Orthobiologic use for equine joint disease

by

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Abstract

Osteoarthritis (OA) is the leading cause of musculoskeletal disability in people and Horses. There is a critical need for better understanding of new therapeutics and their effects on the disease. Orthobiologics are biologically derived products processed and used to promote repair or regeneration of injured musculoskeletal tissues. The overarching aim of this body of work was to investigate the use of orthobiologic therapies by equine veterinarians and gain a better understanding of the effects of certain orthobiologic therapies such as autologous conditions serum (ACS) and autologous protein solution (APS) compared to corticosteroids [triamcinolone acetonide (TA)].

The first presented study is a survey sent to national and international equine practitioners to evaluate their use of orthobiologics. Orthobiologic use has increased among practitioners, with an observed preference for blood-based, mostly point- of-care products to treat acute joint-related pathology compared to past years. However, corticosteroids remain the most widely used intra-articular therapeutic among equine practitioners.

In vitro studies were completed using synovium and cartilage co-culture systems stimulated with IL-1β. The first presented study evaluated the effects of 10% equine serum supplementation compared to serum-free media for culture of synovial tissues. Supplementation of the media with 10% equine serum provided chondroprotective effects more evident over long-term (> 9 days) culture. Based on the results of the study using serum-free media to study OA *in vitro* is recommended.

Using the same *in vitro* model, the effects of ACS and APS obtained from the same horse were compared to a common intra-articular treatment, TA. PGE_2 concentrations in media were

significantly reduced following treatment with APS and ACS, while TA did not reduce PGE_2 significantly.

The effect of ACS and TA were also compared in a *in vivo* synovitis model stimulated with IL-1β. In this study, intra-articular injection of IL-1β with ACS produced the highest total nucleated cell count within synovial fluid, but surprisingly the lowest lameness scores compared to IL-1β alone or IL-1β + TA. The PGE_2 concentration in synovial fluid was lower after ACS and TA administration with IL-1β when compared to IL-1β alone. However, TA with IL-1β caused an increase in cartilage metabolism measured by increased glycosaminoglycans in the synovial fluid compared to PBS, IL-1β alone, ACS alone or in combination with IL-1β.

Results provide evidence that orthobiologics may offer an improved strategy for horses with naturally occurring OA, compared to the standard treatment of TA, by decreasing the concentration of PGE_2, one of the most important pro-inflammatory proteins in OA disease.

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18-S	18S ribosomal RNA
ACAN	Aggrecan
ACPCs	Articular cartilage progenitor cells
ACS	Autologous conditioned serum
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
AGEs	Advanced glycation end products
APS	Autologous protein solution
B2M	Beta-2-Microglobulin
BM-MSCs	Bone marrow-derived stem cells
BMP	Bone morphogenic protein
BS	Betamethasone sulfate
COMPs	Cartilage oligomeric matrix protein
CT	Computed tomography
EMC	Extracellular matrix
GAGs	Glycosaminoglycans
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HA	Hyaluronic acid
IGF-1	Insulin-like growth factor 1
IL	Interleukin
IL-1rap	Interleukin 1 receptor antagonist protein
MMP	Matrix metalloproteinases
MPA	Methylprednisolone acetate

MRI	Magnetic resonance imaging
MSCs	Mesenchymal stem cells
NO	Nitric oxide
OA	Osteoarthritis
OARSI	Osteoarthritis research society international
PAHG	Polyacrylamide hydrogel
PET	Positron emission tomography
PGE_2	Prostaglandin E_2
PGs	Proteoglycans
PRP	Platelet-rich plasma
PSGAG	Polysulfated glycosaminoglycans
qPCR	Quantitative polymerase chain reaction
SCAMP-3	Secretory Carrier Membrane Protein 3
SF	Synovial fluid
SLRPs	Small leucine-rich proteins/proteoglycans
sTNF-R	Soluble tumor necrosis factor receptor
TA	Triamcinolone
TGF-β	Transforming growth factor-beta
TIMP	Tissue inhibitor of metalloproteinases
TNCC	Total nuclear cell count
TNF-α	Tumor necrosis factor alfa
TNF-R	Tumor necrosis factor receptor
TP	Total protein

Chapter 1

INTRODUCTION

Lameness due to joint injury or disease is one of the most prevalent causes of reduced or lost performance, placing a significant economic hardship on the equine industry.[1-3] Osteoarthritis (OA), is the most common chronic degenerative disease of the joint that affects all mammalian species.[4] Treatment of OA makes up the majority of an equine veterinarian's caseload.[5] Multiple tissues such as synovial membrane, articular cartilage, subchondral bone, and peri-articular soft tissue structures participate in the disease process.[6,7] Therefore, it is essential that veterinarians understand joint anatomy and how anatomy contributes to disease progression, to understand the mechanisms of treatment. Intra-articular therapies aim to control ongoing inflammation while minimizing the degenerative changes associated with chronic OA. Two categories of intra-articular therapies are described: symptom-modifying osteoarthritis drugs (SMOAD) and/or disease-modifying osteoarthritis drugs (DMOAD). SMOAD are drugs that decrease the inflammatory process, producing an improvement in clinical signs, while DMOAD not only modulate the inflammatory response but also inhibit or reverse OA progression. Orthobiologics are biologically derived SMOAD processed, used to promote repair or regeneration of musculoskeletal tissue. Since the main hallmark of OA is cartilage loss, an ideal DMOAD would prevent cartilage loss and enhance cartilage regeneration. The studies included here were undertaken to further understand how equine practitioners use intra-articular therapies, with particular focus on the use of orthobiologic therapies; and how these therapies modify the cellular response to protect the synovial environment.

Chapter 2 reviews joint anatomy and pathophysiology of OA, then describes *in vitro* and *in vivo* experimental models of OA. Tools for OA diagnosis and current intra-articular therapies

used in equine medicine are described. Factors that promote inflammation and catabolism that lead to disease progression are described. Understanding natural progression of disease (OA) will help to further explain the proposed mechanism by which different intra-articular therapies treat the disease process. I*n vitro* models of OA are necessary to advance understanding of OA and to evaluate the effects of treatments on synovial cells and/or tissues. Results from these various *in vitro* models allow promising treatments to progress into testing within *in vivo* experimental models prior to testing in clinical trials or using in clinical patients. Researchers have utilized different *in vivo* experimental models, each model having unique advantages and disadvantages, which are explained in this chapter. The most common intra-articular therapies used in horses are described, including corticosteroids, orthobiologics, and synthetic articular therapies. Orthobiologics are defined as biologic treatments derived from cells and/or tissues of the body to treat musculoskeletal injury. Orthobiologic therapies are often used to treat soft-tissue and articular injuries of the musculoskeletal system; however, this review will primarily focus on the intra-articular effects of commonly used orthobiologics in articular injury of the horse. The main *in vitro* and *in vivo* effects observed in corticosteroids, orthobiologics including gene therapy, and synthetic scaffolding products are described in this chapter.

Chapter 3 includes a summary of the experimental objectives and hypotheses addressed in the subsequent experimental chapters. A total of 4 studies consisting of one survey, two *in vitro* studies, and one *in vivo* study were performed to answer the objectives for the body of work presented.

Chapter 4 includes the results of a survey evaluating practitioner use of non-steroidal intra-articular therapies to treat joint disease in horses. The number of different orthobiologic therapies available on the market is increasing, and it seems that these products are gaining

popularity among practitioners and the horse owning public in their use. Although the survey depended on practitioner opinion and recall versus careful review of medical records, this study aimed to understand practitioner use and perception of orthobiologic product efficacy for the treatment of joint disease. Products surveyed for practitioner use data included platelet-rich plasma (PRP), autologous conditioned serum (ACS), autologous protein solution (APS), cellular products, and polyacrylamide hydrogel (PAHG).

Chapter 5 includes a study evaluating the effects of serum supplementation or serum-free media on a long-term (9 days) co-culture model of OA. Co-culture of articular cartilage, synovium, and subchondral bone has been shown to emulate naturally-occurring OA better than single cell and/or tissue (explant) culture due to the significant cross-talk these tissues undergo within the synovial environment with and without disease. Culture media used in *in vitro* OA models has not been standardized, and different additives, including serum, could affect how the tissues respond to induction of OA.

Chapter 6 describes the use of a synovial and cartilage co-culture system stimulated with interleukin 1 beta (IL-1β) and evaluated the effects of common intra-articular treatments triamcinolone acetonide (TA), ACS, and APS. The cellular and cytokine profile of these biological products was measured, and gene expression of synovial tissues was evaluated using qPCR. Additionally, prostaglandin E2 (PGE_2) was measured in the media to evaluate the degree of inflammation present after treatment.

Chapter 7 studies the effects of treatment with ACS and TA in an *in vivo* synovitis model. Synovitis was induced with recombinant equine IL-β in the metacarpo- and metatarsophalangeal (fetlock) joints of horses. In this study, each horse served as its own control. Horses were evaluated clinically before and after induction of synovitis to evaluate clinical effects (reduction

in effusion, lameness, etc). Synovial fluid was collected at times 0, 8, 24- and 48-hours post-injection with IL-1β. Fluid analysis, cytology and cytokine measurement were performed on synovial samples.

Chapter 8 summarizes the presented body of work and discussing experimental conclusions of the included studies. Future directions to build on this body of work are briefly discussed.

Chapter 2

LITERATURE REVIEW

❖ OSTEOARTHRITIS AND JOINT ANATOMY

A diarthrodial joint consists of hyaline cartilage attached to a subchondral bone plate constrained by a joint capsule and synovial fluid.[8] The mechanical integrity and normal cellular processes that maintain homeostasis of subchondral bone, articular cartilage, synovial membrane, and peri-articular soft tissues help maintain normal joint function supporting seamless locomotion (Figure 2.1). Joint disease and, in particular, OA is estimated to be the cause of lameness in 60% of the horse population.[9,10] Previously, OA was considered a disease primarily affecting articular cartilage. However, consideration of the joint as an organ system, in which multiple tissues communicate to support function, has been well accepted. Therefore, any of these synovial tissues can be damaged and perpetuate injury and dysfunction in other synovial tissues.[11,12] The Osteoarthritis Research Society International (OARSI) has defined OA as "a progressive disease of synovial joints that represents failed repair of joint damage that results from stresses that may be initiated by an abnormality in any of the synovial joint tissues, including articular cartilage, subchondral bone, ligaments, menisci (when present), periarticular muscles, peripheral nerves, or synovium".[13] In normal conditions, all joint tissues are in constant metabolic turnover, finding a balance between anabolic and catabolic pathways. OA breaks this equilibrium, and the catabolic pathway is increased, ultimately leading to degeneration of synovial tissues. This could lead to observed clinical signs such as pain, stiffness, lameness, and loss of function in patients.[11] Therefore, it is crucial to understand the role of these structures and how they participate during the disease process in order to choose the best treatment option available.

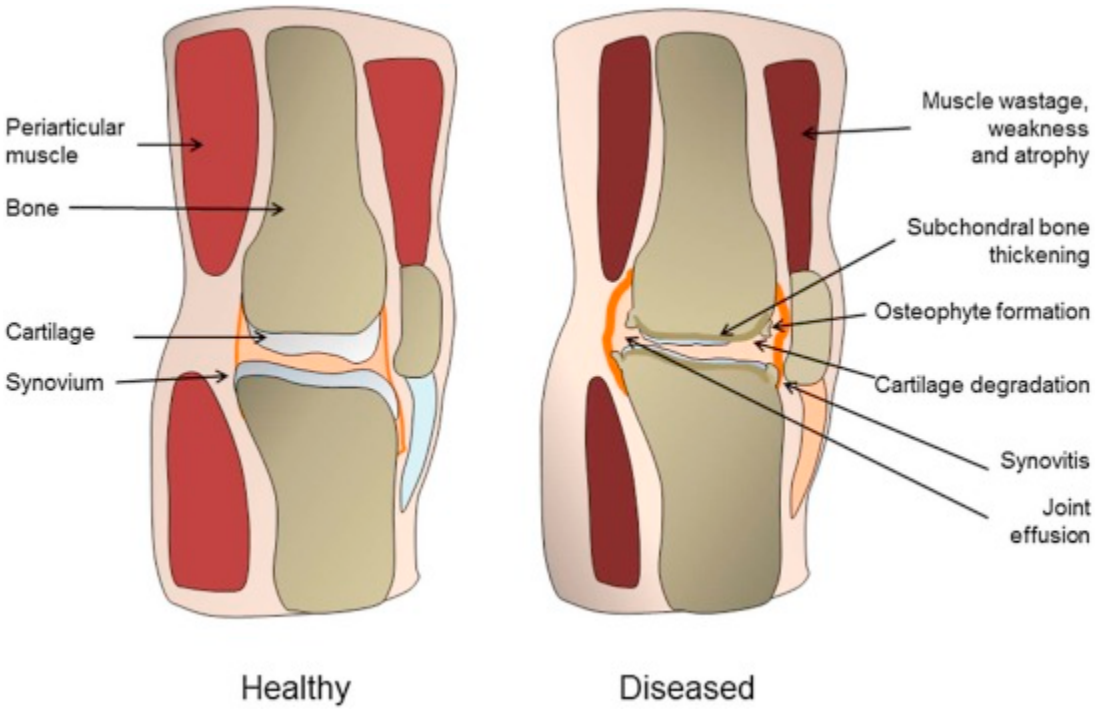

Figure 2.1. Schematic representation of a normal joint (left) and OA joint (right). © Johnson CI, Argyle DJ, Clements DN. In vitro models for the study of osteoarthritis. *Veterinary journal* 2016;209:40-49. Reprinted with the permission of Veterinary Journal. https://creativecommons.org/licenses/by-nc-nd/4.0/

I. The role of the articular cartilage in osteoarthritis.

Hyaline articular cartilage is made up of chondrocytes embedded in an extracellular matrix comprised of water, collagen, and proteoglycans. The primary function of this tissue is to provide a near friction-less gliding surface and provide shock absorption for pain-free locomotion. The articular cartilage has low cellularity, composed mainly of chondrocytes (1-12%) and articular chondroprogenitor cells (ACPCs), which have shown superior chondrogenic

properties compared to bone marrow-derived stem cells (BM-MSCs) *in vitro*.[14] Chondrocytes are primarily responsible for production of extracellular matrix (ECM).[8]

Chondrocytes do respond to inflammatory and mechanical stimuli through production of pro-inflammatory cytokines and enzymatic proteins responsible for initiating and/or perpetuating the inflammatory, degenerative cascade that depletes the extracellular matrix. The water content of the extracellular matrix varies with age of an animal and is between 70-80% of the total weight of cartilage. Approximately 30% of this water is associated with the intrafibrillar space within the collagen, although a small percentage is contained in the intracellular space. The flow of water through the cartilage and across the articular surface helps to transport and distribute nutrients to chondrocytes, in addition to providing lubrication. Additionally, the water flow into the interfibrillar space creates a mechanism that allows the cartilage to withstand significant loads.[15] In early OA, massive loss of proteoglycans (PGs) from the articular cartilage ECM leads to an increase in the proportion and mobility of free water molecules. However, in late stages, there is progressive disorganization of collagen fibers, a decrease in water content, and glycosaminoglycan (GAG) depletion.[16]

The dry weight of the cartilage is comprised of approximately 50% collagen, 35% proteoglycans (PGs), 10% glycoproteins (cartilage oligomeric matrix protein [COMP]) 3% minerals, 1% lipids, and 1% miscellaneous substances.[8]

Collagen fibers provide structure to the articular cartilage. Collagen is comprised of fibril -forming collagens (I, II, III, V, XI) and non-fibril forming collagens.[17] The primary collagen of hyaline (articular) cartilage is type II collagen (makes up 90-95% of extracellular matrix collagen fibrils). Collagen is produced by chondrocytes,[18] and is critical for the mechanical properties of articular cartilage. Cartilage presents shear and tensile properties, mainly attributed to the triple

helix structure of type II collagen, which allows cartilage to withstand compressive loads. The collagen fibers incorporated into articular cartilage also contain collagen types I, IV, V, VI, IX, and XI contributing to only 5-10% of the total collagen composition. These minor collagens are important to help form and stabilize the type II collagen fibril network.[15]

Proteoglycans (PGs) are another essential component of the extracellular matrix attached by a link protein to a hyaluronic acid backbone integrated among type II collagen fibrils. PGs are made up of a core protein, to which glycosaminoglycans (GAGs) bind. The main proteoglycan in articular cartilage is aggrecan, accounting for around 85% of all proteoglycans. An aggrecan monomer is comprised of three different GAGs covalently bonded to a core protein through a tri-saccharide link protein: chondroitin-4-sulfate, chondroitin-6-sulfate and keratan sulfate.[19] GAGs are negatively charged, making them repel one another, but attracting water. The charged repulsion effect combined with the movement of water into the ECM has been described as cartilage swelling pressure, which provides resistance to compressive forces on the cartilage.[20] The remaining 5% of proteoglycans in articular cartilage consist of smaller, non-aggregating proteoglycans such as biglycan, decorin, lumican, chondroadherein, and fibromodulin categorized as small leucine-rich -proteins/proteoglycans (SLRP's). Although the function of these proteins is not entirely understood, SLPRP's play an important role in the regulation and maintenance of the articular cartilage. For example, chondroadherein, a SLRP, has been shown to participate in the modulation of inflammation, downregulating pro-inflammatory cytokines IL-1 and IL-6.[21]

Macroscopically, the cartilage appears to be smooth, demonstrating a glass-like surface. The tissue's low cellularity and absence of direct vascular, lymphatic, and neural supply makes it a unique tissue, challenging to repair. Cartilage thickness is dependent on its anatomical location

and function (weight bearing vs. non-weight bearing). The cartilage is divided into four histological zones[7] (Figure 2.2):

1) Superficial (tangential) zone, which is between 10-20% of the total depth of the cartilage. This zone has an acellular outermost layer (*lamina splendens*). Below this, the superficial zone is characterized by the highest density of chondrocytes. Chondrocytes have a flattened appearance and their axis is oriented parallel to the joint surface. Collagen fibers are oriented parallel as well and are densely packed. This zone is characterized by a high water content and a small amount of PGs.[22] The primary function of this zone is to resist tensile and shear forces.[23] Additionally, most of the water flow from the ECM to the synovial cavity, helping part of the joint lubrication, is produced from this zone.[15]
2) Intermediate (transitional) zone is the most voluminous zone of the articular cartilage (40-60%), characterized by larger, ovoid chondrocytes, lower water content, higher density of collagen and more PGs.
3) Deep zone (radiate) (30%). In this zone chondrocytes are larger and organized in columns oriented perpendicular to the subchondral bone. This zone has the lowest collagen and water content but the highest concentration of PGs. In conjunction with the intermediate zone, it is responsible for resistance of cartilage to compression.[23]
4) The calcified zone is formed by mineralized cells and matrix. This zone is unique because of its matrix composition (higher concentration of type X collagen), mineralization, presence of vessels and the hypertrophic state of the chondrocytes.[24] This zone contains the tidemark, which refers to the junction of the noncalcified and calcified cartilage. Histologic studies have shown perforations present along the tidemark that allow contact (communication) between the non-calcified and calcified zones of hyaline

cartilage and subchondral bone.[25] Recently it has been suggested that this communication between subchondral bone and cartilage could be one of the mechanisms of how initiation and progression of OA.

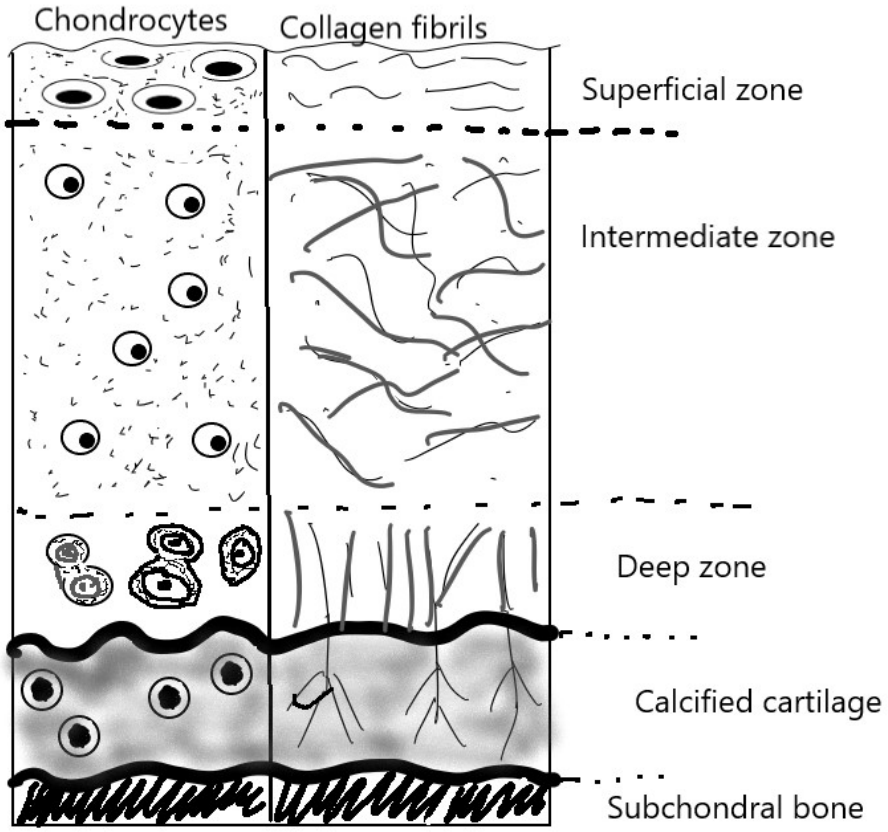

Figure 2.2. Morphology of chondrocytes and orientation of collagen fibrils in healthy articular cartilage.

In humans, it is well recognized that age is a primary risk factor for the development of OA and these age-related effects appear to primarily affect articular cartilage. The mechanism by which aging contributes to an increased prevalence of OA is not wholly understood; however, it is believed to be related to the accumulation of advanced glycation end products (AGEs). AGEs accumulate in human articular cartilage with increasing age and affect its biomechanical,

biochemical, and cellular characteristics. As a result of this accumulation, articular cartilage becomes stiffer, more brittle, and the synthesis of ECM components decreases.[26] AGEs have also been shown to bind to receptors on chondrocytes causing release of pro-inflammatory cytokines like MMP-13, IL-6, and IL-8.[27,28]

During OA, cartilage homeostasis is impaired, and catabolism of the extracellular matrix is upregulated, causing degeneration of articular cartilage with loss of the matrix components responsible for the joint's mechanical integrity, such as type II collagen and aggrecan.

II. The role of the synovial membrane in osteoarthritis.

The synovial membrane (joint capsule) is composed of outer and inner layers. The outer layer is a thick, fibrous layer with many proprioceptive nerves that tightly connect to peri-articular soft tissues such as collateral ligaments that help to maintain joint stability. The inner layer (synovium) is further composed of two layers: an outer subintimal and inner intimal layers. The subintimal layer is made up of loose connective tissue and is well vascularized and innervated. The intimal layer (in contact with the synovial fluid) is thin, with only one to four cell layers and lacks a basement membrane. The lack of basement membrane is critical to facilitate contact between synoviocytes and blood vessels to filter and diffuse plasma components into the synovial cavity.[8] There are three main types of synoviocytes forming the intimal layer. Type A synoviocytes are macrophage-like cells that participate in phagocytosis and/or pinocytosis of cell debris and waste in the joint cavity, possessing an antigen-presenting ability. Type B synoviocytes are fibroblast-like cells, responsible for the secretion of viscoelastic proteins essential for mechanical lubrication of the articular surface by synovial fluid.[29] A third, but minor synoviocyte type has been described, Type C synoviocytes. However,

the phenotype and function of these cells is currently undetermined, and they may represent synoviocytes in phenotypic transition between type A and B.[7] The synovial membrane is highly vascularized, acting as a semipermeable membrane that controls the molecular passage of proteins from the plasma into the synovial space.[30]

The intimal layer is mainly responsible for the content of the synovial fluid. Synovial fluid is described as an ultra-filtrate formed by the passage of plasma through the synovium, with the addition of proteins such as hyaluronan (HA), and lubricin secreted by type B synoviocytes.[31] Synovial fluid provides boundary lubrication to the intra-articular environment and nutrition to the articular cartilage.[8] Boundary lubrication is produced when a fluid film (synovial fluid) is in contact with both joint surfaces allowing frictionless motion to the joint.

Early in the degenerative process of OA, changes in the synovial membrane including hyperplasia, fibrosis, detritus-rich synovial fluid, and inflammation are observed.[32] When the synovial membrane is inflamed and undergoes hyperplasia, the permeability of the synovial membrane is altered. This change in permeability contributes to decreased concentrations of HA and lubricin.[30] In general, synovial fluid in OA becomes less viscous because of the decreased concentration and abnormally low molecular weight of endogenously produced HA.[33] These pathologic changes result in dilution and overproduction of synovial fluid resulting in joint effusion, fragmentation (depolymerization) of hyaluronan by catabolic enzymes, and aberrant hyaluronan synthesis attribute to phenotypic changes in synoviocytes.

Recently in human medicine, it is well recognized that inflammation of the synovial space (synovitis) plays a critical first-step and ongoing role in the pathophysiology of OA,[34,35] similar results have been observed in horses.[11,36] Inflammation of the synovium is an active component of OA, associated with pain and disease progression. The synovium is the most

cellular abundant and permeable structure in the joint. During inflammation, macrophages infiltrate the synovium. These macrophages and synoviocytes produce pro-inflammatory cytokines and catabolic enzymatic proteins that contribute to articular matrix degradation. Therefore, alterations in the synovial membrane can result in decreased concentrations of chondroprotective factors and increased chondro-destructive proteins that contribute to the degradation of the articular matrix. Besides, molecules from degraded hyaline cartilage released into the synovial cavity are likely to perpetuate the synovial inflammation in OA and maintain the disease process (Figure 2.3).

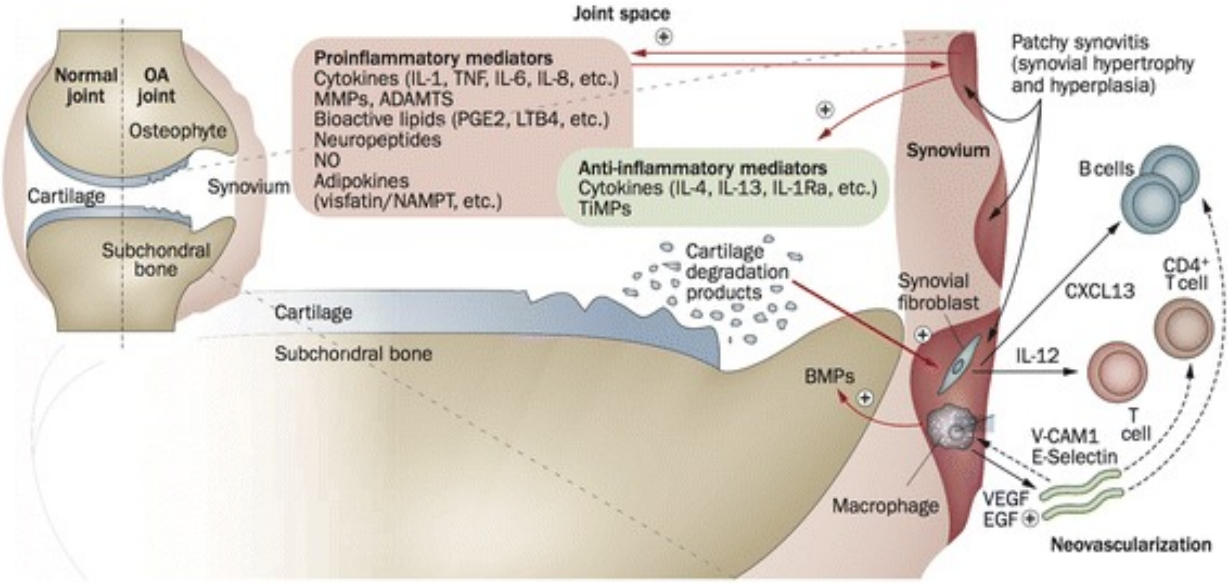

Figure 2.3. Involvement of the synovial membrane in OA pathophysiology. ©Mathiessen A, Conaghan PG. Synovitis in osteoarthritis: current understanding with therapeutic implications. Arthritis Res Ther. 2017;19(1):18. Published 2017 Feb 2. doi:10.1186/s13075-017-1229-9. Reprinted with permission of Arthritis Research & Therapy. https://creativecommons.org/licenses/by/4.0/

III. The role of the subchondral bone in osteoarthritis

The subchondral bone is located deep to the articular cartilage and is attached to it by a layer of calcified cartilage. [8] Histologically is composed of cortical bone with the haversian system (osteons) running parallel to the joint surface, as opposed to diaphyseal bone. The thickness of the subchondral bone plate can be influenced by pathologic conditions and can vary by anatomic location. A study evaluating the subchondral bone thickness of the distal tarsal bones (central tarsal bone, third tarsal bone and proximal aspect of third metatarsal bone) determined that the normal thickness of the subchondral bone in horses at this location was observed to be 2 to 4 mm.[37] However, this subchondral bone thickness is likely different depending on the mechanical forces on the bone due to differing joint biomechanics of other joints in the equid.

