with this disease.

In this work the diagnosis of the disease was designed according to the gold standard in equine practices, and has been previously reported in other clinical trials: Lynn *et al.,* 2004*;* Cayzer *et al.,* 2011*;* Tnibar *et al*., 2015.

The lameness grade has been elected as the primary endpoint since is the most limiting symptom in equine practices. Likewise as previously explained, the objective of

this work was not limited to reduce the degree of lameness, it aimed was to reduce the level of lameness to a non-lame or inconsistent lameness that would allow the horse to restore its normal work. Therefore, it has been considered that those horses that have reduced their lameness to non-lame or inconsistent lameness could have an activity level at least similar to the one before the symptoms appear.

Treatment with MSCs from the umbilical cord have shown great efficacy in the lameness reduction to a non-lame or inconsistent lameness (primary endpoint). This lameness reduction is similar to the one obtained with allogenic chondrogenic induced peripheral blood MSCs in a recent study developed under Good Clinical Practices in Belgium (Broeckx *et al.,*2019).

In addition to the measured objective made by the veterinarians using the AAEP guidelines, a subjective improvement assessment was made by both the owner and the researcher. In this subjective assessment both the owner and the veterinary were asked about the improvement in cold immediately outside the box, during the exercise practice, the degree of improvement in the sport skills and pain sensation.

The mean subjective improvement of the horses treated with EUC-MSC was 65% by the owner and 64% by the veterinarian compared with 28% (owner) and 29% (veterinary) in the placebo group.

In the work of Broeckx *et al.,* (2019) also the owner was asked about the subjective improvement with a 74.8% of overall improvement. This improvement could be considered slightly higher than the one observed with EUC-MSCs. However, it is important to highlight that in the Broeckx *et al,* study only one question was made, “improvement”. In the present study, 4 different questions were made, therefore, the variability in the response is higher and the mean improvement is reduced.

Interestingly, in the study carried out here, the subjective evaluation of the owner was not only made, but also the veterinarian was asked. The high correlation in the results observed between veterinarian and owner is particularly interesting. This fact highlights the high clinical judgment of equine owners in the improvement assessment of their animals.

Another interesting part of this study is the deep investigation made on the possible co-variables involved in the efficacy outcome of EUC-MSCs.

In the bibliography, it has been reported that different co-variables that could affect the effectiveness in OA treatments could exist. Kristiansen *et al.,* (2007) reported that the lameness grade negatively affects the response to the treatment. On the other hand, Dyson *et al.,* (1991) did not find any relationship between lameness grade and

response to treatment. However, the efficacy of EUC-MSC is not affected by the equine lameness grade before product administration.

With respect to the radiographic signs Kristiansen *et al.,* (2007) reported that severe radiographic changes negatively affect the prognosis. In the present study the same seems to occur; however, this difference was not statistically significant (p= 0.0728)

According to the age of the animal the correlation between age and responses to treatment in osteoarthritis (OA) has not been well established in the bibliography. It would be expected that older animals should have worse results although, Kristiansen *et al.,* (2007), reported in their study that younger horses had worse treatment prognosis than adult horses. On the other hand, de Grauw *et al.,* (2014) showed that old horses (more than 13 years old) had a greater incidence of treatment failure when treated with triamcinolone + hyaluronic acid. In this study the efficacy was not affected with the age of the animal.

Another point that could have impacted in the effectiveness of the product from a theoretical point of view could be the chronicity. However, a negative correlation between chronicity and success of the treatment was not reported in Spadari *et al.,* (2014) study, were the efficacy in acute OA was 71.43% vs 95.73% in the chronic OA group. On the other hand, Kristiansen *et al.,* (2007) reported that horses with chronicity ≤ 3 months showed better results than the ones with more lameness chronicity. However in Kristiansen's study horses suffered from several conditions involving tendon and ligament lesions, which could explain the contradictory results obtained when compared with Spadari's work were only OA horses were included.

In addition, the anatomical location of the injury could also affect the efficacy of the product, considering that some joints could have better prognosis than others. However, this hypothesis has not been reported previously in the bibliography. Works from Spadari *et al*., (2014); de Grauw *et al.,* (2014) and Broeckx *et al.,* (2013) where different joints were enrolled, did not show that the efficacy of the treatment could depend on the treated joint.