The subchondral bone provides energy-absorption shielding or minimizing transmitted forces to the articular cartilage. In humans, the haversian construction of subchondral bone makes it up to 10 fold more deformable than diaphyseal bone.[38] Joints can tolerate repetitive loading because of the deformability of the subchondral bone. Subchondral bone responds similarly to diaphyseal bone, remodeling in response to loading forces, coordinating activity of osteoclasts and osteoblasts to remove biomechanically inferior packets of bone and replace it with new bone.[39] During OA, this balance between subchondral bone resorption and generation breaks down. Osteoclasts reabsorb abnormal or damaged bone faster than osteoblastic precursors can replace the lost bone with normal bone, leading to a weaker and osteoporotic subchondral bone plate.[40] In race horses, it has been shown that training inhibits subchondral bone resorption compared to horses at rest. However, a balance between training and rest periods is needed to facilitate subchondral bone repair.[41]

The first change observed to the subchondral bone is sclerosis and thickening of the subchondral bone, producing a decreased elasticity within the subchondral bone plate and trabecular bone.[42] Changes in the subchondral bone affect the ability of the cartilage to withstand mechanical loading, producing horizontal clefts in the cartilage's deep zone.[43,44]. These clefts extend from the deep zone, progressing to the articular surface of the cartilage if mechanical loading continues, perpetuating the OA changes in the joint by altering the natural biomechanical properties of cartilage.[45,46] A study in humans found that *in vitro* co-culture of chondrocytes with subchondral osteoblasts obtained from sclerotic subchondral bone changed the chondrocytes phenotype towards a more hypertrophic phenotype, and stimulated chondrocytes to produce more MMPs and inhibited the aggrecan production.[47] Neoangiogenesis is one of the modifications that occur during OA in the subchondral bone. The new vessel formation penetrates the tidemark and enters into the calcified layer allowing the presence of a higher concentration of protease, MMPs and osteoblasts, and osteoclasts, increasing the inflammatory cross-talk to the cartilage layer from the subchondral bone, perpetuating the OA cycle into the joint.[48] Supporting this, increased neovascularization in the subchondral bone and a higher number of vessels penetrating the tidemark have been associated with more severe OA cartilage changes.[49-51] Osteoclasts during aberrant subchondral bone remodeling in OA produce netrin-1, a protein responsible for increasing innervation of the subchondral bone, causing enhanced sensation and pain in OA.[52]

All these pathologic changes described above explain the importance of the subchondral bone in the pathogenesis of the OA but also how the synovial components participate in maintenance of joint health. Understanding the physiology of the subchondral bone could help veterinarians create appropriate training programs to prevent further changes in the joint.

IV. The role of periosteum and joint soft tissue associated structures in osteoarthritis

The periosteum is the thin outer layer of cortical bone, consisting of two distinct layers: an outer layer predominantly formed by fibroblastic cells and the inner cambium layer, which is highly cellular and is formed primarily by mesenchymal progenitor cells, differentiated osteogenic progenitor cells, osteoblasts and fibroblasts in a sparse collagenous matrix. A well-developed nerve and microvascular network exits along the periosteum, which influences the pain response in OA disease.[53] In humans, with OA, focal pain at the area of osteophyte growth is attributed to the impingement of the richly innervated periosteum by osteophytes.[54] Additionally, it has been reported that chondrocyte precursors are located in the inner cambium layer of the periosteum, which means preserving this tissue could be crucial to maintain joint health.[55]

Soft-tissue structures such as peri- and intra-articular ligaments, menisci (if present), overlying and/or peri-articular inserting musculotendinous units, and the fibrous joint capsule play a role in the stability of the synovial compartment and changes in stability result in synovial inflammation.[7] The contribution of stability to the joint provided by each of these structures differs according to the joint and it's anatomic location. The fibrous joint capsule and peri-articular ligaments are composed mainly of type I collagen and some elastin. These structures are highly innervated and provide most of the synovial innervation, therefore, changes in these structures can result in increased pain. The menisci, crescent-shaped fibrocartilage structures found in the femorotibial joint between the femoral and tibial condyles, can participate in the OA process within the stifle joint. The menisci provide alignment of incongruent bones and even load distribution within the articular environment, and in horses with meniscal degeneration, OA in the stifle has been observed.[56]

Ligamentous or tendinous lesions that produce joint instability and alter normal biomechanics of the joint can trigger OA.[6] Although these tissues are not as metabolically active in OA disease as cartilage, synovium, or subchondral bone, their importance must be considered in the development of OA.

V. Endogenous cartilage repair

"Cartilage once destroyed never heals".[57] This statement was made by Dr. Hunter in 1743, and despite all the research and growing knowledge of joint disease, this statement remains partially true. Damage cartilage has a limited capacity to regenerate. As previously mentioned, cartilage has a reduced endogenous cell population. These cells have a low metabolic rate with limited capacity for outside influence due to a lack of vascular and/or lymphatic supply.[7] Cartilage repair occurs by three mechanisms: 1) intrinsic repair, which depends on the capacity of the chondrocytes to divide and produce new matrix, 2) extrinsic repair, which depends on the mesenchymal progenitor elements of the subchondral bone to help form and fill the defect with fibrocartilaginous tissue, and 3) by producing lips of cartilage from the perimeter of the lesion that extends into the defect, which is known as matrix flow.[58,59]

The depth, size, location (weight-bearing or non-weight-bearing areas), patient age, and concurrent OA could influence endogenous repair.[59] In humans, age has been shown to affect endogenous repair with chondrocytes having reduced ability to synthesize and assemble matrix molecules declining with age.[60] Early research suggested that full-thickness cartilage defects greater than 9 mm in diameter have minimal capacity for repair.[61,62] These lesions heal by ingrowth of subchondral fibrous tissue and formation of fibrocartilage. Partial-thickness defects are believed to have minor capacity for healing, and it has been shown that in rabbits, partial-

thickness cartilage defects present a slightly greater healing capacity by increasing the synthesis of GAGs and type II collagen.[63] How partial-thickness defects heal is not entirely understood , but what we know is that when these defects are present in the equine joint do not necessarily progress or compromise the joint function. Therefore, is no longer recommended to debride these lesions deep to the subchondral bone and only debride surface fibrillation present.[7] Currently, no treatment for full-thickness articular cartilage defects provides reparative tissue similar to hyaline cartilage nor tissue that is similar or better biomechanically than reparative tissue that fills in partial-thickness defects.[7]

Articular cartilage defects are repaired by the formation of fibrocartilage or hypertrophic cartilage. Hypertrophic cartilage is characterized by enlarged chondrocytes that have stopped dividing and accumulate glycogen, lipids, and alkaline phosphatase. Hypertrophy occurs at the expense of ECM production with more type X collagen.[64] Fibrocartilaginous repair tissue contains more type I collagen than type II collagen with reduced aggrecan and chondrocytes content within the extracellular matrix.[64-66] Because of this compositional difference, fibrocartilage has inferior biomechanical properties, further perpetuating OA disease.[66-68] In hypertrophic cartilage repair, a higher number of hypertrophic chondrocytes are present along with greater amounts of short-chain type X collagen.[64] Hypertrophic chondrocytes are not able to produce type II collagen and aggrecan, essential components to maintain the health and biomechanical properties of the cartilage. Contrary, they produce type X collagen and promote ECM mineralization by increasing the production of alkaline phosphatase.[69]

Healing refers to the restoration of the structural integrity and function of the tissue after injury or disease, and despite the attempt of the body to heal a lesion affecting cartilage, the result is still inefficient. Additionally, treatments are lacking that completely modify the disease

and stop its progression, which opens a possible space for orthobiologic therapies. Orthobiologics aim to stop or slow down the progression of lesions and enhance cartilage healing to produce better quality tissue. The healing inefficiency of the body's natural repair and the possibility to enhance repair with orthobiologics has created more interest and produced a large number of studies evaluating the mechanism and efficacy of orthobiologic therapies to treat OA.

❖ PATHOPHYSIOLOGY OF OA

The normal integrity of the joint is crucial to maintaining normal joint function: near-frictionless motion, joint congruency, and force transmission. As mentioned previously, the joint works similar to an organ-like structure, in which all the tissues participate in one way or another during the disease process.[70]

In human OA, the risk factors for development of OA have been classified into two main mechanisms: abnormal loading on normal cartilage or normal loading on abnormal cartilage.[71] In horses, three mechanisms have been identified as being responsible for the pathogenesis of OA: 1) abnormal biomechanical loading on normal cartilage, 2) abnormal change in the subchondral bone, and 3) exposure of abnormal cartilage to normal forces.[7,72-74] Cyclic or athletic trauma, changes in joint congruence (fractures), or loss of joint stability (fractures or ligamentous lesions) can produce abnormal mechanical loads on normal cartilage or subchondral bone, and age or osteochondrosis for example can create an abnormal cartilage surface. These events produce physical cell damage and the joint responds by upregulating pro-inflammatory cytokines and decreasing ECM synthesis. Clinically, this translates into a breakdown of articular cartilage and perpetuation of OA. In the same way, exercise activities can lead to inflammation of the soft tissues as well as remodeling and microfracture formation within the subchondral bone by overloading the joint structures and creating joint instability, affecting the articular cartilage. Also, aging or developmental disease such as osteochondrosis can damage the articular cartilage. Ultimately, abnormal articular cartilage produces more degradative and inflammatory cytokines and decreased synthesis of ECM components, which clinically translates in a breakdown of the articular cartilage, and cartilage breakdown products perpetuate synovial inflammation and disease progression.

Regardless of a causative factor, the damage that occurs to the articular cartilage causes a cascade of changes within the joint.[38,75] (Figure.2.4)

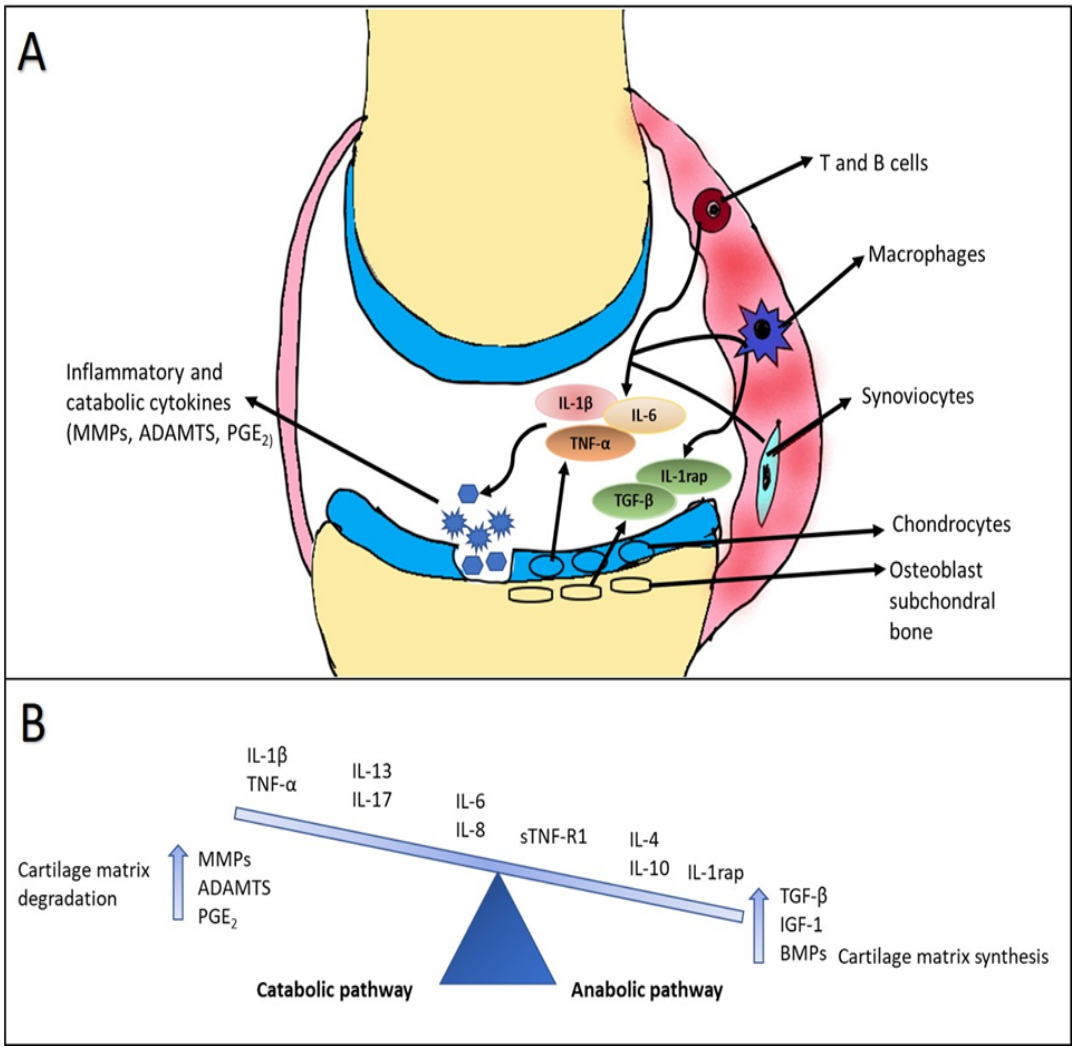

Figure 2.4. A) Simplified OA cytokine cascade. B) Cytokine imbalance during OA.

I. Pro-inflammatory cytokines

OA has been described as a chronic degenerative disease, but it is now known inflammation is present even in the early stages of disease.[76-79] In the diseased joint, damaged chondrocytes, synoviocytes, and monocytes release proinflammatory cytokines, thus causing the joint to enter an inflammatory or catabolic state. While IL-1β and TNF-α are considered the main

pro-inflammatory cytokines of OA,[80-83] other interleukins, proteases, nitric oxide (NO), and prostaglandin E_2 (PGE_2) participate in the inflammatory cascade.[8,71,81,84,85]

IL-1β and TNF-α are the key arthritogenic triggers, and it is thought that these cytokines produce a synergistic effect, enhancing cartilage destruction when combined *in vitro*.[86-88] IL-1β is more potent, requiring only 0.1–1.0% of the TNF-α dose to achieve the same proteoglycan synthesis inhibition in chondrocytes.[89] IL-1β is associated with driving tissue destruction and TNF-α with inflammation.[81,90,91] In all joints, these two cytokines are synthesized by chondrocytes, osteoblasts, synoviocytes, and mononuclear cells. They exert their effect via binding to membrane IL-1 receptor (IL-1R)1 in the case of IL-1β,[92,93] and TNF receptor I (TNFRI) and TNF receptor II (TNFRII) in the case of TNF-α.[94,95] These cytokines regulate other catabolic enzyme and inflammatory cytokine gene expression through signal transduction pathways, such as those regulated by mitogen-activated protein kinases (MAPKs).[96] This pathway also activates the nuclear factor-kappa (NF-kB), which upregulates the release of COX-2, PGE_2, MMPs, and ADAMTS, leading to cellular apoptosis and extracellular matrix degradation.[97] (Figure 2.5)

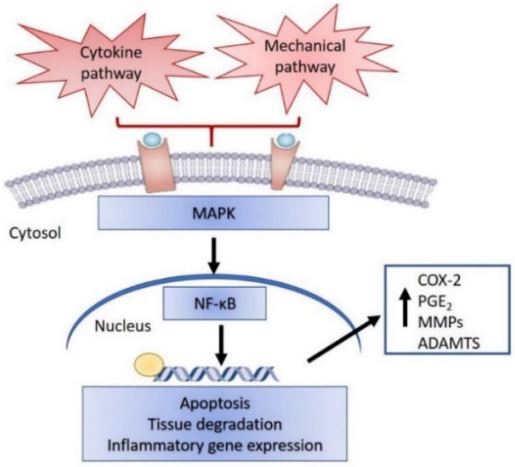

Figure 2.5. Representation of the main activations pathways of MAPK and NF-kB participating in OA.

IL-1β and TNF-α stimulate chondrocytes to release catabolic enzymes such as MMPs, in particular aggrecanases.[81] Multiple MMPs have been described and categorized based on the substrate they tend to degrade, such as collagenases, stromelysins, and gelatinase.[98] Collagenases (MMP-1, 8, -13), gelatinases (MMP-2 and -9), and stromelysins (MMP-3, -10, -11) are all expressed at low levels in healthy joint tissue. However, their production and release are significantly increased in arthritic joints.[99,100] In OA, IL-1β and TNF-α stimulate the production of MMP-1 MMP-3 and MMP-13. These proteolytic enzymes are responsible for cleaving type II collagen and aggrecan. MMPs cleave the three collagen α-chains of the triple helix at 775-776 amino acid residues. This produces articular cartilage depletion interfering with chondrocytes homeostasis.[76,77]

The ADAMTS (a disintegrin-like and metalloproteinase with thrombospondin type 1 motifs) family of proteins include ADAMTS-4 and ADAMTS-5 and are responsible for aggrecan cleavage. Aggrecanase activity is considered a hallmark of cartilage degradation during OA.[101,102] IL-1β and TNF-α have been shown to upregulate the production of ADAMTS-4, but not ADAMTS-5, indicating that gene expression of these aggrecanases might be differentially regulated.[103-105]

Interleukins including IL-6, IL-15, IL-17, IL-18, IL-21, leukemia inhibitor factor (LIF) and chemokines including IL-8 and CCL5 (also known as RANTES) are produced in response to IL-1β and TNF-α stimulation during OA disease.[71,80,81,106,107] These pro-inflammatory cytokines and chemokines are intimately related to the upregulation of MMPs and induction of NO or PGE_2 production, helping to perpetuate the inflammatory response and cartilage destruction in an osteoarthritic joint.[71,81] IL-6 and IL-8 are elevated in patients with OA,[78,108] but they are not directly responsible for initiating or maintaining cartilage degradation. IL-6 appears to have a

dual role in OA, providing some regulation and dampening of the inflammatory process,[77,80,109] and IL-8 promotes the release of MMP-13, attracts and activates leukocytes in the synovium, and induces chondrocyte hypertrophy.[110] IL-6 works synergistically with IL-1[111] to upregulate expression of MMP-1 and MMP-13 in synoviocytes[112] and reduce type II collagen expression in chondrocytes.[113] However, a protective effect of IL-6 has been observed in human synoviocytes and chondrocytes stimulating the production of a natural inhibitor of MMP-1,2,3 and 8, the tissue inhibitory of metalloproteinases 1 (TIMP-1).[114]

PGE_2 is an important inflammatory mediator in OA and is thought to be partially responsible for joint pain in OA.[84,115] An increased PGE_2 concentration has been observed in horses with inflamed joints,[116] and this has been related to an increase in the pain response.[117] IL-1β stimulates the production of PGE_2 by the synovial tissues by stimulating the activity of the cyclooxygenase (COX-2).[118,119] This enzyme simultaneously upregulates the production of NO.[71] Increased concentrations of NO produce chondrocyte apoptosis and matrix degradation.[120] PGE_2 and NO have been mainly characterized as pro-inflammatory mediators, enhancing the activation and production of MMPs and inhibiting the anabolic pathways and production of interleukin 1 receptor antagonist protein (IL-1rap).[81] However, some studies have identified a chondroprotective effects of these mediators.[121,122] In humans, PGE_2 was shown to interfere with IL-1β and inhibited type I collagen synthesis stimulating type II collagen gene expression.[123,124] This dual effect has not been studied in the horses' joints, but in equine MSCs, blocking PGE_2 production significantly increased T-cell proliferation, identifying PGE_2 as one of the main immunomodulatory protein of MSCs.[125]

IL-1β and TNF-α also affect the catabolic pathways of articular cartilage, decreasing the synthesis of essential ECM proteins produced by chondrocytes. Multiple studies have shown that chondrocytes stimulated with IL-1β downregulate of type II collagen synthesis.[123,126,127]

In conclusion, this complex inflammatory cascade produces a critical loss of ECM components, which clinically produces a softening of the cartilage (loss of compressive and tensile strength) and swelling caused by loss of GAG and absorption of water, resulting in cartilage fibrillation and full-thickness erosion.[59]

II. Anti-inflammatory cytokines

IL-4, IL-10, and IL-13 have been classified as inhibitory or anti-inflammatory cytokines since they decrease the production and/or action of the pro-inflammatory cytokines.[80,107,128] The production of IL-4 is related to T cell stimulation and infiltration of the synovium.[129,130] IL-4 is associated with a robust chondroprotective effect and is able to inhibit degradation of PGs and secretion of MMPs.[131-134] In human patients with OA, researchers have found an increased concentration of soluble IL-4 receptor, suggesting that this protein reduces the availability of IL-4 and its effect on chondrocytes.[135] A synergistic effect has been observed between IL-4 and IL-10, preventing chondrocyte apoptosis.[133] IL-10 also downregulates the production of TNF-α and its receptor surface expression in synovial fibroblasts and macrophages *in vitro*.[128,136] IL-10 participates in the downregulation of PGE_2,[137-139] and studies with mice have shown that IL-10 stimulated chondrocyte proliferation and reduced cartilage degeneration.[140,141] IL-13 has been shown to inhibit the production of proinflammatory cytokines such as IL-1β, IL-6, and IL-8 while increasing IL-1rap production.[142]

Another anti-inflammatory cytokine in which equine researchers have focused their efforts is IL-1rap. IL-1rap is a competitive inhibitor that binds to the IL-1 receptor, avoiding receptor engagement and signal transduction to the cell from IL-1β.[143] In humans, a 10- to 100-fold increase in the concentration of IL-1rap to IL-1β is needed to sufficiently block IL-1β from the IL-1 receptor.[144] This ratio has not been described in horses. IL-1rap is produced by many of the same cell types that secrete IL-1, including articular chondrocytes.[107] In humans, IL-1rap can block many of the effects observed during the pathological process of OA, including PGE2 synthesis in synovial cells, collagenase production by chondrocytes, and cartilage matrix degradation.[77]

After exposure to an inflammatory insult, T-cells and activated neutrophils are able to produce soluble TNF- R1 (sTNF-R1), a protein that binds to extracellular TNF-α and blocks its biological activity.[145-149] In humans, two studies reported positive outcomes after using a synthetic anti-TNF-α simulating this soluble receptor for treatment.[150,151] However, there are currently no clinical veterinary studies looking at the effects of anti-TNF-α therapy.

TIMPs are natural inhibitors of MMPs synthesized by numerous cells that include chondrocytes and synoviocytes.[152] These inhibitors bind one-to-one with MMPs to form an inactive complex and include TIMP-1, 2, 3, and 4. TIMP-1, TIMP-2, and TIMP-3 are three out the four natural inhibitors described in the literature involved in joint disease.[7] Although some research has been done targeting TIMPs as a treatment for OA, total inhibition of the MMPs is not desirable as it will alter normal turnover, and more research in this field needs to be done.[153,154]

III. Anabolic cytokines

Although cartilage has poor reparative properties in natural conditions or as a response to an injury or an inflammatory stimulus, production of growth factors by macrophages, synoviocytes and chondrocytes enhances the anabolic pathway.[155] TGF-β is considered one of the most important growth factors involved in cartilage repair and pro-inflammatory cytokines produced during OA can downregulate or reduce the effect of TGF-β, enhancing the catabolic status within the joint.[156,157] TGF-β stimulates chondrocyte proliferation and induces an increased synthesis of PGs and type II collagen.[158-160] TGF-β also downregulates MMPs, counteracting the effect of IL-1β.[160-162] In horses, it has been observed that TGF-β increased production of HA.[163] However, it has been observed in mice that high physiologic levels of TGF-β can produce harmful effects such as increased leukocyte infiltration, synovial fibrosis, and osteophyte formation.[164]

IGF-1 also plays a critical role in cartilage homeostasis and promoting the anabolic pathway, observing that a decreased level of IGF-1 was correlated with more significant cartilage degeneration.[165] Inflammatory products like NO inhibit the anabolic effects of IGF-1[166] such as stimulation of ECM production and inhibition of matrix degradation.[167] In horses, a combination of IL-1rap and IGF-1 gene therapy enhanced the repair of 5 mm full-thickness surgically created articular defects.[168]

Bone morphogenetic proteins (BMPs) belong to the TGF-β superfamily. BMPs and in particular BMP-2 and BMP-7, participate in the chondrocyte regulation.[162] BMP-2 protein is predominantly secreted by osteoblasts, where it has an osteoinductive effect on the subchondral bone. This protein is up-regulated in osteoarthritic chondrocytes and increases the expression of ECM genes like type II collagen.[169] Multiple studies suggest that BMP-7 is the most potent of

the BMP family regarding its matrix protection in OA, increasing the anabolic and anti-catabolic (stimulates ECM synthesis) activities (decreases MMPS and aggrecanases expression) of cartilage and subchondral bone.[162,170]

Research investigating treatments that potentially upregulate the production of these proteins is ongoing since upregulation of ECM components such as type II collagen and aggrecan could help produce tissue with better biomechanical properties than fibrocartilage.

IV. The role of macrophages in osteoarthritis

It has been demonstrated that synovial membrane inflammation can be found in both early and late stages of OA,[34,171-173] and macrophages accumulate within the synovial membrane in patients with synovitis.[174-176] Recently, some studies have focused on the role these macrophages play in OA, finding that macrophages are responsible for the induction of inflammatory mediators, growth factors, and proteinases.[177-179] Macrophages can polarize and present different phenotypes and functions depending on the micro-environment.[180] Classically activated macrophages (M1) are considered to stimulate the inflammatory and catabolic pathways in the joint, while alternatively activated (M2) macrophages are involved in regulatory subsets with anti-inflammatory properties (figure 2.6).[181-183]

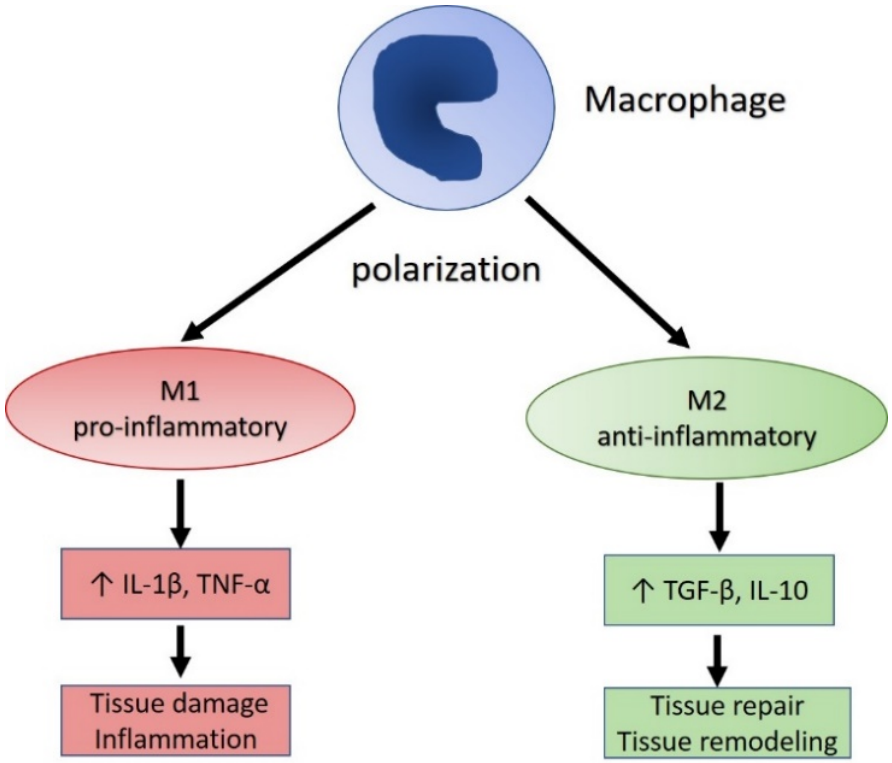

Figure 2.6. Polarization of the macrophages and function in OA disease.

The balance between M1 and M2 macrophages might be distorted in OA, and the degree of imbalance could be associated with the severity of the OA disease.[176] These findings have opened a research pathway in the OA field and possible target to OA treatment.

❖ OA MODELS

In humans, OA is the most common joint disease and a leading cause of disability worldwide, mainly due to pain, the primary symptom of the disease.[184,185] Due to increased life expectancy, OA most commonly affects the elderly population, being the cause of 94% of hip and 97% knee replacements.[186,187] In horses, OA compromises the equine industry, not only due to the costs of treatment, but also as a consequence of a delayed return to athletic performance. Although it is difficult to estimate the exact incidence of OA in horses accurately, many epidemiological studies describe a high prevalence (up to 80%), especially in middle-aged to elder horses.[9,10,188,189] However, OA has been described in young race horses as well (2- and 3-year-old), in which 33% of the study population presented with cartilage and bone lesions consistent with OA.[188]

Both *in vivo* and *in vitro* animal models of OA have been used in the past to investigate OA disease progression and treatment effects. The three main points that research aims to cover are: 1) how to re-establish the balance between anabolic and catabolic pathways of the synovial environment, 2) how to decrease the inflammatory response and 3) enhance the repair process of articular cartilage to restore the normal biomechanical function of the joint. In horses, healthy or naturally-occurring OA tissue can be easily obtained,[190] and the horse provides the closest approximation to humans in terms of articular cartilage thickness, and this approximation is considered key in clinical studies evaluating cartilage healing.[191,192]

I. *In vitro* OA models

The similarities observed between OA pathogenesis in humans and domestic animals means *in vitro* OA animal models are commonly used to study OA for within and cross-species translation of findings.[193] The use of *in vitro* OA models allow researchers to maintain the 3 R philosophy of ethical animal use: reduction, refinement, and replacement.[194] *In vitro* models can be carried out using mono or co-culture of synovial cells or tissues (explants). Cells can be cultured in monolayer, explant, or seeded into engineered 3D- culture models. Each of these models has certain advantages and disadvantages for OA research (Table 2.1).

Table. 2.1. Summary of *in vitro* culture models used to study OA.

In vitro OA models	Advantages	Disadvantages
Monolayer	Easy cellular expansion Homogenous cellular population More straightforward investigation of individual pathways to OA	Altered cellular phenotype Absence of ECM No influence of tissue environment Not grown in 3D as *in vivo*
Tissue explants	Simple, economical, and easy Natural ECM	Cell death at the cut edge Fewer replicates than monolayer cultures Physical attributes may change during culture
Co-culture cells/explants	Allows cross-talk between cell types Explant cultures do not disrupt the natural 3D matrix in which cells are maintained	Altered cellular phenotype Different tissues may have different culture conditions to maintain viability
3D cell culture	Possible culture of different cell lines Allows cross-talk between synovial tissues Allows cells to be grown in 3D environment	Cells need to be isolated and expanded first Proliferation rate is slower compared to 2D models The strength of cultured structure depends on the type of scaffold used

Monolayer cultures are well established and highly reproducible. Since articular cartilage has very low cellularity, monolayer chondrocyte cultures allow an increased number of

chondrocytes to be studied, expanding the cellular response. Monolayer culture studies help to simplify and gain a better understanding of OA pathways, evaluating individual cellular response of chondrocytes or synoviocytes to certain stimuli (cytokine, mechanical, etc.) However, it is essential to consider that cultured chondrocytes and synoviocytes can alter their phenotype in monolayer culture differing from their *in vivo* phenotype.[195] In horses, multiple studies have used monolayer culture of chondrocytes or synoviocytes to evaluate mechanisms of disease and evaluate various treatments for OA.[196-200]

On the other hand, culture of tissue explants in OA studies is an easy and inexpensive technique, which allows maintenance of cells within their matrix. Some of the disadvantages of this type of culture include: enhanced cellular death occurring along the cut edge and on the middle of the explant (depending on the explant thickness), number of explants are limited to the size of the joint, and tissue variability depends on the joint used.[193] Multiple equine studies have been carried out using cartilage, synovial membrane, or subchondral bone explants cultured individually,[201-206]or in combination, with these co-culture systems becoming more popular.[119,207,208]

In vitro co-culture models have been used in an attempt to understand the complex crosstalk present between joint tissues during injury and following therapeutic intervention. Biological crosstalk between cartilage and synovium has been described in humans and other species, but the mechanisms behind the interaction of these tissues and the effect of this cross-talk on ECM turnover are not fully understood. In humans, co-culture studies have proven the essential role of the synovial membrane in OA pathophysiology. Synovial membrane has been described as the primary tissue responsible for production of TNF-α and other cytokine mediators that inhibit chondrocyte GAG production.[209,210] Differences in treatment efficacy of

recombinant IL-1rap has been detected when bovine cartilage was co-cultured with synovial membrane compared to articular cartilage explant monocultures. In this study, synovium produced a protective effect, upregulating the release of anti-inflammatory cytokines such as IL-4 protecting the articular cartilage.[211] In horses, osteochondral explants and synovial membrane co-culture models have been used to study OA *in vitro*.[119,207] In long-term co-culture (tissue cultures maintained for 21 days), co-cultures produced higher MMP-1 production in cartilage (detected with immunohistochemistry techniques) than monoculture of osteochondral explants.

An increased polarization of synovial macrophages towards the M1 phenotype was also observed with tissue in co-culture.[207] Byron and Trahan also reported increased MMP-13 in culture media from osteochondral explants in monoculture compared to co-culture with synovium. Differences observed between monocultures and co-cultures in these studies could be biologically relevant and highlights that co-cultures offer a superior and more realistic model in order to extrapolate to the results of what happens in the joint *in vivo*. In another study evaluating co-cultures of IL-1β cartilage with synoviocytes transduced with IGF-1 and IL-1rap genes compared to untransduced synoviocytes, transduced synoviocytes improved preservation of proteoglycan content of cartilage explants.[212] These results could indicate that targeting to change the gene expression of the synovial membrane could be one of the strategies to treat OA in the future.

Recently, 3D culture systems have become an essential tool in OA research. These culture systems provide a scaffold for previously isolated and expanded chondrocytes that mimic the ECM. In humans using a 3D pellet culture system, chondrocytes were able to be expanded and maintain their chondrogenic phenotype, upregulating their production of type II collagen.[213]

Similar results have been observed in a 3D model in which equine chondrocytes were seeded within hydrogels.[214]

Independent of the type of culture used, two methods of cell/tissue induction of OA have been used *in vitro*: mechanical, load-based models and cytokine stimulation. Mechanical stimuli are essential for cartilage to maintain its homeostatic balance between anabolic and catabolic events in chondrocytes. Mechanical overloading stimulates chondrocytes similar to chondrocyte stimulation with pro-inflammatory cytokines.[215,216] Mechanical stimulation through shear or compressive forces can activate the MAPK and NF-kB pathways, upregulating catabolic pathways.[217] Mechanical load can influence cytokine production by osteoblasts within the subchondral bone plate, which plays a significant role in the establishment and perpetuation of OA. Multiple studies have observed an increased concentration of PGE_2 concentration and IL-6 by osteoblasts subjected to mechanical load when maintained in a monolayer culture. [218-220]

During naturally-occurring OA, synoviocytes, chondrocytes, and macrophages increase their synthesis of catabolic and pro-inflammatory cytokines.[77] As mentioned previously, IL-1β and TNF-α play crucial roles in OA establishment. Therefore, adding these pro-inflammatory cytokines to culture systems can induce a similar cellular response *in vitro*. Although IL-1β at a dose of 10 ηg/ml is the most common cytokine used in equine studies,[91,221-223] TNF-α (10 ηg/ml) or a combination of both have been used by other researchers.[196,207,224,225] Although it makes sense that a combination of both cytokines could mimic the closest scenario to the natural joint environment, further investigation is required in this area. Besides, some in vitro studies have studied the effect of co-culturing healthy cartilage with synovial fluid or synovium from patients with OA, reporting an increase of pro-inflammatory cytokines expression such as IL-1, 6 or 8, proving that a cross-talk between tissues in the joint exists.[210,226]

As previously mentioned, *in vitro* study of OA using articular cartilage and synovial membrane explant co-culture systems offers a superior technique to study the complexity of the OA cascade, including cross-talk between cartilage and synovial membrane while maintaining cells in their natural matrix. Effects of co-culture described above could be relevant and more accurately simulate naturally-occurring OA, requiring further investigation.[119,210,211,221,227]

II. *In vivo* OA models

At present, there is no gold standard animal model used in OA research. Differences in size, anatomy, histology (cartilage thickness), biomechanics, and physiology make translatability between animal models and human disease very difficult. Different pathological features of OA, such as pain, synovitis, cartilage degeneration, and bone remodeling, have been studied in OA animal models. Using animals such as mice, rats, and rabbits is much easier, quicker, less expensive, and these species allow for genetic modification unavailable in larger animals. Large animal models using sheep, goats, pigs, cows, and horses could provide more tissue and similar biomechanical and anatomic properties to humans.[228,229] During experimental design, it is crucial to consider when each species reaches skeletal maturity when choosing the age range to be included in the study.[230,231] Although horses can be difficult and expensive to house, these animals have been shown to resemble human joint anatomy and articular cartilage thickness closely.[191,192] Besides, equids are used for sport; therefore, research to enhance performance in the face of joint injury is essential and has a significant economic impact on the industry. In horses, a wide range of imaging techniques and clinical tests are available, as well as rehabilitation methods, making it much easier to understand OA disease and therapeutic mechanisms of action, extrapolating these results to humans.[228,229,232] The tremendous economic

hardship that OA creates in the equine industry (cost of therapies, loss of performance/training, etc.) has promoted research into OA mechanisms and treatments for use on the species and translation to human disease prevention and treatment.

In humans, the OARSI has created an OA cartilage histopathology grading and staging system.[233] This system allows researchers to classify OA lesions more objectively when using *in vivo* models, making it easier to compare study results. A similar grading system was developed for horses by several researchers for both the articular cartilage and synovium.[173]

An ideal OA animal model has the following five properties:[234]

1) The model should induce consistently reproducible OA in a reasonably short time frame time. In humans, naturally occurring OA can vary greatly between individuals and progression of disease can be a slow process compared to experimental models such as the established ovine meniscectomy model in which OA changes are observed within 3 months of induction.
2) Induced OA changes should progress in a time frame that allows investigation of early, mid, and late pathophysiologic changes.
3) Animals used should be mammalian species, which are economically accessible, easy to house, with well-established and available diagnostic techniques (imagining, synovial fluid analysis, gene expression, or histology, for example).
4) The model should closely resemble pathologic changes and disease progression of human OA, ensuring that all tissues of the synovial "organ" are appropriately included and evaluated.
5) Models should also be developed to simultaneously answer questions relating to the therapeutic modulation effect produced within the different types of joint tissues.

In horses, *in vivo* OA models have been classified as spontaneous or experimentally induced either through mechanical (surgical) or chemical methods (Table 2.2).[228] Some spontaneous models still require human intervention (immobilization of the joint using a cast or pressure applied to the femoral condyle, genetic modification), but this intervention results in spontaneous development of OA. Although these models could more closely represent naturally occurring OA, they require extended periods of time for disease development and result in high variability of disease manifestation, consuming time and expenses.

Surgically induced models are easily reproduced, generating severe lesions in the cartilage or soft tissues that could generate generalized joint disease. These models can be developed in a short and predictable time frame (species dependent), allowing one to study disease progression and treatment effects on disease progression and/or tissue repair. These models work by creating joint instability, altering forces of contact on articular surfaces, resulting in intra-articular inflammation and subsequent matrix degeneration, more closely representing post-traumatic OA. When choosing surgically induced OA models, one must understand joint biomechanics and the recruited forces for model creation. In horses, an induced carpal osteochondral fragment model with controlled post-operative treadmill exercise is the most commonly cited/used.[230] Briefly, arthroscopy of the middle carpal joint is performed to allow removal of an osteochondral fragment (chip) from the radial carpal bone with an osteotome. This creates an 8 mm osteochondral defect on the proximal and distal articular surface of the radial carpal bone. A motorized arthroscopy burr is used to debride the exposed subchondral bone between the fragment and parent bone, creating cartilage debris that will stimulate synovial inflammation along with articular incongruity and instability. The size, location of the fragment, loss of subchondral bone, and subsequent synovitis mimics naturally

occurring OA. Horses are exercised using a standardized treadmill for 70 days, starting at two weeks post-operatively.[235-237]

Chemically induced models consist of injecting a chemical product intra-articularly that produces inflammation, causing direct or indirect extracellular matrix damage and/or chondrocyte toxicity. These models produce rapid disease progression, while induction methods are less invasive and likely more accessible to investigators (does not require an equine surgeon, surgical suite, surgical equipment, etc.). However, chemical models do not entirely correlate with naturally-occurring OA pathogenesis in humans or veterinary species.[238] Chemicals like filipin or sodium monoiodoacetate produce fibrillation along the articular cartilage surface.[239-241] However, products like equine recombinant IL-1β and *E. coli* produce a marked but self-limiting inflammatory reaction within the joint (synovitis) due to global stimulation of joint-related tissues, in particular the synovial membrane.[242] Currently, it is well recognized that synovitis plays a crucial role in OA pathogenesis, setting the stage for initiation and progression of early OA changes.[30] Therefore, studies using these models often focus their evaluation methods on inflammatory mediators in synovial fluids and/or patient assessments (joint effusion and lameness) rather than more characteristic and chronic changes in OA such as articular cartilage degeneration (arthroscopy, MRI, histopathology +/- tissue PCR/protein expression), synovial hypertrophy and/or fibrosis (ultrasonography, histopathology +/- immunohistochemistry, tissue PCR/protein expression), subchondral bone changes including osteophyte formation (radiographs, computed tomography, biomechanical testing of subchondral bone plate, tissue PCR/protein expression).

Table 2.2. Summary of equine *in vivo* experimental models of osteoarthritis.

***In vivo* OA models**	**Specific name**	**Advantages**	**Disadvantages**
Spontaneous	Naturally-occurring OA [243]	Closer to naturally occurring OA Less instrumentation required	Variable in disease manifestation Time-consuming Increased cost
	Lower limb cast immobilization [244]		
	Trauma to the medial femur and tibia [245]		
Surgically induced	Osteochondral fragment and exercise [235-237,246-249]	Rapidly progressing OA Reproducible	Severe lesions More instrumentation and personnel required Inappropriate for pathogenesis studies Increased cost
	Metacarpophalangeal ligament transection [250]		
	Articular groove model [251,252]		
Chemical induced	Filipin (400 µg once a week for 12 weeks)[239]	The most rapidly progressing OA Less invasive procedures required Easy to implement	Inappropriate for pathogenesis studies Response variability
	Sodium monoiodoacetate (0.09-0.16 mg/kg)[240,241]		
	Amphotericin (25 mg IA once every other day for 3 treatments)[253]		
	E. coli lipopolysaccharide (0.125 - 0.5 ng) [242,254,255]		
	IL-1β [242,256,257]		
	Polyvinyl alcohol foam particles[258]		
	Carrageenan[259]		

Other uncategorized models study the development of spontaneous OA and their relationship with increased joint loading. In horses, this has been studied mainly in racing thoroughbreds, where different training regimens are compared with microstructural changes in articular cartilage and subchondral bone post-mortem.[45,260,261]

Although genetic models are not described nor used in horses due to species lifespan, cost, and ethical concerns, this model is used in more sentinel species (i.e. rodent models) for studying the role of specific genes in the OA. Genetically modified mice are commonly used in this type of model. Usually, the desired genetic mutation is designed to protect the synovial environment and/or animal from OA or make the animal more susceptible to OA progression to test certain OA treatments. Consequently, these studies have helped to establish the molecular basis of OA including the effect of proinflammatory cytokines on OA development.[262]

❖ DIAGNOSIS OF OA AND MEASUREMENTS OF DISEASE OUTCOME

Clinically OA is characterized by varying levels of pain, expressed as lameness in horses. The articular cartilage, joint capsule, synovium, peri-articular tendons, peri- or intra-articular ligaments, periosteum, and bone have all been defined as sources of pain in OA. Nociceptors are located within these related joint tissues and respond to mechanical and chemical stimuli.[263] Inflammatory cytokines such as IL-1β, TNF-α, IL-6, IL-17, substance P and PGE_2 have all been shown to contribute to OA-associated pain pathways.[84] Often, clinical signs do not directly correlate with microscopic or macroscopic changes occurring within the joint. Therefore, when diagnosing OA, more than just clinical assessment should be considered when the clinician suspects OA. Additional diagnostic tools used by clinicians could include standard and/or advanced imaging (radiography, ultrasonography, magnetic resonance imaging, computed tomography, nuclear scintigraphy, etc.), synovial fluid analysis and cytology, synovial biomarker analysis, tissue histopathology (+/- immunohistochemistry techniques), and/or arthroscopy. These tools can help clinicians and researchers interpret disease progression and/or efficacy of OA treatments in clinical cases and research.

I. <u>Clinical parameters</u>

Clinical parameters such a degree of lameness, synovial effusion, soft tissue swelling, and range of motion (flexion test) are used to characterize the stage of OA in horses. Loss of performance, lameness, and/or synovial effusion are some of the first clinical signs observed by owners. Synovial effusion can lead to pain, fibrosis of the synovial membrane, and decreased range of motion.[7] After static musculoskeletal examination of the horse, subjective lameness assessment by a trained equine veterinarian is standard. Most commonly, equine practitioners use

the American Association of Equine Practitioners (AAEP) grading scale to categorize lameness. Some studies have demonstrated that subjective lameness evaluation is often biased and can be inconsistent within and between observers, especially when the lameness is mild.[264-266] For this reason, researchers and clinicians use objective systems to measure gait asymmetry (presumed lameness) using kinetic (force plate analysis) and/or kinematics (video capture and/or inertial sensor systems) for assessment. Systems such as the lameness locator (Equinosis Q with Lameness locator® inertial sensor system) are more sensitive and consistent in detecting gait asymmetry in horses than subjective visual lameness evaluation, but these systems still require an experienced clinician to interpret data accurately;[267] therefore, a combination of both subjective and objective assessment leads to the best clinical assessment.[268] Flexion tests are an essential part of the lameness evaluation, identifying joints with a decreased range of motion or a painful response after flexion. Additionally, lameness examination can be accompanied by peri-neural or intra-articular analgesia.[7]

II. Imaging

Radiography is the most common standard imaging modality used in the diagnosis of OA in horses.[229] In horses with OA, the main radiological features include osteophytes, joint capsule distension, joint-space narrowing, subchondral bone sclerosis (with occasional lysis or cyst-like lesions in the subchondral bone) or osteochondral fragmentation. Although there is modest correlation between radiographic and clinical signs, radiography is neither sensitive nor specific for early OA changes,[269] allowing the clinician to assess only mid to late-stage OA without direct visualization of the articular cartilage itself.

Ultrasonography has been used to assess periarticular soft tissue structures. However, this tool can also be used to evaluate synovial membrane, articular cartilage thickness in areas that are accessible via ultrasound, peri- and intra-synovial ligaments (collateral ligaments, menisci, etc.) as well as the presence of osteophytes and/or enthesiophytes.[270]

When using nuclear scintigraphy for diagnosis of OA, methylene diphosphonate (MDP) and hydroxy methylene diphosphonate (HDP) are the common radionucleotides to be used. These compounds are complexed with technetium (^{99m}Tc) and preferentially bind to hydroxyapatite crystals in the bone during osteoblastic activity, allowing detection of bone abnormalities. However, increased radiopharmaceutical uptake of the joint in performance horses may not always reflect pathologic change and can be associated with normal adaptation of bone during training.[271] [272] Recently, another nuclear medicine modality, positron emission tomography (PET), has become available for horses. Compared with traditional nuclear scintigraphy, PET scan offers cross-sectional imaging properties, higher spatial resolution and can quantify biological function of tissues. In a recent study, early OA changes could be identified in the fetlock of Thoroughbred horses in training using PET scans, while MRI did not observe these subtle articular changes in the subchondral bone and proximal sesamoids.[273]

MRI has been considered the gold standard in human medicine to evaluate knee OA providing 3D information of the peri- and intra-articular joint structures.[274] In horses, this modality has been used to evaluate cartilage and subchondral bone in clinical and research cases.[275-278] Equipment availability, expenses, and necessity of general anesthesia for high-field MRI are some of the main disadvantages of this modality in horses. Due to the sheer size and anatomical composition of our equine patients, not all anatomic locations along the extremities can be imaged. For example, the stifle joint can be challenging and often inaccessible for proper

imaging via MRI, despite this joint's use in osteochondral defect research. Although low-field MRI is available for standing and recumbent procedures, it does not provide the same quality image as high field MRI for assessing the articular cartilage.[279] However, low-field MRI is more sensitive than radiography and ultrasound when diagnosing advanced OA of the distal interphalangeal joint.[280]

Computed tomography (CT) is becoming more widely available and more commonly used to diagnose bone and some soft tissue disorders of the horse. CT has been used to characterize the normal subchondral bone density and patterns of articular cartilage degeneration.[281,282] In humans, when MRI is unavailable, computed tomography arthrography (CTA) of the knee has provided accurate information to diagnose cruciate ligament, cartilage, and meniscal injuries.[283] Intra-articular injection of a radiopaque contrast in horses allows identification of cartilage defects and soft tissue lesions in the stifle and carpus that other imaging modalities could not detect.[277,284,285] Another CT modality that has been used mainly in research is high- definition micro-CT systems. This modality can track structural changes in the subchondral bone and cartilage and has become more popular in equine OA research, but it the size of the samples that can be evaluated is limited and it has been mainly used studies using mainly equine limbs *ex vivo*.[286,287]

Arthroscopy evaluation remains the gold standard for defining the degree of osteoarthritic disease in horses.[6] Arthroscopy is a more invasive method to evaluate the synovial space and articular cartilage than the above reference imaging modalities, and the amount of articular surface that can be evaluated is limited.[277] Most commonly, this procedure is performed under general anesthesia, although it can be performed using needle arthroscopy understanding sedation.[288,289]

All these imaging modalities present certain limitations individually, but recent publications have highlighted the importance of using a combination of different imaging modalities to obtain the maximum information regarding the soft-tissue, cartilage and subchondral bone structure within the joint.[273,277,285,290]

III. Histology

Histologic samples of joint tissues, in conjunction with immunohistochemical staining, can classify and measure the degree of joint degeneration. A macroscopic and histological grading system for articular cartilage and synovial membrane was created to quantify OA changes in horses. Macroscopically, this system evaluates the presence and severity of wear lines, fibrillation, erosions, and palmar arthrosis of the fetlock.[173] Microscopically in cartilage, chondrocyte necrosis, clustering, fibrillation/fissuring, focal chondrocyte loss, and safranin O-fast green (SOFG) staining have been used for evaluating joint pathology, while in synovial membrane, cellular infiltration, vascularity, subintimal edema and fibrosis, and intimal hyperplasia are features commonly graded.[173] SOFG stain is a simple histologic technique used to detect proteoglycans in cartilage, but more complicated immunohistochemistry techniques to quantify type II collagen, for example, can be used to evaluate OA in clinical cases and research.

Structural protein (type II collagen or aggrecan), cellular membrane receptors, or cytokines involved in the OA disease could be detected and quantified in joint tissues using immunohistochemistry techniques.

IV. Synovial fluid cytology and biomarker analysis

Normal values for cytologic analysis of synovial fluid in the horse and their changes in joint disease have been reported. Synovial fluid from joints with OA can have greater than 1000 nucleated cells/μL, with less than 15% neutrophils and more than 85% mononuclear cells and a total protein between 0.8-3.5 g/dL.[7] Normal synovial fluid is pale yellow, clear, and free of flocculent debris, and the presence of opacity and flocculent material in the sample indicates joint inflammation. At the same time, during the acute phase of OA (synovitis), the volume of synovial fluid increases and the viscosity decreases.[291] The viscosity of the synovial fluid is directly related to the hyaluronan content.[292] Most of the synovial fluid parameters indicate the relative amount of synovitis in the joint but does not necessarily furnish specific information about the metabolic status of the cartilage.

Biomarkers are defined as is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention".[293] Research into measurement of reliable and accurate biomarkers for OA has been conducted for years, but more recently, newer techniques and/or biomarkers are being used to detect OA early in the disease process.[294] Degradation products of the extracellular matrix have been investigated as biomarkers for OA and can be measured in the serum and synovial fluid. Biomarkers can provide information regarding catabolic or anabolic balance in which the synovial environment is, whether inflammation and cartilage destruction is happening, or the cartilage is attempting to heal. A large number of biomarkers related to OA have been described, and a comprehensive review describing all biomarkers studied in equine is not the focus of this literature review, but the ones that are discussed are important because these

assays are frequently used in OA research, and there are multiple studies in the literature to which results can be compared.

Measurement of lactate dehydrogenase (LDH) and GAG in synovial fluid or culture media are frequently used in equine OA research. LDH is a cytosolic enzyme that is released when the cells die, and its detection has been used to perform cytotoxicity assays.[119] Previous studies have reported increased concentrations of this protein in synovial fluid of patients with OA,[295] and the results of another study suggested that LDH originates from chondrocytes.[296] Increase synovial GAG reflects degenerative cartilage changes associated with OA.[297] Dimethyl methylene blue (DMMB) assay measures GAG concentration via absorbance after conjugation of 1,9-dimethyl methylene blue to GAGs using chondroitin sulfate as a standard. However, the DMMB assay is not very specific because it identifies all GAGs present in synovial fluid regardless of origin.[291]

As mentioned previously, PGE_2 is one of the main inflammatory proteins participating in OA and is associated with intra-articular pain.[84] Previous research suggests that increased PGE_2 is due to synovial inflammation, not articular damage.[298] An increased concentration of PGE_2 in the synovial fluid has been observed in lame horses that responded to intra-articular anesthesia, concluding that there could be a relationship between synovial fluid PGE_2 concentrations and pain.[299] In many studies, the measurement of PGE_2 concentration in synovial fluid has been used to evaluate treatment efficacy regarding a reduction in PGE2 to be related to anti-inflammatory and analgesic effects within the joint.[116,300-302]

The measurement of more specific anabolic or catabolic cartilage biomarkers is available. Increased concentration in synovial fluid of carboxy propeptide of type II collagen (CPII) and chondroitin sulfate (CS) epitopes such as CS-846 indicate synthesis of type II collagen and

aggrecan, respectively, and this can be interpreted as an attempt of the cartilage to regenerate. The presence of these markers indicates an activated metabolic state in cleavage and upregulated repair mechanisms. CPII and CS- 846 have been observed to be elevated in serum and synovial fluid of horses with experimentally and naturally occurring OA. In synovial fluid, CPII was increased in early OA disease and decreased in later stages,[235,303] and CS-846 levels peaked in parallel with GAG release after experimental induction of synovitis.[304] Type II collagen cleavage markers such as carboxy-neoepitope of type II collagen that is exposed after collagenase-cleavage (C2C), carboxy-terminal telopeptide fragments of type II collagen (CTX-II), catabolic collagenase-cleaved type II collagen epitope (C12C) and Col2-1 and Col2-1NO2 have been observed to be increased in the synovial fluid of horses with experimentally induced inflammation or osteochondral fragmentation.[304-307]

COMPs are non-collagenous proteins that form part of the ECM of articular cartilage. Initially, these proteins were thought to be cartilage-specific, but they have been identified in other musculoskeletal tissues like tendons, ligaments, and synovial membranes. Unique fragments of COMP have been identified in inflamed cartilage explants of horses,[308] and a recent publication has found that the measurement of COMP neoepitope in the synovial fluid has the potential to be used as a biomarker for early OA disease.[309]

Biomarkers of bone turnover such as osteocalcin, bone-specific alkaline phosphatase, or type I collagen C-telopeptides (CTX-I) have been used to evaluate the relationship between the subchondral bone changes and OA disease. [294]

❖ CURRENT INTRA-ARTICULAR THERAPIES FOR OA IN HORSES

While the diagnosis of OA is mainly based on clinical and radiological features, lameness represents the first and prevailing symptom that leads owners to seek veterinary advice. OA is a chronic degenerative disease that cannot be cured.[310] The current treatment of OA is based on symptom management, which is primarily pain control, and relies on the combination of non-pharmacological (rest and controlled exercise) approaches and symptom-modifying drugs that are generally tailored to the patient's needs and risk factors. Systemic nonsteroidal anti-inflammatory drugs (NSAIDs) and intra-articular corticosteroids are some of the most common symptom-modifying drugs used by equine practitioners to treat lameness.[311] The ideal treatment to manage OA should help stop or slow down progression of the lesions as well as enhance cartilage healing,[312] which is the primary goal of disease-modifying drugs like orthobiologics therapies.

I. Corticosteroids

Natural corticosteroids participate in diverse cellular functions, including development, homeostasis, metabolism, cognition, and inflammation.[313] Synthetic corticosteroids have a core structure of 21-carbon molecules, bonded in four fused rings (three 6-carbon rings and a 5-carbon ring). These modifications in the chemical structure are responsible for different pharmacological activities, and researchers have aimed to produce drugs with increased glucocorticoid activity and decreased mineralocorticoid activity, producing a more potent anti-inflammatory effect.[314,315]

Corticosteroids exert their anti-inflammatory effects through four mechanisms of action: one genomic and three nongenomic. The genomic mechanism produces modifications of gene

expression by binding to the cytosolic glucocorticoid receptor (cGR) and migrating into the nucleus, up-regulating the expression of anti-inflammatory proteins such as lipocortin 1, IL-10, MAP kinase phosphatase 1; and suppressing the expression of proinflammatory cytokines such as IL-1, IL-2, IL-3, IL-6, and TNF-α.[316] The three nongenomic mechanisms recognized include non-specific interaction with the cellular membrane, non-genomic effects mediated by the cGR, and specific interactions with a membrane-bound glucocorticoid receptor (mGR).[316-318] These three mechanisms have been proposed to explain the rapid anti-inflammatory and immunosuppressive effects of corticosteroids, but their primary effect is related to their genomic mechanisms. Their pain-relieving properties are related to inhibition of phospholipase A2, blocking the arachidonic acid cascade, and selectively inhibiting cyclooxygenase-2 (COX-2) expression.[310] Corticosteroids also can have a dose dependent-effect decreasing MMP activity, either through direct effects of the corticosteroids on MMPs or via inhibition of cytokines that up-regulate MMP production.[319] In articular explants of horses, mRNA expression of MMP-1, -3 and -13 are decreased when adding methylprednisolone acetate (MPA) or triamcinolone acetate (TA) to culture media.[320]

Corticosteroids have a time- and dose-dependent effect on articular cartilage, with beneficial effects occurring at low doses with short-term treatment periods and detrimental effects at high doses with long-term treatment periods. Some authors have identified that the analgesic effect of corticosteroids through dampening of pain could lead to corticosteroid arthropathy or catastrophic fractures.[310,321,322] Actually, a recent study found an increased hazard of post-medication fracture within 56 days after the last intra-articular medication with corticosteroids in horses that received more than three intra-articular corticosteroid injections in

their life.[323] This controversy has initiated research into enhancing the understanding of the ideal dosage and risk-benefit ratio of intra-articular corticosteroids.