According to the bibliography the impact of different co-variables in the efficacy of an intra-articular product in OA diagnosed horses is not clear, therefore, in the present study, the possible impact of co-variables has been deeply investigated.

The election of the co-variables was based in veterinary parameters: clinical experience of the author and bibliographic research, considering that those variables could have some kind of influence in the efficacy of the product. The investigated co-variables were: lameness grade, radiological degree, age, life habits, affected joint, chronicity and activity level. The statistical study performed showed that none of the aforementioned co-variables had influence in the efficacy outcome.

The efficacy of MSCs in horses with mild to moderate OA can be considered to be an efficacy not dependent on environmental or epidemiological factors. Therefore, EUC-MSC can be considered to have a solid efficacy in horses with OA regardless of their age, affected joint, level of sports activity, radiological changes, degree of lameness, etc.

In light of the results, EUC-MSC can be considered a real therapeutic alternative to conventional treatments (COs + HA). As it has been seen, the effectiveness of both products is comparable, however, the safety profile of the EUC-MSC together with its long-time therapeutic action. This provides some medium-term advantages that make the EUC-MSC can be an excellent therapeutic alternative to conventional treatments, especially in sports or young horses where repeated administration of corticosteroids can be widely contraindicated.

According to safety, the health status of the animals was monitored widely throughout the entire study; horses were examined 24h after product administration and at day 14, 35 and 63 by the veterinarian. In addition, the horse owner (or horse caregiver) checked daily the animal and in case signs of illness were observed the veterinary should be called immediately.

During the present study no systemic symptoms related to the administration of EUC-MSCs were detected. No anomalies were detected in the biochemical or haematological values of the horses after the administration of the product. Likewise, no adverse events related to the injection point were detected.

However, there were local orthopaedic adverse events (AE) directly related to the administration of the product.

With ≒10% incidence, horses treated with EUC-MSCs presented an acute inflammation accompanied by local pain and severe lameness, 24 hours after the administration of the product. This phenomenon commonly known as *joint flare* has been previously described in the equine practice after use of cellular products or even conventional medicines (Ferris *et al.,* 2014).

This *joint flare* after the administration of mesenchymal stem cells products have been reported after both autologous and allogenic MSCs with similar incidence. In Ferris´s *et al.* work, the incidence of *joint flare* after autologous bone marrow MSC administration in horses was 9%. In addition, the incidence of *joint flare* reported in the scientific information of two very common hyaluronate sodium for horses Hyvisc® and Hylartin-V® reported and incidence of 12% and 9% respectively (Ferris *et al.,* 2014). This *joint flare* incidence is comparable to the one obtained after EUC-MSCs administration.

It is important to point out that, as described Ferris´s publication, despite the adverse event, the efficacy of the product was not negatively affected. In the present study, 3 out of the 4 horse with *joint flare* of them were classified as therapeutic success (75%); so the efficacy of the product was not negatively affected by the appearance of this AE.

In all the *joint flare* cases registered in the study, horses were treated with NSAIDs for pain control and lameness for 3 days. After 24h the horses presented a substantial improvement and complete resolution 2 weeks after.

Another point of special relevance in the use of allogenic cell products is to clarify the reason for this inflammatory reaction, which could be confused with an immunological reaction to the allogenic use of MSCs by its nature.

However, despite the fact that the exact cause of the *joint flare* is not clear, it seems clear that an immunological reaction to the allogenic use of MSCs is not the cause.

Two reasons support it:

1) the incidence of *joint flare* in autologous products is similar to allogenic products

2) MSCs lack Mayor Histocompatibility Complex II (MHC-II) so immune response is not expected.

Some authors have postulated that it may be due to a reaction to the Fetal Bovine Serum (FBS) used for the cellular expansion in this type of products. In fact, anti-FBS antibodies have been found in horses prior to MSC injection, maybe due to vaccines preparations; however the titres showed no increase after MSCs application (Barrachina *et al.*, 2018). Therefore, the relevance of this is not fully understood.

Another cause that can contribute to *joint flare* is that the joint is a closed space with a barrier (the synovial membrane) that does not allow the proteins to be balanced between systemic circulation and joint causing a greater local inflammation.

In addition, in the experience of the author, also the exquisite sensibility of the equine joints could have an influence in the incidence of *joint flar*e, since in dogs after intraarticular administration of autologous, allogenic or even xenogeneic MSCs, the incidence of *joint flare* is much lower than in horses (unpublished data).Therefore, the cause of *joint flare* can be considered to be multifactorial.