In 1955, hydrocortisone was used intra-articularly for the first time horses,[324] but now, triamcinolone acetonide (TA), betamethasone sulfate (BS), and methylprednisolone acetate (MPA), either alone or in combination with hyaluronic acid, are the most common corticosteroids used intra-articularly in horses.[311] Studies carried out in an experimental osteochondral fragment model previously described, provided compared the intra-articular use of these three products finding that their treatment effects are not equivalent.[248,325,326]

Previous studies in different species indicate that MPA has significant dose-dependent deleterious effects on cartilage, but lower doses demonstrated beneficial effects downregulating MMPs and increasing chondrocyte density.[319] The dose recommended for MPA in horses ranges from 40-120 mg per joint.[327] Protection of articular cartilage against cytokine-induced matrix degradation, improved lameness and synovial membrane histology scores, and significantly reduced PGE_2 concentration in the synovial fluid are some of the beneficial effects of MPA reported.[248,328] However, i*n vitro* and *in vivo* studies have observed deleterious effects after administration of MPA such as decreased GAG content, increased chondrocyte necrosis, decreased articular cartilage thickness, and more extensive cartilage erosions and fibrillation.[203,329-331] These negative effects might have led equine practitioners to use MPA mainly in low-motion joints since articular cartilage preservation is not as important.[311,322,327]

In humans, studies using BS demonstrate time- and dose-dependent deleterious effects on articular cartilage and chondrocytes.[319] The dose recommended for BS in horses ranges from 3-18 mg per joint.[327] *In vitro*, BS at high concentrations (0.1–100 μg/mL) suppressed proteoglycan synthesis by chondrocytes, while lower doses (0.001–0.05 μg/mL) did not produce any

deleterious or beneficial effect.[332] In an osteochondral fragment model, no deleterious effects were observed after treatment with BS.[326] However, there are not many studies evaluating the effects of BS.

According to a meta-analysis evaluating the effects of corticosteroids in humans and other species, TA has shown beneficial and detrimental dose-dependent effects, finding adverse effects on the cartilage with an 18-mg cumulative dose per joint.[319] The dose recommended for TA in horses ranges from 6-18 mg per joint.[327] In the osteochondral fragment model, treatment with TA produced beneficial effects such as improving lameness scores, and increased HA and GAG concentration in synovial fluid. Synovial membrane collected from horses treated with TA had significantly less inflammatory cell infiltration, subintimal hyperplasia, and subintimal fibrosis.[325] In addition, no detrimental effects were observed on the subchondral bone after TA administration.[45] The results of these studies could be why equine practitioners would rather use TA in high-motion joints than MPA.[311] However, in studies carried out *in vitro* TA has had controversial results. TA in IL-1β stimulated chondrocytes did not present any protective effect on the inhibition of the gene expression aggrecan and type II collagen produce by IL-1β,[333] but TA showed chondroprotective effects in LPS and IL-1β culture models.[334,335] Another study found TA was comparable to MPA as far as deleterious effects on cartilage metabolism.[203] Also, *in vivo* TA administration produced an increase of catabolic cartilage biomarkers and downregulation of collagen and aggrecan gene expression,[336,337] which made researchers think that TA could have detrimental effects when used overtime and recommended judicious use. In addition, it is critical to think that even though TA is injected intra-articularly, it can affect the patient systemically. A recent study reported that TA when used intra-articularly improves the lung function of horses with asthma.[338]

In the literature, some reports have associated intra-articular administration of corticosteroids with an increased risk of developing laminitis following administration.[321] However, a more recent retrospective study failed to prove a clear correlation between laminitis and corticosteroids.[339] Bathe reported an incidence of laminitis in 0.15% of horses treated with corticosteroids, with a higher risk in horses treated with high doses of TA (20-45 mg).[340]

The combination of intra-articular corticosteroids with drugs such as HA has been investigated, aiming to mitigate the previously described harmful effects of corticosteroids in articular cartilage. In humans, a combination of corticosteroids and HA produced significant short-term improvement in pain scores compared to each treatment individually.[341,342] In horses, i*n vitro* studies report that a combination of HA and MPA increased PG synthesis,[343] and TA and HA decreased GAG catabolism.[335] Clinically, 60% of equine veterinarians reported favorable outcomes from this combination, but these results were obtained from a survey.[311] More standardized i*n vivo* studies have found contradictive results, where the combination of corticosteroids (BS or MPA) and HA reduced proteoglycan breakdown compared to treatment only with corticosteroids,[344] but TA and HA combined seemed to be less effective compared to horses treated with TA only.[345]

II. Hyaluronan

Hyaluronan (HA) is a protein secreted by the synoviocytes that plays a vital role in the joint. HA works as a boundary lubricant of joint surfaces in synovial fluid and is a major component of the ECM of articular cartilage.[327] Effects of HA within the synovial compartment depend on concentration and molecular weight.[327] It has been observed that a molecular weight of 500 KDa or greater is necessary for enhancing the synovial environment, improving boundary

lubrication and increasing endogenous production of HA.[346] The exact mechanism through which HA exhibits an effect is not known, but curiously, the majority of exogenous hyaluronan is cleared within 96 hours post-injection.[347] Anti-inflammatory effects and disease-modifying effects, such as inhibition of chemotaxis, inhibition of phagocytosis by granulocytes and macrophages, and reduction in the stimulation, proliferation, and migration of lymphocytes, have been attributed to HA in horses.[327] However, in humans, HA has been categorized as a symptom-modifying drug.[327]

The use of intra-articular injections of hyaluronan (HA) aims to return the elasticity and viscosity of the synovial fluid to normal or higher pre-injection levels, and this is known as viscosupplementation.[348] HA has also been extensively researched in multiple species. In humans, HA helped preserve cartilage volume and improve clinical signs up to 1 year in patients with severe knee OA, but these studies were not blinded and lacked controls.[349,350] Although a recent meta-analysis studying medical management of OA found that treatment with HA is associated with limited improvement in pain and an increased risk of complications.[351] As previously mentioned, a disease-modifying effect of HA has been observed in horses. Frisbie et al. found a significant improvement in clinical signs and decreased cartilage fibrillation in horses treated with HA after experimental OA induction, suggesting that HA does alter the disease course.[352] Other in vivo studies have shown weaker disease-modifying effects, mainly finding improvement only in clinical lameness. *In vivo* studies using HA and PSGAG in horses are summarized in table 2.3.

HA can also be used intravenously, producing beneficial effects in the joint. Using the carpal osteochondral OA model, intravenous injection of 40 mg HA weekly for 3 treatments

Table 2.3. Summary of *in vivo* studies that evaluate intra-articular use of hyaluronic acid and/or polysulfated glycosaminoglycans in horses.

Authors/ year	Horses	Lesion	Follow up	Outcome measures	HA concentration	Results	Study limitations	Effect
Frisbie et al. 2009[352]	Blinded study N= 24 horses (n= 8 HA, n=8 PSGAG and n= 8 control)	Surgically induced osteochondral fragment	28 days	Gait and histological analysis	HA 20 mg PSGAG 250 mg	HA decreased cartilage fibrillation PSGAG decreased fibrosis and vascularity	Small number	(+)
McIlwraith et al. 1998[362]	Prospective N= 140 quarter horses	Lameness	9 months	Lameness	HA 40 mg IV	HA trended to race longer, higher speed more money earned	Heterogeneous population	(+)
Gaustad and Larsen 1995[363]	Blinded study N= 77 standardbred	Moderate to severe lameness	7 weeks	Gait analysis	HA 20 mg PSGAG 250 mg	Lameness improvement with HA and PSGAG compared to placebo	Few outcomes measured Heterogeneous population	(+)
Todhunter et al. 1993[364]	Controlled Experimental study N=18 ponies	Surgically induced osteochondral fragment	90 days	Gait, SF, histological, GAG analysis	PSGAG 250 mg weekly for 5 treatments	PSGAG was detrimental reduced type II: type I collagen ratio	Small number	(-)
Peloso et al. 1993[253]	Blinded experimental N= 24 horses	Induced synovitis with amphotericin	23 days	Gait analysis	HA 8 mg, 16 mg and 32mg	No significant difference	Small number	(-)
Yovich et al. 1987[365]	Controlled Experimental study N= 8 horses	Induced synovitis MIA	8 weeks	Gait, histology analysis	PSGAG 250 mg weekly for 5 treatments	PSGAG chondroprotective effect	Small number	(+)
Auer et al. 1980[243]	Experimental study N= 16 horses	Surgically induced osteochondral fragment	2 weeks	Gait analysis	HA 40 mg	Lameness improvement	No control Not blinded Small number	(-)

improved lameness, synovial membrane histology scores and decreased PGE_2 and protein concentration in synovial fluid 42 days after the last treatment.[353]

Despite the positive opinion about the combination of HA and corticosteroids, as previously described, studies have found contradictory results. A recent publication found that when the carpal sheath, medial femorotibial, distal intertarsal and tarsometatarsal joints were treated with MPA and HA, clearance of MPA could be retarded.[354] This needs to be considered when regarding withdrawal times recommended for regulated competitions.

III. Polysulfated glycosaminoglycans (PSGAGs)

The main GAG in commercial PSGAG is chondroitin sulfate (CS), a structural component of the articular cartilage. Some studies have shown PSGAGs downregulate MMPs and PGE_2 and to affect proteoglycan synthesis and degradation.[355,356] PSGAGs have been categorized as a disease-modifying drug, and the therapeutic use of PSGAGs is aimed at preventing or retarding irreversible cartilage degeneration.[357] Early *in vitro* study reported that PSGAG increased collagen synthesis and GAG in both chondrocyte and cartilage explant cultures from normal and osteoarthritic equine samples.[358] However, another study found a dose-dependent inhibition of proteoglycan synthesis, a nonsignificant effect on proteoglycan degradation.[359]

Several studies have evaluated the effects of PSGAG use intra-articularly, and often this drug is compared to HA. A summary of the *in vivo* studies using PSGAG in the horses' joints is shown in table 2.3. This medication can be administered intramuscularly and orally as well, but the beneficial effects have been weakly demonstrated. Trotter et al. did not report significant benefits after administering 500 mg of PSGAG intra-muscularly every 4 days for 7 treatments besides an improved histologic score in the GAG staining on the cartilage.[241]

Adverse reactions have also been reported with intra-articular administration of PSGAG. There is clinical and experimental evidence that PSGAG diminishes the joint's capacity to resist infection.[360] Although this risk has been shown to be avoided by simultaneously administering amikacin (125 mg) intra-articularly.[361] In the last decade equine practitioners have been more inclined to use this medication intra-muscularly than in the joint.

IV. Orthobiologics used to treat OA

Orthobiologics therapy refers to the clinical application of biologically derived materials processed and used to promote repair or regeneration of musculoskeletal tissue.[366] Many studies have focused on their disease-modifying effects, aiming to gain a better understanding of their cellular mechanism and possible therapeutic efficacy in clinical cases. Orthobiologics can be blood or cell derived. Therapies such as platelet-rich plasma (PRP), autologous conditioned serum (ACS), autologous protein solution (APS), mesenchymal stem cells (MSCs), and bone marrow concentrates are categorized as orthobiologics. These products are often used not only intra-articularly but also intra-lesionaly in tendinous and ligamentous injuries.

Platelet-rich plasma (PRP)

Among all blood-derived products available, PRP has been described and used for the longest.[367] PRP refers to a portion of the patient's blood that has at least 1 million platelets per μL (two to six-fold more than whole blood) via centrifugation or filtration.[368] These platelets degranulate after activation, releasing the growth factors (PDGF, TGF-β, VEGF, and IGF-1) contained in them.[369] Increases in cytokines such as IL-1, IL-1ra, IL-4, IL-6, and TNF-α, as well as PDGF and TGF-β have been documented in equine joints treated with PRP.[370-372]

PRP can be produced using commercially available systems or manual laboratory techniques. The cellular and cytokine profiles of PRP have been evaluated, finding high individual variability and differences between the system of preparation used and platelet activation.[373-376] In general, it is thought that platelet plasma products have higher concentrations of growth factors due to their higher platelet concentrations, but this is not consistently supported.[377] A recent *in vitro* study using PRP in tendons reported that it was more important to reduce the number of leukocytes than increase the platelet count to decrease inflammation and enhance matrix gene synthesis.[378] However, this needs to be studied within synovial tissues.

A classification system has been proposed in an attempt to group different PRPs based on their composition, including four major categories: pure PRP with low WBC and collected with anticoagulants (P-PRP), leukocyte-rich PRP with high WBC and collected with anticoagulants (L-PRP), pure platelet-rich fibrin with low WBC and coagulated (PRF), and leukocyte-rich PRF with high WBC and coagulated (L-PRF). This classification may help to interpret the difference in the results found in studies using different types of PRP.[379,380] Although not included in this classification, platelet lysate is another PRP product that has recently been studied. Platelet lysate has shown antibacterial properties.[381] Also, intra-articular injection of platelet lysate in horses with naturally occurring OA produced a decreased concentration of MMP-9 and ADAMTS-5 in synovial fluid at 10, 30, and 60 days post-treatment.[382]

In vitro studies evaluating the effects of platelet-derived products on inflamed joints have shown beneficial effects related to inflammation and matrix catabolism. Multiple studies reported an anti-inflammatory effect, where PRP downregulated expression of MMP-13, stimulated endogenous HA production,[383] decreased chondrocyte apoptosis,[384] inhibited chondrocytes NF-κB activity and decreased expression of COX-2[385] in chondrocytes after

stimulation with IL-1β. Chondroprotective effect by increasing chondrocyte production of PGs and collagen has also been reported.[386] In addition, the efficacy of PRP products could vary upon their cellular (WBC and RBC) concentrations. It has been suggested that the presence of leukocytes could enhance pro-inflammatory and catabolic enzyme production.[387] An i*n vitro* study using human chondrocytes showed that R-PRP produced a better anti-inflammatory effect than L-PRP.[388] However, in patients with knee OA, L-PRP did not produce an upregulation of pro-inflammatory mediators.[387] In horses, L-PRP had an increased concentration of growth factors and anti-inflammatory cytokines compared to P-PRP.[389]

In vivo, PRP has shown variable improvement in patient-assessed functional outcomes and objective disease-modifying measures in human OA. Often PRP has been compared to HA, finding that PRP offers better outcomes than HA, especially for patients with early OA disease.[390] Symptom-related benefits such as pain or lameness reduction have been weakly demonstrated in horses with naturally occurring OA and a summary of the studies using PRP intra-articularly for OA in horses is reflected in table 2.4. A meta-analysis evaluating PRP studies performed in humans and horses confirmed that biased, poorly designed studies, without controls or blinding, and not using standardized outcome measurements, favored the observation of positive results in clinical studies, particularly in equine research.[391] In horses, some studies have evaluated the use of PRP in conjunction with other regenerative therapies such as chondrocytes, MSCs, BMPs and scaffold materials (gelatin/beta-tricalcium phosphate sponges). PRP in combination with these products, have demonstrated disease-modifying effects following intra-articular injection or direct implantation within focal osteochondral defects.[392-396] Currently, no recommendations on volume, platelet concentration, leukocyte concentration, and frequency

Table 2.4. Summary of *in vivo* studies that evaluate intra-articular use of PRP in horses.

Authors/ year	Horses	Lesion	Follow up	Outcome measures	System/ cytology/ activation method	Intervention	Results	Study limitations	Effect
Smit et al. 2019[401]	Controlled cases series N= 10 horses (n= 5 OA, n= 5 no OA)	Mild to moderate OA	14 months	Gait analysis SF	V-PET system /6.21 x 10^5 platelets/µl No activation	Single 4 ml PRP IA injection	No significant difference	Small number Few outcomes measured	(-)
Mirza et al. 2016[402]	Cohort study N = 12 horses	Moderate to severe OA	16 weeks	Radiographs, gate analysis and IA anesthesia	E-PET system / 5.2 × 10^4 to 9.5 × 10^5 platelets/µL No activation	Single 5-10 ml PRP IA injection	Significant improvement lameness	Small number No controls	(+)
Moraes et al. 2015[403]	Control study N =8 horses (PRP vs. Saline)	Healthy joint	1 month	SF analysis	Manual double centrifugation / 423 × 10^3 platelets/µL/ No activation	Single 4 ml PRP IA injection	No differences in IL-1β, Il-1rap, TNF-α, self-limiting inflammatory response after PRP	Small number Healthy joints No blinding	(-)
Pichereau et al. 2014[404]	Case series N= 20 endurance horses	Refractory chronic OA	12 months	Gait analysis SF analysis	Manual double centrifugation/ P-PRP 560 × 10^3 platelets/µL /$CaCl_2$	3 PRP injections with a 15-day interval	80% able to resume work at the same level, significant reduction of IL-1β	Small number No control No blinding	(+)
Textor et al. 2013[372]	Blinded experimental N= 7 horses	Healthy joints	4 days	Gait analysis SF analysis	E-PET/ 542 X 10^3 Platelets/µL / $CaCl_2$ and thrombin	Single 2 ml PRP IA injection	PDGF and TGFβ higher in PRP groups, thrombin increased inflammatory cytokines	Small number Short period Healthy joints	(±)

Authors/ year	Horses	Lesion	Follow up	Outcome measures	System/ cytology/ activation method	Intervention	Results	Study limitations	Effect
Abelanet; Prades 2009[405]	Controlled cases series n = 42 sport horses Chronic OA patients, unresponsive to rest or IA steroid therapy (n = 12)	Chronic (n = 20) and acute (n = 10) cases of OA Healthy (n= 12)	12 - 42 months	Return to athletic performance rate of reinjury	Manual double centrifugation	3 PRP IA injections	No significant differences between PRP and control groups in return to athletic performance. PRP treated horses had a lower rate of reinjury.	Small number Group heterogenicity No placebo control Poor PRP characterization	(±)
Carmona; López; Prades 2009[406]	Case series n = 7 horses	Severe joint disease (OA = 4 and OC = 3)	12 months	Degree of lameness and joint effusion	Manual double centrifugation 259 $X10^3$ platelets/µL / $CaCl_2$	3 PRP IA injections 2 week-interval	Improvement in lameness degree and joint effusion in PRP, more so after last treatment	Small number Poor study design Heterogeneous population No blinding No control Only clinical evaluation	(+)
Carmona et al. 2007[407]	Pilot case series n = 4 horses	Chronic OA	1 year	Gait analysis, US, SF analysis, and clinical evaluation	Manual double centrifugation/ 250 x 10^3 platelets/µL / $CaCl_2$	3 IA injections (10-20 ml) at 2 week-intervals	Significant improvement in lameness degree and joint effusion	Small number No blinding Heterogeneity of lesion treated No US results reported Low platelet count on PRP	(+)

of injection exist, although clinical evidence suggests administration of up to three injections at 2-week intervals should be considered.[367]

Autologous conditioned serum (ACS)

ACS is obtained by aseptic incubation of the patient's whole blood with borosilicate glass beads. The result is a cell-free product with an enriched anti-inflammatory cytokine and growth factor profile (IL-1rap, IL-10, TGF-β, and IGF-1).[397] Leukocyte production of these cytokines and growth factors are thought to be a result of leukocyte interaction with the beads during a 24-hour incubation period.[398] The post-incubation product is recovered by a single centrifugation step and injected into the affected joint. It is believed that ACS exerts its disease-modify effect primarily due to the high concentration of IL-1rap.[399] In humans, *in vitro* experiments have established that a 10- to a 1000-fold increase of IL-1rap to IL-1 is necessary to block all of the available IL-1 receptors that are upregulated during osteoarthritis.[144] Although these numbers have not been confirmed in horses, a recent study found that horses with better clinical outcomes were injected with ACS that contained higher concentrations of IL-1rap, IGF-1, and TGF-β.[400] Significant variability in the cytokine profile contained in ACS processed using different commercially available systems has been reported in horses.[397] This added to considerable inter-individual variation observed using the same product,[397,398,408,409] makes the study of cellular mechanisms of ACS more challenging. Also, it is essential to consider that surgical stress can affect cytokine concentration. Fjordbakk et al. studied the impact of surgical stress on cytokine concentration by correlating serum amyloid A (SAA) concentration with the degree of surgical stress. Horses classified with 'marked' stress (SAA > 200 mg/L) had significantly lower

concentrations of IL-1ra, TGF-β, and IGF-1 than horses classified with 'moderate' or 'mild' stress (SAA < 200mg/L).[398]

It has been shown that IL-1rap progressively increased up to 140-fold with incubation for up to 24 hours.[410] ACS contains not only anti-inflammatory cytokines but also contains pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, and their concentration can be affected by incubation time and surface.[411] Interestingly, a recent study in horses found that incubation time of up to 36 hours did not affect the cytokine profile and whole blood incubated in glass tubes produced a similar cytokine profile to commercial kits.[409] These results suggest that further investigation using incubated serum should be performed.

The cytokine and growth factor concentration of ACS has been compared to platelet plasma products, platelet concentrate, and platelet lysate within the horse.[412] ACS had significantly higher concentrations of TGF-β1, PDGF-BB, and IL-1Ra, but had similar IGF-1 concentrations compared to platelet concentrate. Platelet lysate contained similar concentrations of PDGF-BB but higher IL-1Ra and lower IGF-1 and TGF-β1 concentrations compared to ACS.[412]

Not many *in vitro* studies have evaluated the cellular response of joint tissues to ACS treatment. Interestingly, a study using human cartilage explants obtained from patients with OA did not find any beneficial effect in proteoglycan metabolism of chondrocytes collected from patients that previously received ACS treatment.[413] However, articular cartilage explants in this study were obtained in patients with severe OA that went under total knee arthroplasty, and other authors have suggested that ACS may be more effective in mild to moderate OA cases.[410] In horses, similar results were found in another *in vitro* study, where despite finding a higher

concentration of IL-1rap in media after treatment with ACS, chondrocyte proteoglycan matrix metabolism was not improved compared to autologous unconditioned serum.[414]

Although *in vitro* results have not provided a clear answer into the mechanism of action for ACS, studies in humans and horses have reported positive clinical effects after ACS treatment. To obtain more objective information regarding treatment efficacy, blinded, controlled and randomized studies have been conducted in human medicine, finding that ACS produced a significant improvement in pain scores, range of motion, and decreased joint effusion in patients with OA.[144,415,416] In addition, ACS has produced better outcomes compared to other intra-articular treatments such as betamethasone, HA or PRP in patients with knee OA.[415,417,418] A recent systematic review in humans concluded that despite limited evidence for ACS use intra-articularly, this therapy can improve pain and functionality of patients with mild to moderate OA and may be effective in patients unresponsive to other intra-articular treatment.[419] On the other hand, a prospective long-term study showed that ACS did not prevent or delay surgical intervention (knee arthroplasty).[420] Similarly, ACS has shown mainly improved functional outcomes (reduced lameness) in horses, but minimal disease-modifying effects have been established.[237,421-423] An experimental model of induced carpal OA in horses did not observe any adverse reaction after weekly intra-articular administration of ACS for four consecutive treatments, showing significant improvement in lameness and histologic scoring of the synovial membrane to the control (sham) group.[237]

Often different biologic and/or synthetic therapies are combined to manage OA. Chitosan, a derivative from chitin, is an essential natural polymer that is widely used for cartilage repair. This polymer has been used in studies as a scaffold material, showing positive effects on the proliferation and migration of chondrocytes.[424] A study in rabbits found that the combination

of ACS and chitosan promotes repair of osteochondral defects more effectively than either treatment alone.[425] A recent publication has found that ACS increases chondrogenic differentiation and immunomodulatory activity in human adipose MSCs.[426] This could offer an advantage when injected intra-articularly simultaneously, but further research *in vivo* is required.

It is important to note that the manufacturer recommends dividing ACS to attain 4 ml aliquots per syringe, but in a clinical setting, the volume needed can be influenced by joint pathology, joint volume and clinician preference. Although no standard dose for ACS is universally accepted, Weinberger reported a treatment protocol based on the type of joint medicated based on his personal experience (Table 2.5)

Optimum dose and frequency of ACS administration remains to be determined. In humans, a study noted a strong clinical response in patients with knee OA after intra-articular injection of 1 mL of ACS weekly for 3 weeks.[427] In horses, the most common treatment protocol recommended for ACS is 3 to 5 intra-articular injections spaced 1 to 2 weeks apart.[369,423] This treatment protocol is based on clinical opinion and experience. Recently, a 2-day injection interval was found superior to the traditional weekly injection regime, reducing synovial fluid biomarkers (C12C, CP-II, and CS 846) in horses with natural OA. However, no differences in clinical signs were observed.[408] A summary of studies evaluating ACS use intra-articularly is presented in table 2.6.

ACS is utilized by many veterinary practitioners when there are no owner economic restrictions and/or when patients have become refractory to symptomatic treatment with corticosteroids. The results of a survey distributed to equine veterinarians showed that clinically, most veterinarians felt that the metacarpal/metatarsal phalangeal joint displayed the best response to ACS injection (37.3%), followed by the femorotibial joint (21.6%) and distal

interphalangeal joint (20.3%).[311] In addition to the treatment of OA, equine surgeons have also used ACS in a prophylactic manner following arthroscopic surgery, aiming to reduce the inflammation of the joint during the postoperative rehabilitation period.[369]

Table 2.5. ACS treatment protocol recommended by the manufacturer, according the clinical experience of Weinberger in 262 horses.[423]

Joint	Treatment protocol
Coffin joint	Dose: 4 – 6 ml Number: 2 – 3 times Interval: 8 – 14 days
Pastern joint	Dose: 2 – 4 ml Number: 2 – 3 times Interval: 8 – 14 days
Fetlock joint	Dose: 4 – 6 ml Number: 2 – 3 times Interval: 8 – 14 days
Radiocarpal/ Intercarpal joint	Dose: 4 – 6 ml Number: 2 – 3 times Interval: 8 – 14 days
Elbow joint	Dose: 4 – 6 ml Number: 2 – 3 times Interval: 8 – 14 days
Shoulder joint	Dose: 4 – 8 ml Number: 2 – 3 times Interval: 8 – 14 days
Tarsometatarsal and distal intertarsal joint	Dose: 1 – 2 ml Number: 2 – 3 times Interval: 8 – 14 day
Tarsocrural joint	Dose: 6 – 8 ml Number: 2 – 3 times Interval: 8 – 14 days
Femorotibial and femoropatellar joint	Dose: 4 – 8 ml Number: 2 – 3 times Interval: 8 – 14 days
Hip joint	Dose: 4 – 8 ml Number: 2 – 3 times Interval: 12– 21 days

Table 2.6. Summary of *in vivo* studies that evaluate intra-articular use of ACS in horses.

Authors/ year	Horses	Lesion	Follow up	Outcome measures	Protocol	ACS /cytokine analysis	Results	Study limitations	Effect
Marques-Smith et al. 2020[400]	Cases series N= 20 horses	Mild to moderate OA	48 days	Gait and SF analysis	3 IA injection 2-weeks interval	Arthrex/ IL-1rap IGF-1 TGF-β	58% responded to ACS treatment with a higher concentration of IL-1rap and IGF-1	Small number No control	(±)
Lasarzik et al. 2018[408]	Randomized study N = 12 horses	Advance OA	42 days	SF analysis	(1) 3 IA injection at weekly intervals (2) 3 IA injection at 2 days intervals	Arthrex/ Not measured	ACS increased IL-1rap in SF, decreased of cartilage biomarkers	Small number No controls Few outcomes measured	(+)
Schneider and Veith 2013[428]	Case series N = 36 horses	OA in 19 cases and 18 soft tissue	3-6 months	Gait analysis and clinical signs	4 ml IA injection 4 times weekly	Goldic / Not measured	Lameness and joint effusion improvement	No controls No blinding Few outcomes measured	(+)
Jöstingmeier et al. 2010[421]	Randomized study N = 54 (27 treated with ACS and 27 with HA and corticosteroids	OA coffin joint	6 months	Gait analysis	2-5 injections	Orthogen/ Not measured	ACS produced a stronger reduction in lameness	Few outcomes measured No blinded Few outcomes measured	(+)

Authors/ year	Horses	Lesion	Follow up	Outcome measures	Protocol	ACS /cytokine analysis	Results	Study limitations	Effect
Weinberger 2008[423]	Case series N = 262	OA Lameness unresponsive to IA corticoid or HA	6-12 weeks	Gait analysis	2-3 injections 8-14 days intervals Joint dependent (table 2.6)	Orthokine/ Not measured	Lameness resolution in 221 at 6 weeks and 178 at 12 weeks	Heterogenous population and treatment No blinded No control Few outcomes measured	(+)
Osterdahl 2008[422]	Case series N= 20 horses	Refractory chronic OA unresponsive to IA PSGAG or HA	3 months	Gait analysis	2-3 injections 8-14 days	Orthokine/ Not measured	Lameness resolution in all PSGAG failures and 7/10 HA failures	Small number No control No blinding Heterogenous population	(+)
Frisbie et al. 2007[237]	Blinded randomized experimental study N = 16 horses	Surgically induced osteochondral fragment model	70 days	Gait, radiography, SF, histological and GAG analysis	6 ml 4 IA injections weekly	Orthokine/ IL-1rap	Lameness and histology scores improvement	Small number	(+)

Autologous protein solution (APS)

APS is a newer biologic that combines the beneficial effects of ACS (anti-inflammatory cytokines) and PRP (growth factors). APS preparation is relatively simple and requires 52 ml of peripheral blood added to 8 ml of anticoagulant (ACD-A) followed by a double centrifugation process. The first centrifugation produces platelet-rich plasma using the APS separator device. The product is then transferred to the APS Concentrator device containing polyacrylamide beads that stimulate leukocyte production of anti-inflammatory cytokines (Figure 2.7). APS has an elevated (concentrated) leukocyte count 12.1x and platelet count 1.6x higher than whole blood in horses.[429] In contrast to ACS, this product does not require incubation, making this intra-articular biologic more convenient for equine practitioners.

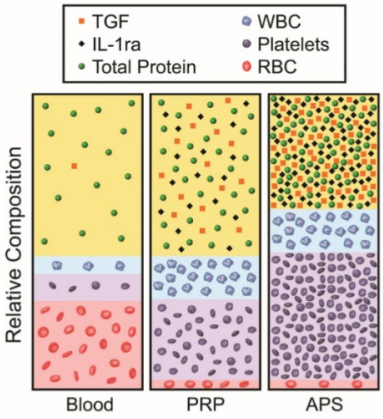

Figure 2.7. Illustration demonstrating the relative composition changes in blood components before and after processing whole blood using the separator and concentrator APS devices. © Muir, S. M., Reisbig, N., Baria, M., Kaeding, C., & Bertone, A. L. (2019). The Concentration of Plasma Provides Additional Bioactive Proteins in Platelet and Autologous Protein Solutions. *The American Journal of Sports Medicine*, *47*(8), 1955–1963. https://doi.org/10.1177/0363546519849671. Reprinted with permission of the American Journal of Sports Medicine.

The cytokine profile of APS has been studied in humans more extensively. An increased concentration of anti-inflammatory cytokines (IL-1rap, sIL-1RII, sTNF-RI, and sTNF-RII), pro-inflammatory cytokines (IL-1β, IL-6, and IL-8), and growth factors (PDGF-AB, PDGF-BB, IGF-1, EGF, TGF-β1) have been measured compared to whole blood in humans. APS obtained from patients with OA produced an increased concentration of anti-inflammatory cytokines compared to pro-inflammatory cytokines.[430] In horses, an increased concentration compared to whole blood of IL-1rap, IL-10, sTNF-RI, TGF-β, TNF-α, and IL-1β has been measured.[196,429] One study compared the cytokine profiles of APS to ACS, finding similar profiles despite a significant increase in the concentration of TGF-β in APS.[196] Another study found that there was a positive correlation between the WBC concentration and the ratio of IL-1rap: IL-1β in APS. This ratio was positively correlated with the improvement of pain scores in patients with OA.[431] A recent study where APS and PRP were compared in healthy humans and horses found that the concentrator device containing polyacrylamide beads was more efficient at increasing IGF-1 concentration, resulting in a higher concentration in APS than PRP. Although APS from humans and horses had an increased concentration of IGF-1, TGF-β, and IL-1rap; interestingly, the concentrations were always higher in humans than horses.[432]

APS has been shown to have certain disease-modifying effects *in vitro*. In bovine articular cartilage explants, APS was more effective than recombinant antagonists (IL-1rap and soluble TNF receptor I) in preventing cartilage matrix degradation. APS reduced the SOFG staining and enhanced chondrocyte cellularity and cell division.[433] In humans chondrocytes, APS also was able to downregulate the MMP-13 concentration in media after stimulation with IL-1β and TNF-α. In unstimulated chondrocytes, APS stimulated chondrocytes to produce MMP-13.[88] In a co-culture model using human articular cartilage and synovial membrane obtained from

patients undergoing knee replacement, APS showed chondroprotective effects against apoptosis and destructive chondrocyte clustering, a reduction in cartilage matrix degradation, and produces a temporary anti-inflammatory effect, that could help to mitigate OA disease progression. In the same study, the authors also reported that a double dose of APS six days apart seemed to produce a more significant chondroprotective effect than a single dose.[434] APS has not only shown cellular modification in joint tissues but also in the macrophages. Culture macrophages with IL-1β resulted in an increased production of IL-8, and APS treatment downregulated the production of IL-8 and TNF-α and increased the concentration of IL-1rap and sTNF-RI in media.[435] In horses, only one *in vitro* study has been conducted until the present work, where APS-treated chondrocytes had increased concentrations of chondroprotective (IL-1rap and IL-10) and modulatory (IL-6) cytokines. In this study, ACS and APS profile was also compared. Although TGF-β concentration was significantly higher, ACS and APS presented a very similar cytokine profile.[196]

Looking at clinical data, in humans, APS has been shown to be a safe therapy that could improve the pain scores after intra-articular treatment.[431,436,437] However, these studies lack controls and are not blinded or randomized. More recently, a pilot multicenter, double-blind, randomized, saline-controlled trial was conducted to assess clinical outcomes of APS at a 1-year follow-up. This study reported significant improvement in pain scores and MRI evaluation, between 6 to 12 months post-treatment.[438] Another prospective human study found that patients with moderate synovitis on baseline MRI appeared to have better response to the APS treatment showing increased functional improvement and better pain relief. In addition, they reported that moderate temporary flares can be expected without affecting clinical outcomes.[439] In horses, improved functional outcomes have been demonstrated following patient assessed long-term

follow-up (12 months) in naturally occurring OA.[429] This study did not report any complications after treatment to APS, but to the authors' knowledge and experience, APS can produce a self-limiting flare that most commonly resolves with NSAID administration in horses as it does in humans.

Cellular therapies: Mesenchymal stem cells (MSCs) and cellular concentrates

Currently, there are different cellular therapeutic options in the market to treat OA in horses. Cellular therapeutics can be differentiated into the following categories: 1) cells (stem/stromal and/or progenitor) contained within tissue particles, which are typically shipped directly from the company, 2) progenitor and stem/stromal cell concentrates, which are obtained after harvesting tissue (adipose or bone marrow) and concentration of the cells from the tissue via centrifugation with or without prior tissue digestion (i.e., adipose-derived stromal vascular fraction or bone marrow aspirate concentrate), and 3) cultured cells, which are obtained after harvesting tissue (adipose, bone marrow, blood, etc.) and sending the tissues to a commercial laboratory for culture. The cultured cells would then be shipped back to the practitioner for injection at least two weeks or more after the tissue harvest.

Bone marrow aspirate concentrates (BMAC) and an adipose-derived stromal vascular fraction (ADSVF) are a slightly more straightforward technique to obtain a high concentration of MSCs without the requirement of cell culture techniques. BMAC contains a concentrated mononuclear cell population, in which MSCs are present in a very small proportion (0.001-0.01%). Human BMAC has been shown to be a source of high concentration of growth factors, PDGF, TGF-β, and bone morphogenetic proteins 2 and 7.[440] PRP has been shown to have a higher TGF-β1 and PDGF-BB, but similar IGF-1 concentration compared to BMAC.[441]

However, this same laboratory showed similar concentrations of TGF-β1 and PDGF-BB between PRP and BMAC using the same processing and assay methodology, but different horses, highlighting the role of patient variability in comparing these products between studies.[442] In a model where 6 microfractures were surgically created with an awl in the lateral trochlear ridge of horses, treatment of the defect with direct implantation of BMAC increased the type II collagen content of the repair tissue (making the repair tissue more like hyaline cartilage) and improved collagen fiber orientation of the repair tissue compared to the sham-operated contralateral limb.[443] This same group of investigators has reported similar positive results after a year follow-up where larger osteochondral defects were created.[444] ADSVF contains heterogeneous cell populations such as mesenchymal progenitor/stem cells, preadipocytes, endothelial cells, pericytes, T cells, and M2 macrophages.[445,446] A study comparing the use of ADSVF to MSCs in surgically created osteochondral defects reported the MSCs reduced lameness, PGE_2 concentration and synovial effusion more efficiently than ADSVF.[447]

Mesenchymal stem cells are multipotent adult stem cells that are present in multiple tissues, including umbilical cord, bone marrow and fat tissue. Mesenchymal stem cells can self-renew by dividing and can differentiate into multiple tissues including bone, cartilage, muscle and fat cells, and connective tissue.[448] Cultured mesenchymal stem cells (MSCs) have been applied in the attempted management of several joint conditions and the initial goal of restoring damaged/lost tissues due to their unique tri-lineage differentiation properties (osteogenesis, chondrogenesis, and adipogenesis).[449] In an *in vivo* study with mice, MSCs have shown an affinity for damaged joint tissue homing and participating in the repair of cruciate ligaments, menisci, and cartilage lesions.[450] Considering that MSCs can differentiate to chondrocytes, the initial hope for research in this area was that MSCs could be injected into the damaged tissue and

then engraft, differentiate to chondrocytes, and restore the cartilage lesion and joint surface. This outcome has not come to pass despite years of research on MSCs therapy for various orthopedic conditions. One reason may be differences in the capacity to chondrogenically differentiate among MSCs derived from different patients and tissue sources, cells can tend to hypertrophy during differentiation, and the phenotypic stability of mature chondrocytes remains challenging to ensure.[451] Lately, several equine studies have focused on understanding which source of MSCs could provide better chondrogenic differentiation and whether it is better to let these cells differentiate within the injured microenvironment or be stimulated to differentiate prior to tissue implantation to enhance tissue repair.[452-454]

Equine MSCs have been established from tissues including bone marrow, adipose tissue, peripheral blood, umbilical cord, amniotic membrane, tendon, synovial fluid and membrane, and the mammary gland.[446,455-458] Bone marrow and adipose tissue are easily collected, and MSCs from these tissues can be used as nonexpanded cells or can be cultured and expanded.[367] When MSCs are cultured and expanded, the cultured product contains less heterogenic populations than uncultured cells obtained from the same tissue with higher therapeutic cell dosages (10 to 50 million in a 10 to 50 mL joint),[459] requiring a minimum of 2 to 3 weeks for expansion.

Though MSCs maintain the ability to differentiate into various mesenchymal phenotypes, MSCs also exert a paracrine effect on cells within the joint, especially macrophages.[460,461] MSCs express a variety of chemokines and cytokines that aid in repairing degraded tissue, restoration of normal tissue metabolism, and, most importantly, counteract inflammation.[462] Still, it is not completely clear when MSCs should be injected. In an inflammatory environment, MSCs upregulate their production of anti-inflammatory and immunomodulatory mediators, but during acute and severe inflammation, MSCs can be overwhelmed, reducing their chondrogenic

capacity. According to some experimental and clinical animal models, MSCs injected 1-week post-injury, following resolution of the acute inflammatory response is recommended.[460]

In the last two decades, extensive research has been performed to evaluate MSC treatment of OA. *In vitro*, experimental *in vivo*, studies and clinical trials have been evaluated their use. In horses, MSCs research has also represented a point of interest in the scientific community and many studies have been produced to understand their effect on treating OA.

Equine MSCs have been chondrogenically differentiated *in vitro,* finding differences in chondrogenesis between the two main tissue sources of horse MSCs. Studies have observed that bone marrow-derived MSCs (BM-MSCs) may have higher chondrogenic capacity (greater matrix protein production) compared to adipose-derived MSCs (AD-MSCs).[463,464] These observed differences may be due to different methods of cellular expansion. TGF-β induces chondrogenesis in BM-MSCs, while to induce chondrogenic activity in AD-MSCs, it is necessary to be cultured with both TGF-β and BMP-6.[463] Another *in vitro* study found that AD-MSCs primed with interferon-gamma (IFNγ) were able to suppress T-cell proliferation showing enhanced immunomodulatory capacity, and this priming enhanced the chondroprotective effect of these MSCs on stimulated cartilage explants.[465]

Multiple studies have been carried out to evaluate the effect of MSCs used intra-articularly to treat OA. Multiple factors can affect the isolation and proliferation of MSCs *in vitro,* such as individual variability, age, or type of culture. Studies have shown that some horses could not be suitable donors since MSCs isolated from these patients did not proliferate or presented little to no osteogenic and chondrogenic potential.[466] Therefore, the possibility of creating an allogeneic MSC (different donor than the recipient) bank has powered some research in the last years. In an *in vitro* study, autologous and allogenic equine studies demonstrate that

autologous and allogeneic MSCs injected into healthy joints can induce mild, transient inflammation consistent with a joint flare.[467-469] Two *in vivo* studies found no difference in this post-injection inflammatory reaction when using autologous or allogeneic MSCs,[470,471] but other studies have observed more significant post-injection inflammatory reaction when using allogeneic MSCs.[469,472] Schnabel et al. reported that allogenic MSCs expressing major histocompatibility complex type II (MHC-II) produced an increased inflammatory response in the host post-injection recommending that MHC II negative MSCs should be used for allogeneic applications.[473] The results of this study could potentially explain the diversity in the inflammatory response between autologous and allogenic in horses, but unfortunately, MHC-II expression in MSCs was not measured in many of these studies. A recent publication corroborates the importance of MHC expression, reporting that re-exposure to allogenic MSCs produces antibodies that target MSCs expressing MHC-II.[474]

Intra-articular injection of scaffold-free and MSCs within scaffolds have been investigated for treatment of both experimental and naturally occurring OA, paying particular attention to the type of tissue created to repair cartilage defects.[447,475-481] Most of these studies reported beneficial effects attributed to MSC therapy on OA of patients, primarily within the acute treatment period, improving clinical signs and showing limited, but improved cartilage healing. A recent study evaluated the effect of the combination of BM-MSCs with PRP. PRP alone produced a thicker repair tissue compared to the combination with BM-MSCs, and BM-MSCs stimulated bone formation in some cartilage defects.[481] A summary of the studies performed in horses using intra-articular MSCs or BMAC is in table 2.7.

In general, both in horses and humans, despite the beneficial results shown, many authors agree that studies evaluating use of MSCs for treatment of OA are not well-designed to obtain

objective conclusions. This problem is also observed in the study of many other orthobiologic therapies. Many of the studies we have available present a high risk of bias and a lack of consistency regarding MSCs preparations, and thus a lack of reproducibility of the reported outcomes.[482] This should move the scientific community to aim for control, blinded, standardized prospective studies that will provide more objective and reliable results.

V. Synthetic scaffolding joint therapies

Numerous synthetic scaffolding agents have been used to augment cellular implantation or improve frictional or mechanical properties of the cartilage surface, such as polymers or collagen-based scaffolds. In particular, polyacrylamide hydrogel (PAHG) is a synthetic scaffold that is currently often used by equine practitioners to treat OA. PAHG is a synthetic material consisting of 97.5% sterile water and 2.5% cross-linked polyacrylamide, which has been used for years in human medicine for soft-tissue augmentation.[483-485] PAHG is a biocompatible, nonimmunogenic, non-toxic polymer gel with a permanent and stable augmentative effect due to constant molecular water exchange with its host tissue.[486] Previous studies have shown that PAHG allows cellular ingrowth and integrates into the soft-tissues, producing minimal foreign body reaction.[484,485,487]

Viscosupplementation with hyaluronic acid therapy is a widespread practice to treat osteoarthritis (OA) in humans and horses.[311] Results of the viscosupplementation therapy are expected to depend upon the rheological properties and molecular weight of the hyaluronan preparation.[488] Initially, PAHG was created as a prosthetic device for supplementing, augmenting, or replacing cartilage within the joint.[489] PAHG presents similar viscoelastic

Table 2.7. Summary of *in vivo* studies that evaluate intra-articular use of MSCs or BMAC in horses.

Authors/ year	Horses	Lesion	Follow up	Outcome measures	Product	Results	Study limitations	Effect
Colbath et al. 2020[471]	N=8 horses	Experimental synovitis IL-1β	7 weeks	Clinical assessment, gait and SF analysis	Allogeneic and autologous BM-MSCs	Ineffective in reducing the inflammatory response	Small number Severe synovitis produced by IL-1β	(-)
Barranchina et al. 2018[492]	N=18 horses	Experimental synovitis amphotericin B	6 months	Clinical assessment, radiographic, US and MRI, SF, histological analysis and gene expression	Allogeneic BM-MSCs single and repeated IA injection	Improvement of clinical signs, upregulation type II collagen downregulation inflammatory cytokines	MHC-matching was not assessed between donors and receptors	(+)
Chu et al. 2018[444]	N=8 horses	Surgically induced osteochondral fragment model	12 months	Arthroscopy, MRI, histological analysis	SmartPrep 2 BMAC	Improvement of cartilage healing and subchondral changes	Small number	(+)
Goodrich et al. 2016[481]	N= 12 horses	Surgically induced osteochondral fragment model	12 months	Arthroscopy, MRI, histological analysis	PRP ± autologous BM-MSCs	PRP produced thicker repair tissue and BM-MSC stimulated bone formation	No control	(±)

Authors/ year	Horses	Lesion	Follow up	Outcome measures	Product	Results	Study limitations	Effect
Williams et al. 2016[469]	N=6 horses	Experimental synovitis LPS	72 hours	Clinical assessment, gait and SF analysis	Allogeneic umbilical cord MSCs	Decreased inflammation	Small number Short term synovitis	(+)
Broeckx et al. 2014[477]	N =165 horses	Chronic OA	18 weeks	Return to work	Allogenic PRP + peripheral blood MSCs With or without chondrogenic induction	1.8% synovitis in the first week, improved return to work at 18 weeks chondrogenic MSCs resulted in a higher return to work in distal limb	No control Heterogenous population Few outcomes measured	(±)
Ferris et al. 2014[480]	N= 33 horses	Stifle lameness OA or meniscal injuries	6-63 months	Return to work	Autologous BM-MSCs	Increased return to work than horses with only arthroscopic debridement	No control Heterogenous population Few outcomes measured	(+)
Yamada et al. 2013[475]	N=8 horses	Surgically induced osteochondral fragment model	150 days	Gait, SF, histological analysis	Autologous AD-MSCs	No clinical improvement but MSCs decreased PGE_2 and improved repair tissue	Small number	(+)
Nicpon et al. 2013[493]	N= 16 horses	Tarsometatarsal and distal intertarsal OA	180 days	Gait and SF analysis	Autologous AD-MSCs	No change in lameness at 30 days but reduced at 60 and 180 days	Short term evaluation Few outcomes measured	(±)

Authors/ year	Horses	Lesion	Follow up	Outcome measures	Product	Results	Study limitations	Effect
McIlwraith et al. 2011[478]	N= 10 horses	Surgically induced osteochondral fragment model	12 months	Gait, MRI, SF, histological analysis	Autologous BM-MSCs	No clinical or histological improvement signs but increased aggrecan content and tissue firmness	No control Small number	(±)
Fortier et al. 2010[443]	N= 12 horses	Surgically induced microfractures	8 months	Arthroscopy, histological Analysis, standard MRI and MRI analysis of GAG and Collagen	SmartPrep 2 BMAC	Improvement of cartilage healing increased type II collagen and improved collagen architecture	Small number	(+)
Frisbie et al. 2009[447]	N= 24 horses	Surgically induced osteochondral fragment model	70 days	Gait, SF and histological analysis	AD-MSCs or ADSVF	No significant improvements, MSCs decreased PGE_2 more than PGE_2	No blinding	(-)
Wilke et al. 2007[494]	N= 6 horse	Surgically induced osteochondral fragment model	8 months	Arthroscopy, SF and histological analysis	Autologous BM-MSCs	Improved early chondrogenesis, arthroscopic scores, type II collagen	Small number	(+)

properties to the normal synovial fluid and hyaluronic acid,[490] has longer-lasting viscous effects, and is a non-degradable material.[491]

Recently, two experimental studies in horses have investigated tissue distribution and intra-articular effects of PAHG in healthy and OA joints of horses, observing that PAHG initially incorporated within the interstitial space of the synovium and is then incorporated within synoviocytes by 14 to 30 days post-injection, forming a sub-synovial layer without deleterious effects.[495,496] Following injection, these studies report an increased viscosity of synovial fluid after injection with PAHG. However, this was macroscopically assessed by subjective assessment and no objective measurements were done. Currently, there are no *in vitro* or *in vivo* studies that evaluate alterations in synovial fluid composition and mechanical properties following incorporation of PAHG into the synovial membrane. It is speculated that incorporation of PAHG into the synovial membrane creates an added synovial layer that acts as an immune barrier. Potentially, this could decrease cross-talk between the synovium and the cartilage during OA progression, slowing down the propagation of inflammation. However, no studies have identified which cellular mechanism are modified by the treatment with PAHG to reduce the cellular inflammatory response.

Certain disease-modifying effects have been attributed to PAHG treatment after evaluating results of a caprine OA model.[497] In this study, transection of the medial collateral ligament, bisection of the medial meniscus, and partial-thickness cartilage defects of the medial tibial plateau were performed on 6 goats bilaterally. One of the joints was injected with PAHG and the contralateral with saline working as a control. Histological analysis seven months after treatment revealed that the synovial membrane from joints treated with PAHG was thickened due to increased angiogenesis, collagen and synovial cells. Harvested synovial membrane from

joints with PAHG was more elastic, and the MRI results showed progression of OA compared to the saline group.[497]

Despite the lack of scientific evidence regarding the exact mechanism of PAHG,[498] intra-articular use of PAHG has been reported in humans and horses.[499-504] In horses with naturally-occurring OA, PAHG treatment produced an improvement of lameness and decreased joint effusion in some cases up to 24 months with a single injection.[500] However, it is essential to note that no controlled clinical trials have been performed. Recommended by the manufacturer doses depending on the joint injected are presented in table 2.8 and a summary of the studies evaluating PAGH in horses intra-articularly is reflected in table 2.9.[505] Although no adverse reactions have been reported in horses; in humans, there is a case report where PAHG produced an acute, severe inflammation consistent with a foreign body immune response that required arthroscopic debridement, resulting in significant patient morbidity.[506]

Table 2.8. PAHG treatment protocol that is recommended in the literature.[505]

Joint	Treatment protocol
Coffin/pastern joint	Dose: 1– 2 ml
Fetlock joint	Dose: 2 ml
Radiocarpal/ Intercarpal joint	Dose: 2 ml
Tarsometatarsal and distal intertarsal joint	Dose: 1 ml
Tarsocrural joint	Dose: 2 ml
Stifle per compartment	Dose: 3-4 ml
Hip joint	Dose: 3-4 ml
Vertebral joints	Dose: 1 ml cervical/ 0.5 ml thoracic and lumbar
Temporomandibular joint	Dose: 1 ml

Table 2.9. Summary of *in vivo* studies that evaluate intra-articular use of PAHG in horses.

Authors/ year	Horses/ PAHG	Lesion	Follow up	Outcome measures	Results	Study limitation	Effect
de Clifford et al. 2019[503]	N= 49 horses Arthramid	Mild to severe OA	24 weeks	Gait and radiographic analysis	Significant lameness improvement at 1, 12 and 24 weeks	No control No blinded Heterogenous population Few outcomes measured	(+)
McClure and Wang 2017[504]	N= 28 horses/ Noltrex	Chronic OA	90 days	Gait analysis	Significant lameness improvement	No control No blinded Short-term Heterogenous population Few outcomes measured	(+)
Tnibar et al. 2015[500]	N= 43 horse Arthramid	Chronic OA	24 months	Gait and clinical evaluation	Significant lameness improvement	No control Heterogenous population Few outcomes measured	(+)
Tnibar et al. 2014[507]	N= 40 horses Arthramid	OA fetlock	6 months	Gait analysis	Significant lameness improvement compared to HA and TA group	No control groups Few outcomes measured	(+)
Janssen et al. 2012[501]	N=12 horse Arthramid	Refractory OA	3 months	Gait analysis	Significant lameness improvement	No control No blinded Heterogenous population Few outcomes measured	(+)

VI. Gene therapy

Gene therapy offers novel approaches to medical management of OA. Researchers are testing several approaches to gene therapy, including replacing a mutated gene that causes disease with a healthy copy of the gene, inactivating, or "knocking out" a mutated gene that is malfunctioning or introducing a new gene into the body to help fight a disease. This last approach is the most commonly investigated to treat OA. Gene transfer can be used to modify the intra-articular environment by transferring genes encoding therapeutic molecules to intra-articular tissues to become endogenous sites of therapeutic protein synthesis.

Briefly, the sequences of cDNA in which genes encoding potential therapeutic products are included within a viral plasmid.[508] This viral plasmid can be used to transduce chondrocytes or synoviocytes *in vitro* and inject the cells intra-articularly, or the viral plasmid can be injected directly into the joint and modify the cells *in vivo*. Regardless of the technique used, the final goal would be to obtain genetically modified cells that are directed to overproduce and continuously secrete the transgenic protein into the synovial fluid and surrounding tissue. In this manner, the diseased joint becomes an endogenous site of sustained, elevated drug production, eliminating the need for a repeated application while providing the greatest concentration of the protein specifically at the site of disease. Some *in vitro* studies have shown that chondrocytes are efficiently transduced with growth factor genes, but their protected position in the extracellular matrix makes a lot more challenging gene transduction *in vivo*.[509] Therefore, synoviocytes have become an easier and more effective cellular targets of gene therapy *in vivo*, which seems a wise direction to move forward considering the crucial role of the synovium in the OA pathogenesis.

Many genes have a therapeutic potential in OA, including IL-1Ra, TNF soluble receptors, IL-4, IL-10, IGF-1, TGF-β, or type II collagen or COMP.[510] Basically, two strategies can be used in gene therapy: 1) chondroprotection by blocking inflammatory cytokines, and 2) stimulate cartilage repair and regeneration. Genes can be readily transferred to the synoviocytes or chondrocytes *in vivo* or *ex vivo* using a variety of different viral and non-viral vectors. This technique is what is called genetic transduction. Various viral vectors have been utilized for intra-articular gene therapy, including adenovirus, retrovirus, lentivirus and adeno-associated virus (AAV).[511] The adenoviral vector does not require mitosis to transduce cells *in situ* successfully, and it has resulted in significant elevations of protein IL-1rap or IGF-1 for 14-21 days in horses.[512-514] However, adenoviral vectors can cause significant inflammation due to immunogenic stimulation. Ideally, the vector and genetically modified cells must avoid recognition and elimination by the receptor immune system to produce a long-term effect.[515] AAV is a nonpathogenic, non-enveloped, single-stranded DNA parvovirus that has become increasingly popular in horses due to its small size, small immunogenic presentation, and lack of initiating viral-based diseases.[511]

The clinical benefits of IL-1rap have been previously demonstrated by administering recombinant IL-1ra protein in multiple species,[211,516,517] and gene therapy in horses targeting IL-1rap has shown promising results (Table 2.10). Administration of adenovirus as a vector with equine IL-1rap gene produced dose-dependent increases in IL-1rap levels in synovial fluid. However, the highest viral dose tested, 5×10^{11} viral particles, induced a more marked acute synovitis.[512] Most of the studies produced in horses using gene therapy have investigated the effects of IL-1rap and IGF-1 genes either by direct intra-articular injection of the vector or by injecting transduced chondrocytes. One of these studies has shown that most of the transgene

expression is originated from the fibroblast in the synovial lining by using fluorescent tagged genes.[518] A recent publication achieved increased concentrations of IL-10 maintained for up to 84 days. IL-10 has the potential to modulate the articular inflammatory response, thereby protecting cartilage from degradation and osteoarthritis.[519]

Gene therapy could potentially play an essential role in the treatment of OA in the future. However, the large number of interacting loci participating during OA and patient variability observed make immensely challenging the clinical application of gene therapy.

Table 2.10. Summary of *in vivo* studies that evaluate intra-articular use of gene therapy in horses.