In the author's experience, this incidence can be significantly reduced if prior to the administration of EUC-MSC a single dose of NSAIDs is administered to the horse

(unpublished data). The use of NSAIDs as preventive of *joint flare* adverse event in advance therapies have been previously use in Broeckx *et al.,* 2019.

By last, both the efficacy and the safety have been evaluated in a long-term study. Although it is true that this part of the study was not carried out under good clinical practices and that the veterinarian was not blind and therefore certain bias could occur, it is indisputable that the efficacy of the EUC-MSC is long-term. 70% of the horses did not relapse in their symptoms or need re-treatment in the following 12 months after EUC-MSC administration. According to the safety none of the animals suffered adverse event suspected of being related to EUC-MSC along the 2-years of the follow up. However, the results from this 2 years follow-up study are interesting since it is important to highlight that the use of concomitant treatments, change in routine habits, and change of activity level was not registered so their impact in the efficacy could not be ignored.

8. CONCLUSIONS / CONCLUSIONES

8. CONCLUSIONS

In this work, we can conclude that:

1. The robust design of the present clinical study, following the European directives and regulations related to the design and conduct of clinical trials, together with an execution without relevant deviations has allowed to obtain solid and reliable results. The use of EUC-MSC in horses with mild to moderate OA has proven effective in reducing lameness in horses under field conditions. 72% of the horses treated with EUC-MSC presented a reduction in the degree of their lameness (non-lameness or inconsistent lameness) 35 days post administration onwards.

2. The EUC-MSCs have proven to be safe in their intra-articular use. According to the data obtained, the EUC-MSCs are safe in their allogeneic use, presenting no serious or systemic adverse effects in any treated animal. However, local adverse effects such as joint effusion and lameness have been detected with a relatively high incidence (10%) after product injection. These local inflammations, commonly known as *Joint Flare*, are self-limiting, do not negatively affect the effectiveness of the cells and do not leave sequel in the horse.

3. The present work has shown that the efficacy of the EUC-MSC in reducing equine lameness is consistent and is not affected by epidemiological factors such as the age of the horse, the affected joint, the chronicity of symptoms, the level of activity sports, etc.

4. The EUC-MSC can be considered an innovative alternative treatment to conventional treatments since its high efficacy combined with high safety profile highlight EUC-MSC as a clear therapeutic alternative for horses with mild to moderate OA.

CONCLUSIONES

De este trabajo se pueden extraer las siguientes conclusiones:

1. El robusto diseño del presente estudio clínico, siguiendo las directivas y normativas europeas relativas a diseño y ejecución de ensayos clínicos, unido a una ejecución sin desviaciones relevantes al protocolo han permitido obtener unos resultados sólidos y confiables. El uso de EUC-MSC en caballos con OA de leve a moderada ha demostrado ser eficaz en la reducción de la cojera en caballos en condiciones de campo. El 72% de los caballos tratados con EUC-MSC presentaron una reducción del grado de su cojera a partir de 35 días post administración, quedando sin cojera o con una cojera inconsistente.

2. Las EUC-MSC han demostrado ser seguras en su uso intra-articular. Según los datos obtenidos, las EUC-MSC son seguras en su uso alogénico, no presentando efectos adversos graves ni sistémicos en ningún animal tratado. Sin embargo, se han detectado efectos adversos locales como efusión articular y cojera con una incidencia relativamente alta (10%) después de la aplicación del producto. Estas inflamaciones locales, comúnmente conocidas como *Joint Flare*, son autolimitantes, no afectan negativamente la efectividad de las células y no dejan secuelas.

3. El presente trabajo ha demostrado que la eficacia de las EUC-MSC en la reducción de la cojera equina es consistente no viéndose afecta por factores epidemiológicos como la edad del caballo, la articulación afectada, la cronicidad de los síntomas, el nivel de actividad deportiva, etc.

4. Las EUC-MSC pueden ser consideradas un tratamiento innovador alternativo a los tratamientos convencionales ya que su alta eficacia unida a su baja tasa de efectos adversos posicionan a las EUC-MSC como una clara alterativa terapéutica para caballos con OA de leve a moderada.

9. BIBLIOGRAPHY

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