Authors/ year	Horses/ vector and gene	Lesion	Follow up	Outcome measures	Results	Study limitation	Effect
Moss et al. 2020[519]	Controlled experimental study N= 12 horses AAV5-IL-10	Healthy	84 days	Clinical, lameness, SF and histological analysis	Significantly increased in IL-10 concentration in SF	Use in healthy joints Small number	(+)
Nixon et al. 2018[520]	Controlled experimental study N= 4 HdAd-IL-1rap	Surgically induced osteochondral fragment model	72 days	Clinical, lameness, SF and histological analysis	Improved lameness and histological scores but not significantly increased in IL-1rap concentration	Small number	(±)
Watson et al. 2018[518]	Controlled experimental study N= 20 horses AAV2.5- EqIL-1rap	Surgically induced osteochondral fragment model	12 weeks	Clinical, lameness, SF, arthroscopy, MRI and histological analysis	Increased IL-1rap concentration in SF and improvement lameness histologic scores	Small number	(+)
Goodrich et al. 2015[521]	Controlled experimental study N=6 horses scAAIL-Eq1ra	Surgically induced osteochondral fragment model	276 days	Clinical, lameness, SF, arthroscopy, and histological analysis	Increased IL-1rap concentration in SF	Small number	(+)
Ortved et al. 2014[522]	Controlled experimental study N=8 horses rAAV5-IGF-1 modified chondrocytes	Surgically induced osteochondral fragment model	8 months	SF, arthroscopy, and histological analysis	Better histological scores more chondrocytes and type II collagen	Small number	(+)

Authors/ year	Horses/ vector and gene	Lesion	Follow up	Outcome measures	Results	Study limitation	Effect
Menendez et al. 2011[523]	Controlled experimental study N=5 horses Ad-BMP-2 Ad-BMP-6	Surgically induced osteochondral fragment model	54 weeks	CT, quantitative MRI, PCR and histological analysis	BMP-6 produced a higher concentration of GAG in the repair at 52 weeks but not clear long-term effects evidence	Small number	(±)
Goodrich et al. 2007[514]	Controlled experimental study N=6 horses adIGF-1 modified chondrocytes	Surgically induced osteochondral fragment model	8 months	Clinical, lameness, SF, arthroscopy, and histological analysis	Improved early cartilage healing (four to nine weeks) and to a lesser degree in a long-term	No blinding Small number	(+)
Goodrich et al. 2006[513]	Controlled experimental study N=14 horses adIGF-1 different concentrations	Healthy joints	28 days	Clinical, lameness, SF, arthroscopy, and histological analysis	Direct injection of 20 and 50 × 1010 AdIGF-I resulted in significant elevations of IGF-I in synovial fluid for approximately 21 days	No blinding Small number Use in healthy joints	(+)
Morisset et al. 2007[168]	Controlled experimental study N=8 horses adEq-IGF-1 adEq-IL-1rap	Surgically induced osteochondral fragment model	16 weeks	SF and histological analysis	increased proteoglycan and type II collagen and increased concentration of IL-1rap in SF during the first 3 weeks	Small number No blinding Few outcomes	(+)
Frisbie et al. 2002[512]	Control experimental study N= 16 horses Ad-EqIL-1rap	Surgically induced osteochondral fragment model	70 days	Clinical, lameness, SF, and histological analysis	Increased IL-1rap for 28 days, improved lameness and histologic scores	Small number	(+)

Chapter 3

STATEMENT OF OBJECTIVES

The general purpose of this body of work was to further investigate if equine veterinarians use orthobiologics therapies for the treatment of intra-articular joint disease, report their clinical experience with orthobiologic therapy and gain a better understanding of their cellular mechanism compared to more traditional treatments as corticosteroids. The use of orthobiologics in veterinary medicine has become more popular in the last decade. However, there is a lack of objective information regarding their *in vitro* and *in vivo* effects. Differences between products could help determine in which musculoskeletal injuries certain treatments may be more effective and, which treatment protocols are ideal for varying synovial pathology (acute vs. chronic, ideal dosage or treatment repetitions). Currently, there are no clear guidelines available for equine practitioners. The specific objectives were to understand use of orthobiologics by practitioners and objectively evaluate treatment effects of commonly used orthobiologics compared to standard treatments such as corticosteroids.

❖ Specific objective 1: To investigate how equine practitioners use nonsteroidal intra-articular therapies (NSIATs), specifically PRP, ACS, APS, cellular products, and PAHG, through a survey sent to national and international equine practitioners. The survey provides information about the use of NSIATs, subjective clinical efficacy of these products, treatment protocols commonly employed, and complications associated with product use. The hypothesis was that NSIATs are more frequently used by equine practitioners than previously reported in the literature.

❖ Specific objective 2: To develop an *in vitro* synovial membrane and articular cartilage co-culture system to be used for long-term culture (9 days). This model would mimic OA through stimulation of the tissues with equine recombinant IL-1β stimulation. Evaluation of the maintenance of tissues in culture with two different media, serum-free media and media with 10% equine serum was performed. Tissue viability and histologic (structural changes) were analyzed and compared. The hypotheses were that tissues would maintain their viability in long-term co-culture and that serum would have a protective effect on stimulated inflammatory and catabolic gene expression. If equine serum offers a chondroprotective effect, but tissue viability is maintained, eliminating serum from OA co-culture would be recommended to investigate treatment effects.

❖ Specific objective 3: Evaluate horse differences in cellular composition and concentration of important cytokines and growth factors of ACS and APS. Currently, only one study has directly compared the compositional profile of ACS and APS. Identified differences in composition could help understand differences in identified treatment effects. The hypothesis was that ACS and APS obtained from the same horse would have different cellular and cytokine profiles.

❖ Specific objective 4: To investigate the effects of ACS, APS, and TA on inflammatory and catabolic gene expression in an IL-1β stimulated cartilage and synovial membrane co-culture model of OA and to investigate differences in the effect of different concentrations of ACS and APS (25% v/v vs. 50% v/v). Although some studies have evaluated the effect of different orthobiologics *in vitro*, none has compared their effects on traditional treatment of corticosteroids. Corticosteroids and in particular TA, are the most commonly used intra-articular treatment used by equine practitioners. Finding differences in their treatment effects may help to better understand their clinical use. The hypotheses were that IL-1β would produce an

inflammatory response in the co-cultured articular cartilage and synovial tissue and TA would reduce expression and production of inflammatory proteins more effectively than orthobiologics (ACS and APS). However, orthobiologics would protect matrix gene expression more effectively than TA. Another hypothesis was that 50% v/v concentration would produce a more marked chondroprotective effect compared to 25% v/v in the ACS and APS treatments.

❖ Specific objective 5: To investigate the use of equine recombinant IL-1β intra-articularly in the horses' fetlock to stimulate a mild, self-limiting synovitis. This protein has been used in other synovitis models such as the middle carpal and tarsocrural joints, but no reports using IL-1β in the fetlock are available. Clinical signs and cellular characteristics of synovial fluid were evaluated to assess the degree of inflammation produced. The hypothesis was that IL-1β will be able to induce synovitis in normal metacarpi/metatarsi phalangeal joints (MC/ MTPJ). This will be a moderate, self-limiting synovitis showing a maximum effect at 8 hours post-injection resolving within 24-36 hours post-injection.

❖ Specific objective 6: To compare the clinical and biochemical effects of TA and ACS in an *in vivo* synovitis model stimulated with equine recombinant IL-1β. It is clearly accepted that the synovial membrane plays a crucial role in the pathogenesis of OA. Finding a therapeutic option that helps to control early synovitis could offer advantages in the early stages of OA. The hypotheses were that ACS and TA would improve the clinical signs (decrease heat, joint circumference, pain upon passive flexion and lameness) of synovitis to the same degree. However, administration of ACS will cause a significant reduction of inflammatory mediators and catabolic enzymes in synovial fluid compared to administration of TA.

Chapter 4

Survey: Clinical usage of non-steroidal intra-articular therapeutics by equine practitioners

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Abstract

There are several non-steroidal intra-articular therapeutics (NSIATs) available for use by equine practitioners for the treatment of performance-limiting joint-related pathology. Information is limited on perceived clinical efficacy, recommended treatment protocols, and associated complications.

Our objective with this cross-sectional survey was to investigate the current clinical usage of NSIATs by equine practitioners. An electronic cross-sectional convenience survey inquiring about the use of steroidal and NSIATS (platelet-rich plasma, autologous conditioned serum, autologous protein solution, cellular therapies, and polyacrylamide hydrogel) was distributed internationally to equine practitioners. A total of 353 surveys were completed. NSIATs were used by 87.5% of the participants. Corticosteroids and hyaluronic acid remain the intra-articular therapeutic of choice among practitioners, followed by autologous conditioned serum, platelet-rich plasma and autologous conditioned protein. Polyacrylamide hydrogel was the least used. Practitioners were more likely to use NSIATs if their caseload was greater than 50% equine ($P < 0.001$), they treated more than 10 horses intra-articularly per month ($P < 0.001$), and horses treated were considered English sport horses ($P = 0.02$). Years in practice and practice location did not influence the use of NSIATs. One of the most common reasons why NSIATs were chosen was to treat acute articular pathologies. As survey limitations, answers to questions regarding clinical response and complication rates were based on subjective estimation and practitioners recall, not clinical records.

In conclusion, corticosteroids remain the most widely used intra-articular therapeutic. Among the NSIATs, blood-based products are more commonly used by practitioners, followed

by cellular and synthetic products. Equine practitioners frequently use NSIATs, choosing to treat acute joint pathology more than previously reported.

Introduction

Within the equine industry, lameness as a result of musculoskeletal pain, particularly osteoarthritis (OA), has a significant economic impact and is one of the top reasons for veterinary evaluation and treatment.[1,188] Several different intra-articular medications are available to equine practitioners to help alleviate musculoskeletal pain, especially when initial rest and systemic anti-inflammatory therapy is unsuccessful.[310]

Currently, the mainstay of intra-articular therapy is modification of disease symptoms through transient reduction of inflammation via administration of corticosteroids with or without hyaluronic acid.[311] Recently, non-steroidal intra-articular therapies (NSIATs), such as biological and synthetic products, have become more popular. NSIATs have been shown to possess limited disease-modifying properties, such as slowing down disease progression and enhancing the quality of repair tissue.[196,237,312,429,447,477,524]

Equine practitioners have used NSIATs for years, [525] and currently, there are many options available on the market. In the literature, there is limited information regarding the clinical experience of practitioners with these products, such as their product preference and treatment protocol. [311,525] This survey was not a hypothesis-driven study. The objectives of this study were: 1) to explore how equine practitioners use NSIATs, specifically autologous conditioned plasma (also known as platelet-rich plasma, PRP), autologous conditioned serum (ACS), autologous protein solution (APS), cellular products (i.e., stem/stromal/progenitor cell therapy), and polyacrylamide hydrogel, and 2) to observe if NSIATs are more frequently used by

equine practitioners than previously reported in the literature. The survey would provide information as to which NSIATs are more commonly used by equine practitioners as well as subjective clinical efficacy, treatment protocols commonly employed, and complications associated with product use.

Material and Methods

The project was reviewed and approved by the Institutional Review Board for the Protection of Human Subjects in Research (18-486 EX 1811). An electronic questionnaire (Qualtrics XM software, Provo, Utah, USA) inquiring about the use of 5 different NSIATs was distributed internationally to equine practitioners between January 2019 and October 2019. The survey was distributed to an estimated 10,000 equine practitioners. The questionnaire link was distributed to members of the American Association of Equine Practitioners through the Spur of the Moment Newsletter. The link was also distributed by the European College of Veterinary Surgeons to their Diplomats. Equine practitioners at 26 of 30 USA veterinary schools were contacted by email addresses obtained through university websites. Through a collaboration with Zoetis, the survey link was also distributed to equine customers in the USA who purchased pain and sedation products commonly used for lameness workup, diagnosis, and treatment. Lastly, the questionnaire was distributed through equine practitioner groups on social media (Facebook groups: Equine Vet-2-Vet and Equine lameness vets). Each participant was given a unique identifier based on email and IP address, to avoid duplication of answers. The survey additionally collected demographic information about each practitioner's practice and experience (geographic location, primary equine discipline treated, years in practice, frequency of intra-articular injection).

The survey contained a total 59 questions, with a combination of multiple-choice and rank questions, inquiring about PRP, ACS, APS, cellular therapies and polyacrylamide hydrogel. Within the survey, brief descriptions of each NSIAT were provided before specific questions (Table 1). If the practitioner did not use a product, product questions were eliminated from the survey. Questions regarding the use of NSIATs included: rank and justification of product preference, clinical usage, subjective assessment of clinical efficacy, treatment protocol used, and frequency of an observed inflammatory response (joint flare) after intra-articular administration. A copy of the survey has been provided, supplementary item 1. Before distributing the survey to the public, it was tested and evaluated by ten individuals that did not include the investigators. Answers from these individuals were not included in the results.

Statistical analysis

Survey data were summarized and reported using percentages and/or rankings. Chi-Square analyses (Qualtrix XM software)[a] were conducted to evaluate the influence of practitioner geographic location (USA vs. non-USA), years of experience, lameness caseload, number of horses injected intra-articularly on a monthly and yearly basis, and primary discipline treated. Effect of allogenic or autologous cell therapy on flare rate and the practitioner's geographic location (USA vs. non-USA) with use of polyacrylamide hydrogel was evaluated using a chi-square analysis as well. Significance was set at $P < 0.05$. For questions in which practitioners were asked to rank responses, the response with the lowest average number (ranked as 1) was reported as the preferred choice, followed in descending order to the least preferred response. Participants were required to rank at least 3 options, the median and the interquartile range were calculated for each NSIAT and reported.

Table 4.1. Brief description of each NSIATs provided to practitioners prior to questioning.

NSIAT	NSIAT Description
Autologous Conditioned Plasma (ACP)	PRP is a product obtained from the horse's blood. The blood is filtered or centrifuged to obtain plasma with an increased number of platelets rich in growth factors.
Autologous Conditioned Serum (ACS)	ACS, also known as IRAP (Orthokine® vet irap 10 or 60, IRAP II™ System), is obtained from the horses' blood following collection into specialized syringes and whole blood incubation. The serum is then collected and administered, or aliquots are frozen for subsequent injection.
Autologous Protein Solution (APS):	APS (i.e., Pro-Stride™ APS) is an autologous product obtained from the horse's blood. The blood is first processed using a kit and centrifugation to obtain plasma with concentrated platelets. This plasma is then harvested and processed in a kit that allows exposure of the cellular components of the plasma to polyacrylamide beads, enhancing their production of anti-inflammatory proteins during a second centrifugation cycle.
Cellular Therapeutic	Cellular therapeutics would include the following products: • Cells (stem/stromal and/or progenitor) contained within **tissue particles** (i.e., Pulpcyte® Vet Graft). These products are typically shipped directly from the company. • Progenitor and stem/stromal cell **concentrates**. These products are obtained after harvesting tissue (adipose or bone marrow) and concentration of the cells from the tissue via centrifugation with or without prior tissue digestion (i.e., Adipose-derived stromal vascular fraction or bone marrow aspirate concentrate). • **Cultured** cellular therapy. These products are obtained after harvesting tissue (adipose, bone marrow, blood, etc.) and sending the tissues to a commercial laboratory for culture. The cultured cells would then be shipped back to the practitioner for injection at least two weeks or more after the tissue harvest.
Polyacrylamide Hydrogel	Polyacrylamide Hydrogel (PAHG, Noltrex™ Vet, or Arthramid®Vet, Aquamid®) is a synthetic product injected intra-articularly. It is incorporated into the synovial lining and provides enhanced viscoelasticity to the synovial fluid.

Results

A total of 473 equine practitioners participated. Three hundred fifty-three surveys were completed, and 120 surveys were partially completed. Three hundred fifty-three completed surveys were included in the results (75%).

Demographics

The majority of participants indicated that their caseload was greater than 75% equine (315/353; [89.2%]), 27/353 [7.7%] reported having a caseload between 25-75% equine, and the remainder 11/353 [3.1%] reported having a caseload less than 25% equine. From these answers, 87/353 [24.6%] participant caseload consisted of 75-100% lameness, 113/353 [32%] 50-75% lameness; 103/353 [29.2%] 25-50% lameness, and 50/353 [14.2%] < 25% lameness. Participants whose caseload consisted of more than 50% lameness were more likely to use NSIATs compared to practitioners with a lameness caseload less than 50% ($P < 0.001$).

English sport horses were more commonly treated by participants, followed by recreational riding horses, western performance horses, racehorses, endurance horses, and other disciplines such as gaited, draft, retired, and geriatric horses. English sport horse practitioners were more likely to use NSIATs compared to other disciplines ($P = 0.02$). Participants treating pleasure horses were less likely to use NSIATs ($P = 0.04$) than participants that practiced on other disciplines.

The majority of survey participants practiced in the USA (293/353[83%]; 80/353 [22.6%] southeastern USA; 54/353 [15.3%] northeast USA.; 65/353 [18.4%] midwestern USA.; and 94/353 [26.6%] western USA). The remainder of participants (60/353 [17%] practiced internationally, including Europe, Canada, Australia, and the Middle East (Figure 4.1).

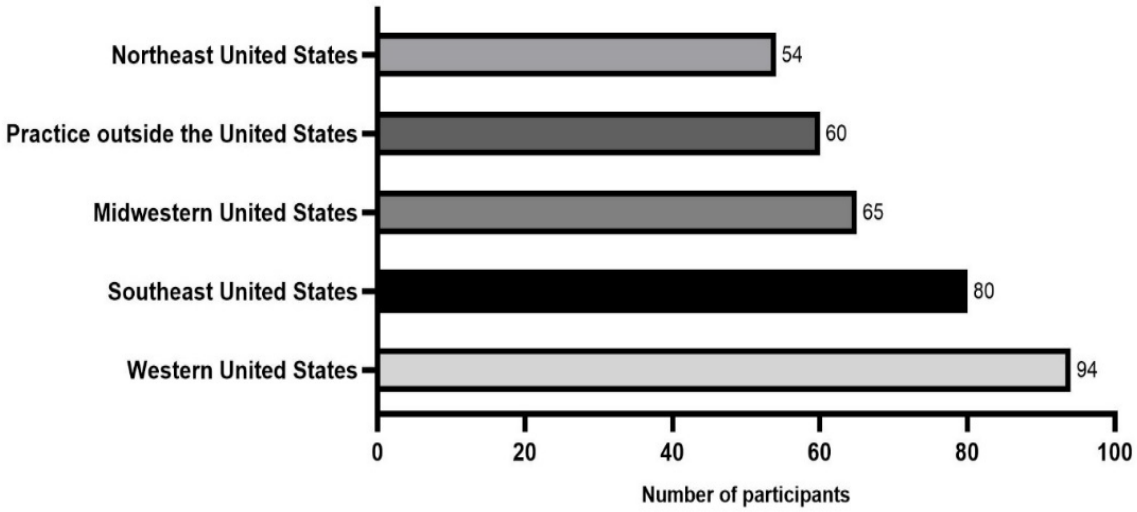

Figure 4.1. Histogram showing the geographic distribution from a total of 353 equine practitioners that answered the survey.

The majority of participants had been in practice for greater than 20 years (143/353 [40.5%]), with 96/353 [27.2%] between 10 to 20 years, 75/353 [21.3%] between 5 to 10 years, and 39/353[11.1%] practicing less than five years. The use of NSIATs was not affected by participants' geographic location (USA residents vs. non-USA residents) (P =0.6) or years of experience (P = 0.1).

Injection Frequency

Participants were asked to estimate the number of horses in which they perform joint injections (steroidal and non-steroidal products) per month (Figure 4.2). Seventeen/353 [4.8%] did not perform joint injections, 69/353 [19.5%] injected less than 5 horses/month, 80/353 [22.7%] injected between 5 to 10 horses/month, 85/353 [24.1%] injected between 10 to 20 horses/month, 65/353 [18.4%] injected 20 to 50 horses/month, and 37/353 [10.5%] injected more

than 50 horses in a month. Participants that treated more than 10 horses intra-articularly per month were more likely to use NSIATs (P = 0.001).

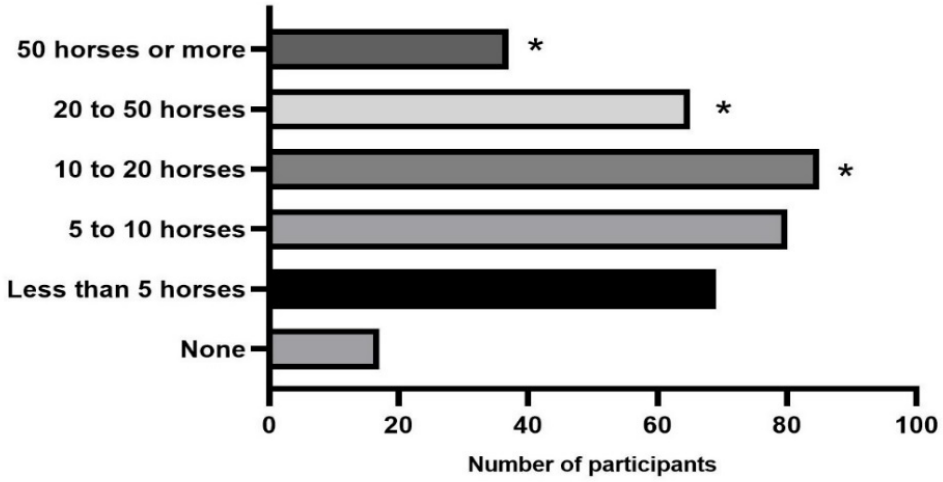

Figure 4.2. Response count of the number of horses injected per month by participants. * Denotes a significant difference between participants using NSAITs (P < 0.001).

Use of NSIATs

Of 336 participants who perform intra-articular injections, 291 used NSIATs in their practice (291/336 [87.5%]), while 42/336 participants [12.5%] did not use these products. Of the participants who did not use NSIATs, 22/42 [52.4%] did not have the product or equipment for processing available in their practice. Twenty/42 [47.6%] did not use NSIATs, no reason was given. Participants that injected 10 or more horses per month were more likely to use NSIATs compared to participants that performed intra-articular injections in less than 10 horses per month (P < 0.001).

Thirty-eight / 291 [12.9%] participants that used NSIATs estimated that they injected less than 5 horses per year with NSIATs, 39/291 [13.4%] used NSIATs in less than 10 horses per year, 83/291 [28.5%] used NSIATs in 10-20 patients per year, 66/291 [22.5%] used NSIATs to

inject between 20-50 horses per year, and 65/291 [22.3%] used NSIATs in more than 50 patients a year., and 66/291 [22.5%] used NSIATs to inject between 20-50 horses per year.

The participants were asked to rank at least three products in order of their preferences but were instructed no to rank products that they did not use. The three most popular therapies chosen by the participants were corticosteroids (210/291 [72.2%]), hyaluronic acid (195/291 [67%]), and ACS (90/291 [30.9%]). According to the median value of rank, intra-articular therapies were preferred by participants in the following order (reported as median rank ± interquartile range): Corticosteroids (1 ± 1), hyaluronic acid (2 ± 0), ACS (4 ± 2), APS (4 ± 2), PRP (4 ± 2), cellular therapies (5 ± 2), and polyacrylamide hydrogel (6 ± 3) (Figure 4.3 and table 4.2).

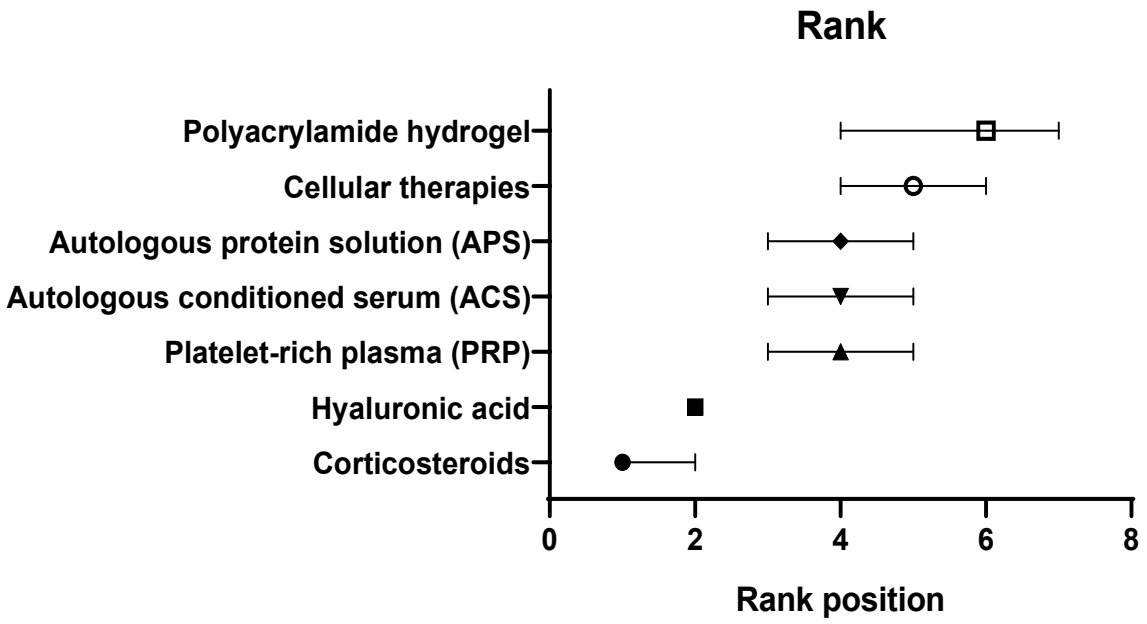

Figure 4.3. Ranking of the most preferred intra-articular therapy by 291 participants. Symbols are representing the median and the whiskers the interquartile range (IQR) of the ranking obtained from each intra-articular therapy.

Table 4.2. A total of 291 participants ranked NSIATs according to their preferences. The table indicates the number of practitioners that ranked a product from 1 to 7, the median obtained, and the number of times the product was ranked.

Rank / Product	1	2	3	4	5	6	7	Median	Total times product was ranked
Corticosteroids	210	41	15	3	3	6	20	**1**	**291**
Hyaluronic acid	44	195	20	3	8	18	5	**2**	**289**
Autologous conditioned plasma (PRP)	4	11	56	75	51	25	15	**4**	**237**
Autologous conditioned serum (ACS)	7	8	90	72	46	23	4	**4**	**250**
Autologous protein solution (APS)	10	12	76	31	36	28	25	**4**	**218**
Cellular therapies	4	16	13	45	48	52	31	**5**	**209**
Polyacrylamide hydrogel	12	8	21	28	28	33	68	**6**	**200**

The three most common reasons for the use of NSIATs were scientific data and articles published regarding product safety and efficacy (105/291 [36.1%]), personal experience with the product (80/291 [27.5%]), and specific conditions being treated (49/291 [16.8%]).

When participants were asked to rank which NSIAT they preferred regardless of client preference or product availability, ACS (96/291 [33%]) and APS (91/291 [31.3%]) were the top choices, followed by PRP (35/291 [12%]), cellular therapies (22/291 [7.6%]), and polyacrylamide gel (17/291 [5.8%]). Thirteen /291 [4.4%] selected other therapies as their first option, indicating a preference to use polyglycan and/or polysulfated glycosaminoglycans (Adequan®). For polyglycan and polysulfated glycosaminoglycans (Adequan®), practitioners did not specify if they were used intra-articularly or systemically. Although this was not specifically asked, in the comment section, participants explained that the main reasons not to

choose NSIATs were economic constraints and lack of standardized studies with results regarding product efficacy. Participants with a reduced lameness caseload reported economic difficulties in purchasing equipment to provide NSIATs, preferring to send these cases to a referral institution to be treated with these products.

Autologous Conditioned Plasma or Platelet-rich plasma (PRP)

Survey results for PRP are summarized in table 3. From the 291 participants that used NSIATs, 225 (77.3%) used PRP, while 66 (22.7%) did not. One hundred and eighty/ 224 [80.4%] participants used commercial kits processed by centrifugation, 18/224 [8%] processed PRP using commercial filtration kits, 17/224 [7.6%] processed PRP by manual centrifugation, and 9/224 [4%] sent out their blood samples to an outside laboratory or referral center to process PRP.

Arthrex ACP® Double Syringe System (69/224 [30.8%] and Restigen PRP® (62/224 [27.7%]) were the commercial kits most commonly used by participants, followed by Harvest® SmartPrep® System (22/224 [9.8%]), E-PET™ Equine Platelet Enhancement Therapy (19/224 [8.5%]), Magellan® Autologous Platelet Separator System (19/224 [8.5%]), and GPS® III Platelet Concentration System (5/224 [2.2%]). Regarding the activation method, 177/224 [79%] of the participants did not activate their PRP before administration, 18/224 [8%] activated platelets with calcium chloride, 9/224 [4%] activated platelets with extracorporeal shockwave therapy, and 9/224 [4%] activated platelets with freeze/thaw cycle(s).

The three most common reasons participants chose PRP were the treatment of ligament or tendon lesions (150/224 [67%]), acute articular pathology 25/224 [11.2%], and chronic articular pathology (23/224 [10.3%]).

One hundred and twenty-one / 224 [54%] of participants used systemic anti-inflammatory medication (flunixin meglumine or phenylbutazone) when administering PRP, while 103/224 [46%] did not use any of these medications simultaneously. Ninety-nine/224 [44.2%] participants ensured that the horse was not currently receiving a long-term sedative such as reserpine before collecting and processing PRP, while 125/224 [55.8%] did not ask about this regularly.

The majority of participants did not combine PRP with other intra-articular medications or products (161/224 [71.9%]). Twenty-three/224 of participants [10.3%] used antibiotics such as amikacin with PRP, 9/224 [4%] combined PRP with hyaluronic acid, 9/224 [4%] combined PRP with other cellular therapies, and 4/224 [1.8%] combined PRP with corticosteroids. Twenty-eight /224 [12.5%] of participants did not use PRP intra-articularly.

Regarding intra-articular treatment protocols, 61/196 [31.1%] of participants repeated injections based on short-term clinical response, 56/196 [28.6%] used PRP as a one-time injection, 42/196 [21.4%] repeated injections every 1-2 weeks for a total of 3 treatments, 22/196 [11.2%] repeated injections based on long term clinical response, and the remainder of participants 15/196 [7.7%] used different personalized protocols (Figure 4.4 A).

When evaluating the subjective assessment of clinical response to PRP, thirteen / 196 [6.6%] estimated that their patients had a 90% or greater improvement, 64/196 [32.7%] participants reported 75-90% improvement in their patients, 90/196 [45.9%] estimated 50-75% improvement in their patients, and 29/196 [14.8%] reported less than 50% improvement (Figure 4.4 B).

Participants were asked about their impression regarding the incidence of acute joint flares after intra-articular PRP treatment. Eighty-eight of 196 [44.9%] participants reported no

incidence of joint flare in their patients, 76/196 [38.8%] participants observed joint flare in less than 1 horse per 50 injected (2%), 18/196 [9.2%] participants estimated joint flare in less than 1 horse per 20 horses injected (5%), 11/196 [5.6%] participants estimated joint flare in less than 1 horse per 10 horses injected (10%), and 3/196 [1.5%] observed joint flare in more than 1 horse per ten horses injected (Figure 4.4 C).

Autologous conditioned serum (ACS)

Survey results for ACS are summarized in table 3. From the 291 participants using NSIATs, two hundred (68.73%) used ACS, while 91 (31.27%) did not use ACS in their patients. The commercial kits most commonly used by equine participants were the Orthokine® vet IRAP (87/200 [43.5%] and Arthrex-IRAP II™ System (94/200 [47%]), while MediVet ACS was minimally used (5/200 [2.5%]), and the rest of the participants did not specify a kit used (14/200 [7%]).

The three most common reasons for participants to choose ACS were for treatment of acute articular pathology (71/200 [35.5%]), treatment of chronic articular pathology (70/200 [35%]), or for post-operative therapy (28/200 [14%]).

Regarding combinations of ACS with other therapies, 147/200 [73.5%] participants did not combine ACS with any other product; while 25/200 [12.5%] combined ACS with antibiotics like amikacin, 17/200 [8.5%] combined with hyaluronic acid, 7/200 [3.5%] combined with other NSIATs and 4/200 [2%] combined with corticosteroids.

The most common ACS intra-articular treatment protocol was repeated injection every 1-2 weeks for 3 treatments (152/200 [76%]). The next most frequent treatment protocols in descending order were: repeated injections within 3 months based on short-term clinical

response (28/200 [14%]), repeated injections within 6 months to a year based on long-term clinical response (9/200 [4.5%]), and one-time injection or various diverse protocols based on disease 11/200 [5.5%] (Figure 4.4 A).

Overall subjective clinical outcome assessment for ACS, 30/200 [15%] participants observed a 90% or more clinical improvement after treatment, 93/200 [46.5%] observed 75-90% improvement in their patients, 58/200 [29%] observed 50-75% improvement, and 18/200 [9.5%] considered that improvement was 50% or less improvement (Figure 4.4 B).

Regarding the incidence of joint flare post-ACS administration, 103/200 [51.5%] reported no observed flare after ACS treatment, 77/200 [38.5%] observed joint flare in 1 per 50 horses injected, 12/200 [6%] observed joint flare in less than 1 horse per 20 horses injected, 3/200 [1.5%] observed joint flare in less than 1 horse per 10 horses injected, and 5/200 [2.5%] observed joint flare in more than 1 horse per 10 horses injected (Figure 4.4 C).

Autologous protein solution (APS)

Survey results for APS are summarized in table 3. A total of 137/291 (47.1%) participants that used NSIATs used APS, while 154/291 (52.9%) did not use APS. During the time the survey was done, Pro-Stride® was the only brand available on the market for processing APS in horses.

The three most common reasons for the use of APS were the treatment of acute articular pathology (54/137 [39.4%]), treatment of chronic articular pathology (51/137 [37.1%]), and for other unspecific disease processes (13/137 [9.5%]).

APS was mainly used alone (119/137 [86.9%]), but 13/137 [9.5%] of participants combined APS with antibiotics like amikacin, and 5/137 [3.6%] combined APS with hyaluronic

acid. All the participants that used APS intra-articularly processed one kit for small volume synovial structures such as coffin, fetlock, or tarsometatarsal joint. For large volume synovial structures, such as the stifle, 77/137 [56.2%] processed 1 kit, 52/137 [37.9%] processed 2 kits, 2/137 [1.5%] processed more than 2 kits, and 6/137 [4.4%] indicated that they do not use APS for this purpose.

Eighty-eight / 137 [64.2%] participants used systemic anti-inflammatory medications when administering APS, while 49/137 [36.8%] did not. Eighty-four / 137 [61.3%] did not check with clients to see if the horse was or had been on long-term sedatives, while 53/137 [38.7%] ensured horses were not receiving these drugs before collecting blood to process APS.

Regarding intra-articular treatment protocols for APS, 56/137 [40.9%] respondents repeated injections within 6 months to a year based on a long-term clinical response, 45/137 [32.8%] used APS as a one-time injection, 28/137 [20.4%] repeated injections within 3 months based on short-term clinical response, and 5/137 [3.7%] used APS as a repeat injection every 1-2 weeks for a total of 3 treatments. The remainder of participants (3/137 [2.2%]) varied the protocol depending on the disease (Figure 4.4 A).

Overall subjective clinical outcome assessment for APS, 40/137 [29.2%] participants observed greater than 90% improvement, 68/137 [49.6%] observed 75-90% improvement, 23/137 [16.8%] observed 50-75% improvement, and 6/137 [4.4%] observed less than 50% improvement (Figure 4.4 B).

Eighty-two/137 [59.8%] participants had not observed acute joint flare after treatment with APS, 4/137 [29.2%] observed joint flare in less than 1 horse per 50 injected, 11/137 [8%] observed joint flare in less than 1 horse per 20 injected, and 4/137 [3%] observed joint flare in less than 1 per 10 horses injected (Figure 4.4 C).

Cellular therapies

Survey results for cellular therapies are summarized in table 3. From the 291 participants using NSIATs, 142 (48.8%) used cellular therapies, while 149 (51.2%) do not. One hundred and fifteen / 142 [81%] of participants preferred to use autologous cells/tissues, while 24/142 [19%] used allogenic cells/tissues. Eighty-nine/ 142 [62.7%] participants obtained cells from bone marrow, 28/142 [19.7%] from adipose tissue, 21/142 [14.8%] from umbilical cord, 3/142 [2.1%] peripheral blood, and 1/142 [0.7%] from synovial tissue.

The three most common reasons participants used cellular therapies were the treatment of ligament or tendon lesions (71/142 [50%]), treatment of acute articular pathologies (25/142 [17.6%]), and for post-operative treatment (21/142 [14.8%]).
Seventy-nine/142 [55.6%] practitioners did not combine cellular therapies with other products, 26/142 [18.3%] used cellular therapies in combination with hyaluronic acid, 20/142 [14.1%] used cellular therapies with PRP, 6/142 [4.2%] used cellular therapies with ACS, 7/142 [4.9%] used cellular therapies with antibiotics, and 5/142 [3.52%] did not use cellular therapies intra-articularly.

The cellular intra-articular treatment protocol most commonly used by participants was a one-time injection (72/137 [52.6%]). This protocol was followed in frequency by repeated injections based on short-term clinical response (34/137 [24.8%]), repeated injections every 1-2 weeks for a total of 3 treatments (15/137 [11%]), and repeated injections based on the long-term clinical response (8/137 [5.8%]). The remainder of participants (8/137 [5.8%] varied the protocol depending on disease treated (Figure 4.4 A).

Overall subjective clinical outcome assessment of cellular therapies, 8/137 [5.8%] participants observed greater than 90% improvement, 59/137 [43.1%] observed 75-90%

improvement, 48/137 [35.0%] observed a 50-75% improvement, and 22/137 [16.1%] observed less than 50% improvement (Figure 4.4 B).

Regarding the incidence of joint flare post-administration, 63/137 [46%] reported they had not observed joint flare after treatment with cellular therapies, 49/137 [35.8%] observed joint flare in 1 per 50 horses injected, 19/137 [13.8%] observed joint flare in less than 1 horse per 20 horses injected, and 6/137 [4.4%] observed joint flare in less than 1 horse per 10 horses injected. There was no difference between reported flare rate and the use of allogenic vs. autologous cellular therapies (P = 0.8) (Figure 4.4 C).

Polyacrylamide hydrogel

Survey results for polyacrylamide hydrogel are summarized in table 3. One hundred and four / 291 (35.7%) participants used polyacrylamide hydrogel intra-articularly, while 187 (64.3%) did not. Noltrex™ was the brand most commonly used (61/104 [58.7%]), followed by Vet Arthramid® (38/104 [36.5%]) and VetAquamid® hydrogel reconstruction (5/104 [4.8%]). Participants practicing outside of the USA were more likely to use polyacrylamide hydrogel compared to participants practicing in the USA (P <0.001). Some practitioners practicing in the USA reported difficulties in acquiring this product.

The most common reason for the use of polyacrylamide hydrogel was to treat chronic articular pathologies (75/104 [72.1%]) and severe osteoarthritis unresponsive to other treatments (17/104 [16.3%]).

The most common intra-articular treatment protocols in descending order were a one-time injection (47/104 [45.2%]), repeated injections based on long-term clinical response

(37/104 [35.6%]), and repeated injections based on short-term clinical response (17/104 [16.3%]) (Figure 4.4 A).

Regarding subjective clinical outcome assessment of polyacrylamide hydrogel administration, 18/104 [17.3%] participants observed 90% or more improvement, 36/104 [34.6%] observed 75-90% improvement, 33/104 [31.7%] observed 50-75% improvement, and 17/104 [16.4%] observed improvement in less than 50% of patients (Figure 4.4 B).

Sixty-five / 104 [62.5%] reported no acute joint flare after treatment with polyacrylamide hydrogel, 26/104 [25%] observed joint flare in less than 1 horse per 50 injected, 10/104 [9.6 %] observed joint flare in less than 1 horse per 20 injected, and 3/104 [2.9%] observed joint flare in less than 1 per 10 horses injected (Figure 4.4 C).

Table 4.3. Summary of each NSIAT included in the survey from a total of 291 participants to the survey.

	PRP	ACS	APS	Cell Therapy	Polyacrylamide hydrogel
Number of responding practitioners using product for musculoskeletal injuries	224/291; 76.9%	200/291; 68.7%	137/291; 47.1%	142/291; 48.8%	104/291; 35.7%
Number of responding practitioners using product IA	196/291; 67.4%	200/291; 68.7%	137/291; 47.1%	137/291; 47.1%	104/291; 35.7%
Top 2 reasons for practitioner use of the product	1- Ligament/ Tendon pathology (150/224; 66.9%) 2- Acute articular Pathology (25/224; 11.2%)	1-Acute articular Pathology (71/200; 35.5%) 2- Chronic articular pathology (70/200; 35%)	1- Acute articular pathology (54/137; 39.4%) 2- Chronic articular pathology (51/137; 37.2%)	1- Ligament/Tendon Pathology (71/142; 50%) 2- Acute articular pathology (25/142; 17.6%)	1- Chronic Articular Pathology (75/104; 74.3%) 2-Severe OA unresponsive to other treatments (17/104; 16.3%)
Most frequent products used in combination for IA injection	None (161/224; 71.9%)	None (147/200; 73.5%)	None (119/137; 86.9%)	None (79/142; 55.6%)	Not asked

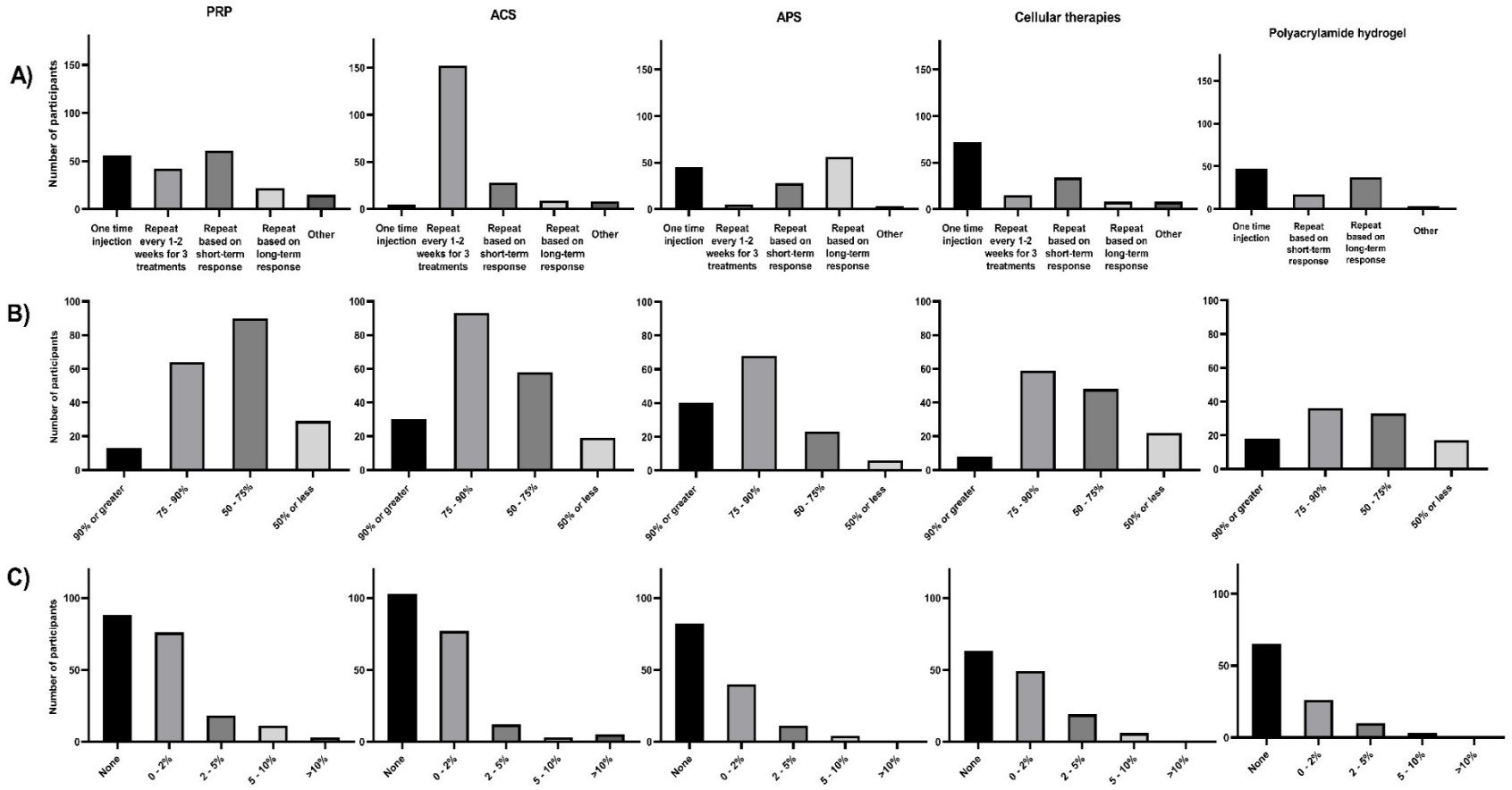

Figure 4.4. Summary of each NSIAT (columns) included in the survey from a total of 291 participants to the survey. A) Treatment protocol, B) Subjective clinical improvement and C) Flair rate observed after intra-articular treatment.

Discussion

The results of our study show that NSIATs are frequently used by equine practitioners and participants were familiar with the different modalities of NSIATs available on the market. Of the 353 practitioners surveyed, 291 (87.5%) use NSIATs. However, when asked which intra-articular therapy they prefer, corticosteroids and hyaluronic acid remained most popular.

Within NSIATs, ACS and PRP were the most commonly used, followed by APS, cellular therapies and polyacrylamide hydrogel. Practitioners with a higher lameness caseload and those that performed intra-articular injections in more than 10 horses/month were significantly more likely to utilize NSIATs. The most cited reason why practitioners did not use NSIATs was the economic limitations of the client. Commercial kits to process products such as PRP, ACS, or APS often require specific centrifuges, which makes the purchase and use of these products difficult for the veterinarian with a low lameness caseload. Discipline, as previously reported, still influences the use of NSIATs, as English sport horse practitioners were more likely to use NSIATs compared to other disciplines.[311]

According to a previous survey, corticosteroids with or without hyaluronic acid were the most common therapies used by members of the American Association of Equine Practitioners (AAEP).[311] In a 2009 survey of equine practitioners, Ferris et al. reported that 54.1% of the participants used ACS intra-articularly when horses were unresponsive to corticosteroid treatment or cost was not an issue for the client. Based on our survey results, it appears that practitioners (68.73%) are using ACS more often than in the past, followed by PRP (67.35%). Additionally, it appears that practitioners are now selecting them for the treatment of acute joint disease rather than advanced OA. NSIATs have been available on the market for longer

compared to the 2009 survey, this might have increased practitioner and owner awareness and willingness to use these products.

In another survey of equine practitioners in 2018, PRP and ACS were considered two of the top ten rehabilitation modalities for musculoskeletal injuries.[525] PRP was used in 98.9% of the cases to treat tendon or ligament injuries, while ACS was more frequently used in the joint postoperatively (55.3%) or to maintain performance (32.3%).[525] Similarly, in our survey, PRP and cellular therapies were more frequently used to treat soft tissue injuries (tendons or ligaments), while products such as ACS and APS were used to treat joint disease. Interestingly, cellular therapies were more frequently chosen as intra-articular treatment during the post-operative period compared to other therapies; for example, some practitioners commented about the common use of mesenchymal stem cells (MSCs) in stifle injuries. The stifle is a unique joint in which soft tissue structures (meniscus and ligaments) are contained within the synovial space.[526] Use of MSCs for meniscal injuries is likely influenced by a previous publication reporting improved outcomes in horses with stifle injury that were treated with arthroscopic exploration and debridement followed by intra-articular MSCs administration.[480]

Cellular products rely on cell-to-cell communication as well as autocrine and paracrine signaling to exert their effects on the tissue's microenvironment in which they are injected. The cellular and molecular mechanisms of MSCs produce their immunomodulatory effect have not been fully clarified yet. A recent publication has shown that MSCs can maintain their anti-inflammatory properties despite being metabolically inactive.[527] However, the authors believe that for these products to be effective, cellular viability, as well as function should be maintained. Few studies have evaluated the effects of other therapies on cellular properties *in vitro*. These studies have shown beneficial and deleterious effects on cellular products, and practitioners

should be aware of detrimental effects to prevent reduced efficacy and/or death of the cellular product. Most participants in our study (55-85%) did not combine cellular products with other intra-articular medications. When hyaluronic acid was added to MSCs *in vitro*, cellular viability, and chondrogenesis were enhanced.[528] However, in another *in vitro* study, no differences in cellular viability or increased production of transforming growth factor-beta was observed when MSCs were cultured with hyaluronic acid.[529] Beneficial effects have been observed when combining PRP and MSCS. In an *in vitro* study, PRP enhanced proliferation and chondrogenesis in cultured MSCs,[530] and *in vivo*, horses with naturally occurring OA treated intra-articularly with MSCs combined with PRP showed clinical improvement compared to either product alone.[477] However, Goodrich et al. reported that the combination of MSCs and PRP enhance bone formation instead of cartilage in osteochondral defects created on the lateral trochlear ridge of the stifle.[477] Although no practitioners reported combining corticosteroids with cell therapies, it is important to mention that adding methylprednisolone or triamcinolone to MSC cultures *in vitro* resulted in the rapid death of MSCs.[531]

Some practitioners use antibiotics intra-articularly when performing joint injections. Studies investigating the effects of antibiotics (aminoglycosides and fluorinated quinolones) at clinically extrapolated doses added to MSC cultures have shown deleterious effects with marked reduction in cellular viability.[529,532] A recent study that evaluated the effects of clinically relevant doses of antibiotics on chondrocytes *in vitro* resulted in significant cellular death.[533] In this survey, 7/142 [4.9%] of participants that used cellular therapies reported using them in combination with antibiotics. Though the effects of these products in combination *in vivo* have not been investigated, a combination of cellular products and antibiotics is not recommended. When using blood or tissue-based products, practitioners should be aware of the positive and

negative effects other therapeutics can have on the therapy administered. Approximately 8.5-10.3% of the practitioners combined products like PRP, ACS, and APS with antibiotics. These blood-based products are acellular or have few cells (red and white blood cells). The effect of antibiotics on these products for treatment of inflamed synovial tissues has not been evaluated. The authors recommend caution with the use of antibiotics in combination with NSIATs that are obtained from blood and tissues, particularly if cellular processes are how these products are thought to exert their effects within the synovial environment.

Surveyed practitioners were questioned about their use of NSAIDs when using PRP and APS as these products exert some of their effects through platelet concentration and release of growth factors.[524] This question was not asked with the rest of therapies included in the survey (ACS, MSCs, and polyacrylamide hydrogel). In the author's experience, some practitioners elect not to use NSAIDs at the time of PRP administration due to concerns in reducing the inflammatory response within the microenvironment of the diseased tissue, possibly reducing the reparative cellular response that is stimulated with injection of PRP. However, no further studies have been performed to answer this question. On the other hand, few studies have evaluated the simultaneous administration of NSAIDs when preparing blood-derived therapies. PRP obtained from horses receiving ketoprofen achieved higher platelet counts than PRP obtained from horses not receiving ketoprofen.[534] Although no growth factors were measured in that study, high platelet counts have been correlated with high concentrations of transforming growth factor-beta, insulin-like growth factor, and platelet-derived growth factor in PRP preparations.[376,535] A recent study evaluated the effects of NSAIDs on platelet aggregation and function. This study found that administration of firocoxib, flunixin meglumine, or phenylbutazone had no effect on platelet aggregation or function.[536] Incubation of NSAIDs or corticosteroids with blood prior to

processing did not affect concentration of inflammatory (interleukin -1β) or anti-inflammatory proteins (interleukin 1 receptor antagonist protein) in APS.[537,538] However, these were *ex vivo* studies, and results should be confirmed with *in vivo* experiments to evaluate changes in product efficacy. An *in vitro* study where NSAIDs have shown a dose-dependent effect on cultured MSCs.[539] A low dose of flunixin meglumine and meloxicam had positive effects on cell proliferation and migration, while a high concentration of these drugs and phenylbutazone produced a significant decrease in cellular viability and proliferation.[539] Although the effect of the NSAIDs on some NSIATs is not completely clear *in vivo*, there is no indication that NSAIDs cannot be simultaneously administered with NSIATs.

A recent publication reported that horses receiving reserpine (a long-term sedative), had hypercoagulable blood, especially when attempting to produce autologous biologic products.[540] Reserpine produces a detrimental effect, significantly increasing platelet aggregation, thus it is recommended to harvest blood for biological processing before using this medication.[540] In our survey, more than 50% of the participants that use PRP and APS did not ask their clients if their horses had received reserpine before blood collection. Considering the effect of this drug on platelet function, equine practitioners should include this question prior to blood collection and processing of blood-based NSIATs, particularly if the horse is on stall rest at the time of collection.

When APS is processed, an average of 3 mL of final product is obtained per kit. In our survey, practitioners were questioned how many kits were used when considering the size of the joint. The majority used one kit for smaller joints and 37.9% used two kits for treatment of larger joints. This is different from what it was used in a clinical study, where horses with naturally occurring OA were treated with two kits per joint independent of the volume of joint being

treated.[429] Although practitioners have not reported worse outcomes when using a single kit, further investigation to evaluate a possible dose-effect of this drug is warranted.

Our results indicate that practitioners outside the USA more frequently used polyacrylamide hydrogel than practitioners in the USA. Investigations into the use of polyacrylamide hydrogel have been primarily based out of Europe, [500,541] where it has been available on the market for a longer time period than in the USA. These factors indicate that practitioners outside the USA are likely more familiar with the product and have had more opportunities to use this product than practitioners in the USA.

This study has several limitations that warrant further discussion. Data on the number of practitioners that saw the link to the survey posted on social media, received and reviewed the email, and opened the Spur of the Moment newsletter and viewed the link but did not respond is not available, so response rate could not be calculated. Despite the different avenues used to reach as many practitioners as possible, our response rate could be considered low. The reported use of use NSIATs could be higher or lower than the actual use of NSIATs among equine practitioners surveyed in this study. Practitioners that use NSIATs were likely more willing to take time to complete the survey. Investigators ensured that participants were aware that they did not have to use or be familiar with NSIATs to answer the survey. These participants were asked questions on demographics and use of steroidal and NSIATs. Participants were not required to move forward within the survey to answer therapeutic-specific questions if they responded that they did not use NSIATs. Answers to questions regarding clinical response and complication rates were based on estimation and practitioner recall, and these were subjective impressions, not based on clinical records. The authors only questioned participants on the rate of observed joint

flare to provide more standardized options across products to the questionnaire; however, the authors recognize that other complications occur with intra-articular injections.

This survey provides information on the clinical use of NSIATs by equine practitioners, illustrating that NSIATs are used routinely to treat joint pathology. However, practitioners still have questions about the efficacy of these products and ideal treatment protocols in horses. Research investigating the disease-modifying effects of these products and investigations into best practices for how and when these products should be used and needed.

Acknowledgments

The authors would like to thank Dr. Chelsea Klein for her assistance with survey questions and each of the equine practitioners who took time to participate in the survey as well as AAEP, ECVS, and Zoetis for their help in distributing the electronic link to the survey.

Chapter 5

Culture media supplemented with 10% equine serum provided chondroprotection in an *in vitro* long-term cartilage and synovial membrane co-culture of OA

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Abstract

OA has been extensively studied *in vitro, ex vivo* and *in vivo* in horses. However, no single method of *in vitro* modeling is considered the gold standard for OA research. The use of co-culture systems allows cross-talk between the synovial membrane and cartilage to emulate natural OA. Culture media used in these systems may be supplemented with serum. No studies have evaluated the effect of culture in serum-free media (SF) vs. media supplemented with equine serum (ES) on co-culture of synovial membrane and cartilage tissue explants. The study objective was to evaluate the effects of equine serum supplementation on induced production of inflammatory and catabolic mediators from articular cartilage and synovial explants while in co-culture.

Cartilage and synovial explants were harvested from the stifle of 5 horses, placed in co-culture, stimulated with IL-1β (10 ng/ml) and maintained in culture for 3, 6 and 9 days in 10% ES or SF. At each time point, media was harvested for analysis of cellular viability (Lactate dehydrogenase, LDH) and elution of glycosaminoglycans (Dimethylene Blue Binding Assay, DMMB). Tissue explants were harvested for histopathologic and gene expression analysis. No differences in the cell viability was observed between SF and ES groups. SF culture produced an upregulation of TNF-α in synovial membrane and ADAMTS-4 and 5 in articular cartilage at 9 days of culture. ES produced an upregulation of aggrecan expression in cartilage at 9 days of culture. No differences in tissue viability were found between culture media, but SF media produced a higher glycosaminoglycan concentration in media at 3 days of culture.

The addition of 10% ES produced a slight chondroprotective effect in an inflamed co-culture system. This effect should be considered when designing studies evaluating treatment of serum or plasma-based orthobiologics *in vitro*.

Introduction

Osteoarthritis (OA) is a leading cause of disability in humans and domesticated animals, which has an enormous negative impact on healthcare costs worldwide.[185,189,542,543] Understanding the pathogenesis of this chronic degenerative disease and its multifactorial etiology could lead to the development of effective disease-modifying treatments

OA has been extensively studied *in vitro, ex vivo* and *in vivo* through experimental but translational animal models and in species-specific clinical trials. Similarities observed in the pathogenesis of OA in humans and domesticated animals have lead researchers to identify translatable animal models for both *in vitro* and *in vivo* research to enhance our understanding of OA.[193] A variety of methods have been used *in vitro* to investigate further OA and treatment effects of certain therapies on cells or tissues of the synovial environment. However, no single method of *in vitro* modeling is considered the gold standard for OA research.[193] When choosing an in vitro model for OA research, one must consider the type of cultured cell/tissue to be used, the number of cells/tissues to be included in the culture, culture conditions and method for induction of disease. Co-culture of synovial-derived cells or tissues better emulates the *in vivo* synovial environment by allowing cross-talk between these cells/tissues that are responsible for propagation of OA.[544] When using a co-culture model one must consider requirements of each tissue to maintain optimal cell viability and/or metabolism.[545] Some studies have evaluated viability of synovial and tissue explants alone, but little objective information is available that evaluates the optimum use of media for maintenance after induction of OA.

The most common source of serum used in animal tissue culture is fetal bovine serum (FBS), but other sources such as bovine calf, adult horse, and human serum can be added to culture media.[546] It is well known that serum represents a fundamental source of nutrients,

cytokines and adhesive molecules necessary to support *in vitro* cell growth, proliferation and metabolism.[547,548] However, there are some disadvantages from the addition of serum to culture media such as physiologic variability, higher risk of contamination, availability, and/or increased cost.[549] Also, FBS may contain lipopolysaccharide despite being certified endotoxin free and other xenogeneic antigens that can modify the phenotype of cultured cells and increase the risk of rejection by the host in regenerative medicine applications.[550] Alternatively, serum-free media can be used for tissue culture. Although cell proliferation is often slower in serum-free media,[549] previous studies have been able to maintain MSC chondrogenesis and cellular viability.[551,552] However, to our knowledge no studies have compared the use of serum-free media or equine serum supplemented media when culturing equine chondrocytes and/or synoviocytes.

In the literature there is no consensus among researchers in regard to serum supplementation when studying OA *in vitro*. In addition to media composition, no consensus for other culture components including the type of cells and/or tissues to include, culture conditions and OA induction is reported. Choosing an appropriate culture media with supplements that support and maintain cell viability and metabolism is crucial to effectively measure treatment effects. Certain blood-based treatments (orthobiologics) used for treatment of OA likely contain similar proteinaceous compounds for support cellular growth and proliferation that are contained in standard serum supplements for use in culture. These blood-based products have been shown to have weak chondroprotective properties. Therefore, standard serum supplementation may limit cellular responses to OA induction. Serum free culture of cells has been advocated for cells produced for human and animal medical use to reduce or limit cellular inoculation of the patient with xenogenic proteins that could possibly elicit an adverse reaction. On serum free media other nutrients such amino acids, growth factors or vitamins could be added to the culture to support

cellular viability, proliferation, and metabolic processes. The objective of this study was to evaluate the effects of commercially available equine serum supplementation on induced inflammatory and catabolic mediators of articular cartilage and synovial explants in co-culture stimulated with IL-1β. The hypothesis was that the addition of equine serum (10% v/v) would not improve cellular viability, but it would downregulate expression of inflammatory cytokines and decreasing the glycosaminoglycan (GAG) concentration in media. If commercially available equine serum supplementation offers chondroprotective effects, researchers should consider eliminating serum supplementation in *in vitro* OA culture models when studying intra-articular treatments to ensure a robust response.

Materials and Methods

Subjects

This study was performed following the Institutional and NIH guidelines for the Care and Use of Laboratory Animals, and the study was approved by the Institutional Animal Care and Use Committee (IACUC) at Auburn University (14-259). Five adult American Quarter horses (1 mare and 4 geldings, aged 13.6 ± 3 years) free of systemic disease and euthanized for reasons unrelated to the study were used. Horses were excluded from the study if they had a history of lameness isolated to the stifle.

Synovial membrane and articular cartilage harvest

Following euthanasia, synovial membrane and articular cartilage were aseptically harvested from the femoropatellar and femorotibial joints. Tissues from horses with gross signs of osteoarthritis, including cartilage erosion, score lines, discoloration, or fibrillation[553] were not

used. The synovial membrane was evaluated for gross signs of synovitis such as hyperemia, hypertrophy or fibrosis, and horses showing any of these changes were not included in the study. A 4 mm diameter disposable biopsy punch (Integra, Saint Priest, France) was used to obtain the cartilage and synovial membrane explants (Figure 5.1). Sixteen cartilage explants were obtained from the medial femoral condyle of each horse. The synovial membrane was dissected from the fibrous joint capsule, and 32 explants were obtained. Synovial membrane was further dissected under a dissecting microscope to ensure that only synovial membrane was present (Figure 5.2).

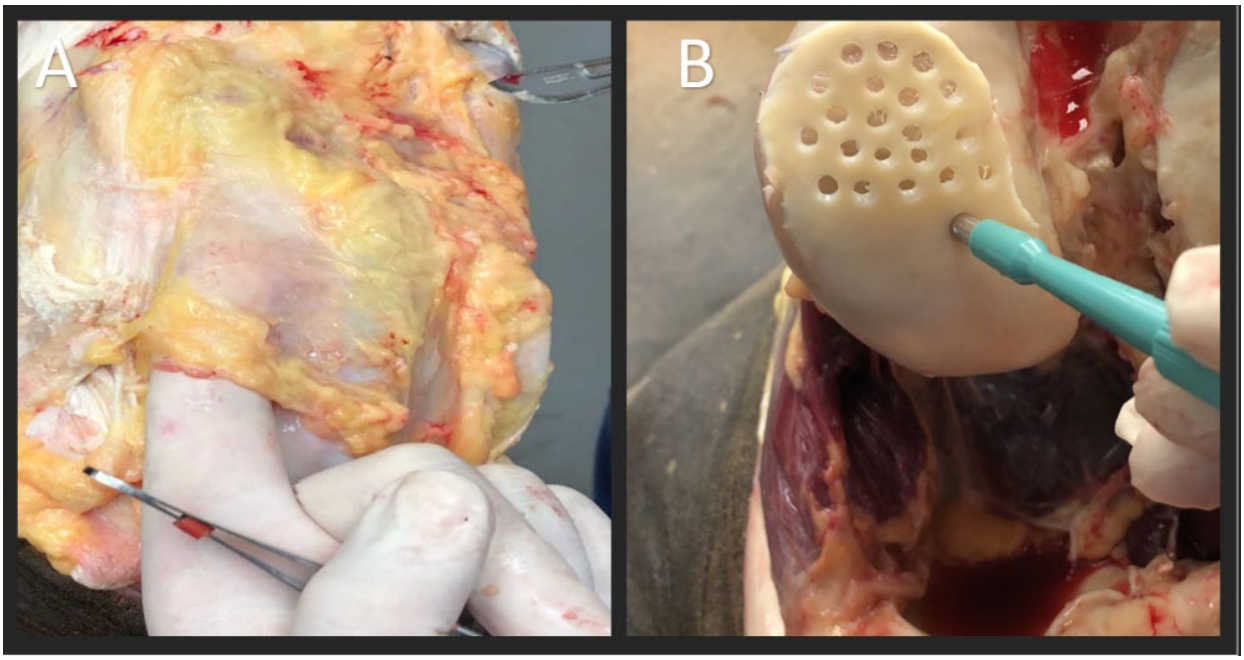

Figure 5.1. A) Macroscopic dissection of the synovial membrane. B) Articular cartilage explant harvesting from the medial condyle of the femur.

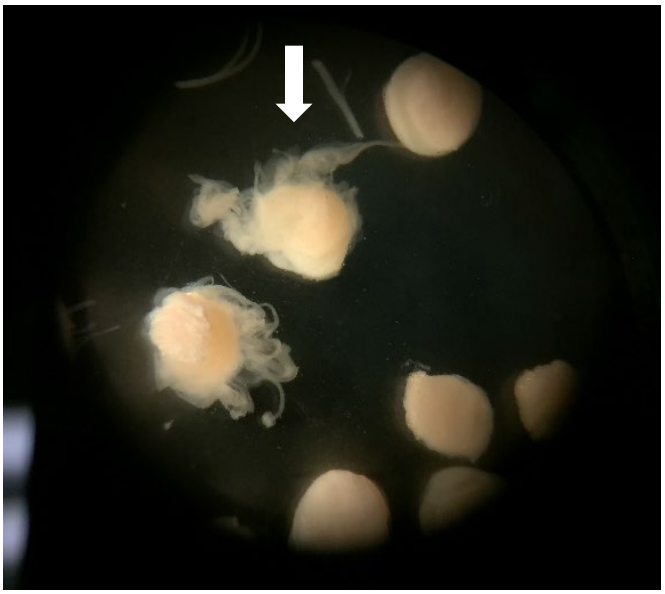

Figure 5.2. Visualization of the synovial membrane under the dissecting microscope (arrow pointing the synovial villi).

Co-culture system:

A 12-well plate with a hanging insert with a 3.0 µm pore size (VWR, Radnor, PA) was utilized for this study. Two synovial membrane explants were placed in the lower well, while one cartilage explant was placed in the upper well (Figure 5.3).[554] Co-cultures were performed in quadruplicate.

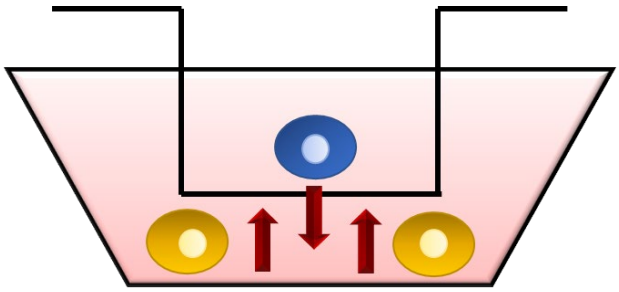

Figure 5.3. Diagram of the co-culture system. One 4 mm articular cartilage explant was placed in the insert, while two 4 mm synovial membrane explants were placed in the well.

Explant co-cultures were divided into two treatment groups: 1) Dulbecco's modified eagle media (DMEM), high glucose (4500mg/L) with 0.25gm of L-glutamine and sodium bicarbonate, 50 µL of streptomycin (100µg/ml) and penicillin (100µg/mL), 1% insulin-transferrin-selenium-sodium pyruvate, 20 µg/mL ascorbic acid (BioWhittaker; Lonza, Basel, Switzerland) serum-free (SF) media and 2) the same DMEM described, supplemented with 10% equine serum (ES). Incubation was maintained at 37°C and 5% CO_2 room air incubator for 24 hours in DMEM culture media according to the allocated treatment group (SF and ES) to allow tissue acclimatization. After 24 hours, all the co-cultures were stimulated with interleukin-1β (10 ng/ml) (R&D Systems, Minneapolis, MN). Cultures were maintained for 3, 6, and 9 days. Media were refreshed every 3 days throughout the culture period along with IL-1β. Media were harvested at each time point (0, 3, 6 and 9 days) from both treatment groups (SF and ES), snap-frozen in liquid nitrogen and stored at -80°C for batch analysis at a later date.

Gene expression in cartilage and synovial membrane

Frozen cartilage samples were added to TRIzol reagent (Invitrogen, Carlsbad, CA) then pulverized with a tissue homogenizer (Polytron, Thomas Scientific, Swedesboro, NJ). Synovial membrane samples were homogenized in TRIzol using a bead homogenizer (TissueLyser LT, Qiagen, Germantown, MD) at 50 oscillations per minute for 20 min. To isolate RNA, a chloroform extraction protocol was performed. Briefly, 200 µl of chloroform were added to the samples, mixed vigorously, and incubated at room temperature for 10 min. The mix was centrifuged at 17,000 RCF for 15 min at 4°C. The supernatant was saved (approximately 500 µl), while the remaining pellet was discarded. Equal parts of isopropanol were added and incubated at -20°C for 15 min, followed by centrifugation at 17,000 RCF for 20 minutes. The supernatant

was decanted, and 1 mL of cold 75% ethanol was added and spun at the same speed for 10 min. The supernatant was decanted again, and once the ethanol was evaporated entirely; the pellet was re-suspended on 40 µl of nuclease-free water. Nucleic acid concentrations were determined using a spectrophotometer at 260/280 nm (DeNovix, Wilmington, DE). RNA was stored at -80°C until qPCR analysis. RNA was reverse transcribed to cDNA using iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad, Hercules, California). Relative gene expression of IL-1β, MMP-3, MMP-13, IL-6, IL-8, ADAMTS-4, and ADAMTS-5 (in synovial membrane) and type II collagen (COL2A1), aggrecan (ACAN), TNF-α, IL-1β, IL-6, IL-8, ADAMTS-4, and ADAMTS-5 (in articular cartilage) was calculated. All primers were derived from the Equus caballus genome (GenBank) and designed using the NCBI-Primer-BLAST **(**Table 5.1).

Primer efficiencies were determined using 2-fold dilutions of cDNA, and efficiencies calculated for all the primers ranged between 98-102.5%. All the qPCR experiments were performed in triplicate using SYBR Green Master Mix (PerfeCTa SYBR Green FastMix, Quantabio, Beverly, Massachusetts). The thermocycler (CFX 96 Thermocycler Bio-Rad Hercules, California) was heated at 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 55°C for 30 s, and 70°C for 30 s, followed by a melting curve analysis. The relative gene expression was calculated by the comparative threshold cycle method (ΔΔCt method). Reference genes used were 18s and GAPDH for synovial and articular cartilage These genes were selected by evaluating the stability of various reference genes with equine tissue (18-S, β2M, GAPDH, SDHA, HPRT1, SCAMP-3, and β-actin). ΔΔCt values for all these genes were calculated under different culture media conditions in the 5 horses, and the two genes for each tissue with the least amount of change in gene expression were chosen.[555] Differences in gene expression between

treatment groups and time points were determined as fold change of relative gene expression compared to control tissues harvested at time 0.

Table 5.1. Equine primer sequences used for gene expression analyses.

Gene	**Primer sequence**	
18 small ribonucleic acid (18S)	Forward	5′- GCCGCTAGAGGTGAAATTCT-3′
	Reverse	5′- TCGGAACTACGACGGTATCT−3′
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Forward	5′-TGTCATCAACGGAAAGGC-3′
	Reverse	5′-GCATCAGCAGAAGGAGCA-3′
Interleukin-1 β (IL-1β)	Forward	5′-GCGGCAATGAGAATGACCTG-3′
	Reverse	5′-AGCCACAATGATTGACACGA-3′
Interleukin-6 (IL-6)	Forward	5′-AACAGCAAGGAGGTACTGGCA-3′
	Reverse	5′-CAGGTCTCCTGATTGAACCCA-3′
Interleukin-8 (IL-8)	Forward	5′-AGGGACAGCAGAGACAGAGACACAAG-3′
	Reverse	5′-TACAACCGCAGCTTCACACA-3′
Matrix metalloproteinase 3 (MMP- 3)	Forward	5′-GGCAACGTAGAGCTGAGTAAAGCC-3′
	Reverse	5′-CAACGGATAGGCTGAGCACGC-3′
Matrix metalloproteinase 13 (MMP-13)	Forward	5′-GTCCCTGATGTGGGTGAATAC-3′
	Reverse	5′-ACATCAGACAAACTTTGAAGG-3′
ADAM metallopeptidase with thrombospondin type 1 motif 4 (ADAMTS-4)	Forward	5′-GCTGTGCTATTGTGGAGGATGATGG-3′
	Reverse	5′-CCAGGGAAAGTCACAGGCAGATG-3′
ADAM metallopeptidase with thrombospondin type 1 motif 4 (ADAMTS-5)	Forward	5′-GGTGCAGAACATCGACCAGA-3′
	Reverse	5′-AAGAAACCGTCGAGACCACC-3′
Aggrecan (ACAN)	Forward	5′-CCTTGACTCCAGTGGTCTTATC-3′
	Reverse	5′GTCGTGGACCACCTAATTCTATC-3′
Type II collagen (COL2)	Forward	5′-GCCCGTCTGCTTCTTGTAATA-3′
	Reverse	5′-CGTGACTGGGATTGGAAAGT-3′

Lactate Dehydrogenase (LDH) Assay

LDH release when cell death occurs, and concentration was measured to analyze cell viability. Concentrations of LDH in media were determined with a commercially available assay (Roche, Basel, Switzerland). Briefly, 100 µL of sample media was incubated with 100 µL of reaction mixture containing diaphorase/NAD+, iodotetrazolium chloride, and sodium lactate in 96-well plates in the dark at 25°C for 30 min. Cytotoxicity was quantified as a measure of LDH activity by measuring absorbance at 492 nm on a microplate reader (SpectraMax ID3, Molecular Devices, Sunnyvale, CA, USA). Samples were measured in triplicates.

1,9-dimethyl methylene blue assay (DMMB)

DMMB assay was used to measure the glycosaminoglycans (GAG) concentration in media as a biomarker of cartilage extracellular matrix damage. Media were digested in papain (0.5 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) at 65°C for 4 h. The 1,9-dimethyl methylene blue dye (Sigma-Aldrich, St. Louis, MO, USA) was prepared following the method of Farndale et al.[556] The standard curve was created by preparing solutions containing 0 to 65 µg of chondroitin sulfate (Sigma-Aldrich, St. Louis, MO, USA). Briefly, using a 96-well flat-bottom transparent plate (Stellar Scientific, Baltimore, MD, USA) 50 µl of diluted digested media (1:4 dilution in distilled water) was mixed with 200 µl of DMMB dye (1 mN hydrochloric acid, 0.06‰ DMMB, 40 mM glycine, 27 mM NaCl pH 3) and the plate was shaken on a horizontal orbital microplate shaker (0.12" orbit) at 500 RPM for 5 secs. Measurement of total GAG content was performed by a direct spectrophotometric method using optical density measured at 525 nm on a microplate reader (SpectraMax ID3, Molecular Devices, Sunnyvale, CA, USA).[557] Samples were measured in triplicate.

Histology analysis

For histology, cartilage and synovial membrane explants were fixed in 10% phosphate-buffered formalin, embedded in paraffin, cut into 5 mm sections, and mounted on coated glass slides. Sections are stained with hematoxylin and eosin (H&E) or stained with hematoxylin and 0.1% aqueous safranin O. Tissue sections were examined via light microscopy by a board-certified pathologist. The OARSI histopathology score system for OA in horses previously described was used.[173] For synovial membrane, cellular infiltration, vascularity, intimal hyperplasia, subintimal edema, and subintimal fibrosis were graded from 0 (normal) to 4 (Table 5.2). For cartilage, microscopic characteristics such as chondrocyte necrosis, cluster formation, fibrillation, focal cell loss, and SOFG stain uptake were graded from 0 (normal) to 4 (Table 5.3). For articular cartilage and synovial membrane, the sum of the score from the 4 parameters evaluated provides a total score from 0 to 20.

Statistical analysis

Normality was determined using the Shapiro–Wilk test and visual examination of Q-Q plots. Data that were not normally distributed were log-transformed prior to analysis. Differences observed in the data between time points and the effect of serum supplementation were assessed using mixed model analysis of variance. The linear model specified culture group, time, and interaction between group and time as fixed effects and horse was identified as the random effect. Post hoc analysis was performed using pairwise comparisons of groups applying Tukey's test. All analyses were performed using JMP Pro 13 (Cary, NC, USA). Significance was set at $P < 0.05$.

Table 5.2. Microscopic grading system for synovial membrane histology created by the Osteoarthritis Research Society International (OARSI).[173]

Synovial membrane parameter	Grade	Description
Cellular infiltration	0	No mononuclear cells in the section
	1	Occasional small areas of mononuclear cells throughout the section
	2	Mild presence of mononuclear cells in 25% of the section
	3	Moderate presence of mononuclear cells in 25-50% of the section
	4	Marked presence of mononuclear cells in greater than 50% of the section
Vascularity	0	Normal
	1	Slight increase in vessels in focal locations throughout the section
	2	Mild increase in number and dilatation of vessels in focal locations throughout the section
	3	Moderate increase in number and dilatation of vessels in up to 50% of the section
	4	Marked increase in number and dilatation of vessels in greater than 50% of the section
Intimal hyperplasia	0	None
	1	Villi with 2-4 rows of intimal cells within the section
	2	Villi with 4-5 rows of intimal cells over 25-50% of the section
	3	Villi with 4-5 rows of intimal cells over 50% of the section
	4	Villi with 5 or greater rows of intimal cells over 50% of the section
Subintimal edema	0	No edema
	1	Slight edema detected within section
	2	Mild edema within 25% of the section
	3	Moderate edema within 25-50% of the section
	4	Marked edema in greater than 50% of the section
Subintimal fibrosis	0	Normal
	1	Slight increase in fibrosis within the section
	2	Mild increase in fibrosis in 25% of the section
	3	Moderate increase in fibrosis in 25-50% of the section
	4	Marked increase in fibrosis in greater than 50% of the section

Table 5.3. Microscopic grading system for articular cartilage histology created by the OARSI.[173]

Articular cartilage parameter	Grade	Description
Chondrocyte necrosis	0	Normal section without necrosis
	1	No more than one necrotic cell located near the articular surface per 20x objective
	2	1-2 necrotic cells located near the articular surface per 20x objective
	3	2-3 necrotic cells located near the articular surface per 20x objective
	4	3-4 necrotic cells located near the articular surface per 20x objective
Cluster (complex chondrone) formation	0	No cluster formation throughout section
	1	Two chondrocytes (doublets) within same lacunae along superficial aspect of the articular cartilage section
	2	2-3 chondrocytes (doublets & triplets) within same lacunae along superficial aspect of the articular cartilage section
	3	3-4 chondrocytes within same lacunae along superficial aspect of the articular cartilage section
	4	Greater than four chondrocytes within same lacunae along superficial aspect of the articular cartilage section
Fibrillation/fissuring	0	No fibrillation/fissuring of the articular cartilage surface
	1	Fibrillation/fissuring of the articular cartilage restricted to surface and superficial zone
	2	Fissuring that extends into the middle zone
	3	Fissuring that extends to the level of the deep zone
	4	Fissuring that extends into the deep zone
Focal cell loss	0	Normal cell population throughout the section
	1	A 10-20% area of acellularity per 20x field
	2	A 20-30% area of acellularity per 20x field
	3	A 40-50% area of acellularity per 20x field
	4	A greater than 50% area of acellularity per 20x field
SOFG stain uptake	0	Normal staining
	1	Less than 25% loss of staining characteristics
	2	25-50% loss of staining characteristics
	3	50-75% loss of staining characteristics
	4	Greater than 75% loss of staining characteristics

Results

Gene expression in cartilage and synovial membrane

Synovial membrane

Gene expression was assessed in synovial membrane at 0, 3, 6, and 9 days of co-culture. From baseline, the gene expression of IL-1β, IL-6, IL-8, MMP-3, MMP-13, ADAMTS-4, and ADAMTS-5 was increased at 3, 6, and 9 days of culture, but no significant differences were observed between time points, except at 9 days of culture in SF media in which TNF-α was significantly upregulated compared to baseline ($P = 0.024$) (Figure 5.4).

No significant differences were observed between ES media and SF media in the gene expression of IL-β, IL-6, IL-8, MMP-3, MMP-13, ADAMTS-4, and ADAMTS-5 at any time points. The expression of TNF-α was significantly increased at 9 days ($P = 0.027$) of culture in the SF group (634-fold) compared to the ES group (22-fold).

Articular cartilage

Gene expression was assessed in articular cartilage at 0, 3, 6, and 9 days of co-culture. From baseline, the gene expression of IL-1β, TNF-α, IL-6, IL-8, ADAMTS-4, and ADAMTS-5 was increased at 3, 6, and 9 days of culture, but no differences were observed between time points after stimulation, except the SF group at 9 days of culture, in which the expression of ADAMTS-4 ($P = 0.007$) and ADAMTS-5 ($P = 0.002$) were significantly upregulated compared to baseline. The expression of type II collagen and aggrecan (ACAN) were downregulated. However, no significant differences were observed between time points after stimulation, except the ES group at 9 days of culture in which ACAN expression was significantly increased compared to 3 and 6 days of culture ($P = 0.017$) (Figure 5.5).

When comparing groups, gene expression of ADAMTS-4 and ADAMTS-5 was significantly increased at 9 days of culture in the SF group ($P = 0.002$ and $P = 0.006$, respectively) compared to the ES group at 9 days. In addition, gene expression of ACAN was significantly increased at 9 days of culture in the ES group ($P = 0.025$) compared to the SF group.

LDH assay

No differences in the LDH concentration were observed between the SF and ES groups at any time point. Increased cellular death occurred, detected by a measured increase in LDH concentration at each time point in both groups, increasing over time (Figure 5.6). In the SF media, LDH detection was higher at day 9 compared to day 3 and 6 ($P < 0.0001$ and $P = 0.001$, respectively). In the ES media group, the LDH detection was significantly higher on day 9 than on day 3 ($P = 0.002$).

1,9-dimethyl methylene blue assay (DMMB)

The SF group produced the highest GAG concentration at day 3 (125.68 ± 58.31 μg/ml), while ES group was at day 6 of culture (84.95 ± 30.26 μg/ml). There was no difference in the GAG concentration between time points within each treatment group (SF vs. ES) (Figure 5.7). However, a significant difference between treatments was found ($P = 0.017$). SF group at day 3 produced higher GAG concentration in media compared to the ES group ($P = 0.023$).

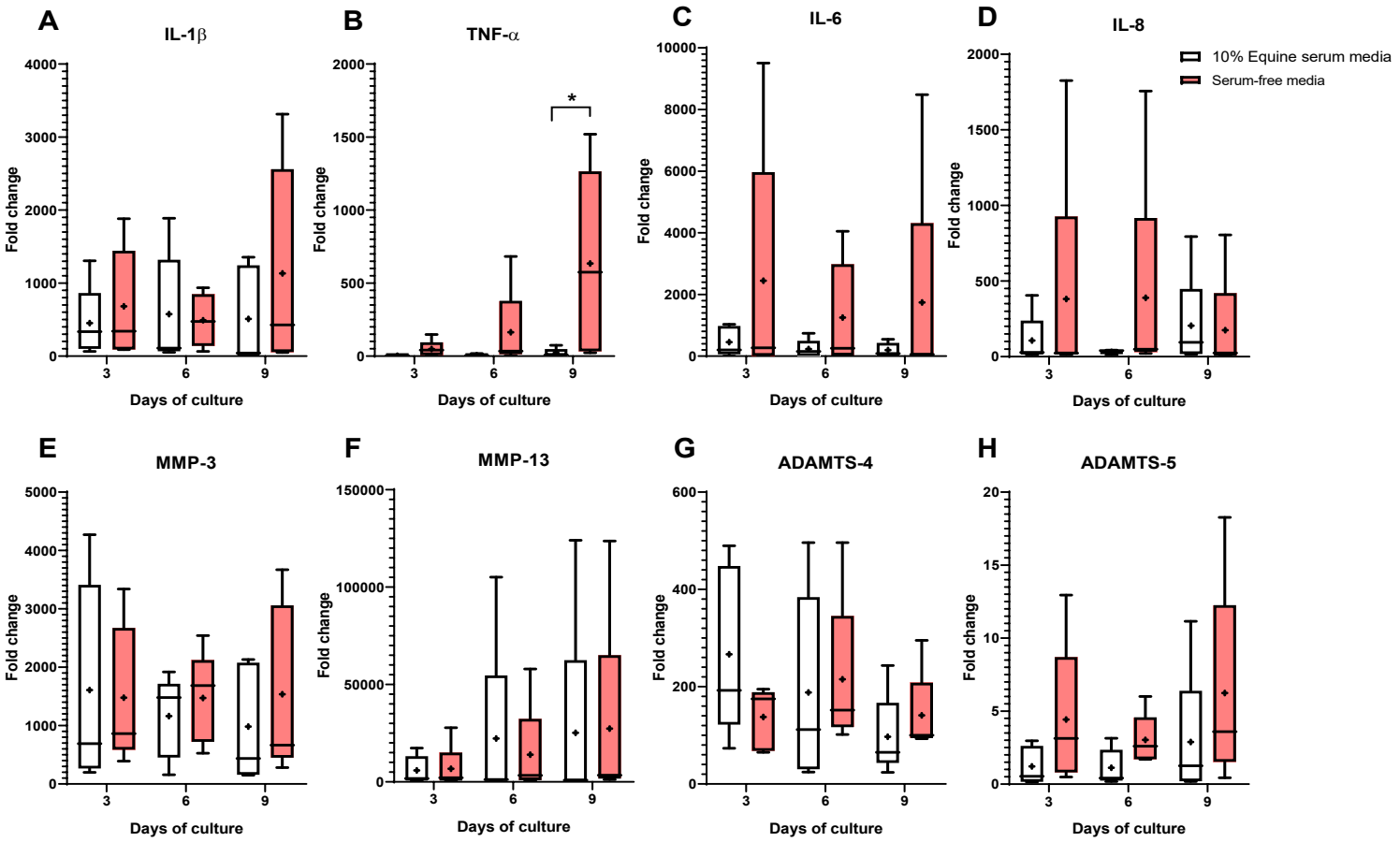

Figure 5.4. Fold change in relative gene expression comparing tissue at the harvesting time (T0) with IL-1β stimulated synovial membrane in co-cultures with 10% equine serum and serum-free media. (A) IL-1β, (B)TNF-α (C) IL-6, (D) IL-8, (E) MMP-3,(F) MMP-13, (G) ADAMTS-4, and (H) ADAMTS-5. The boxplots represent the interquartile range (IQR) of n = 5. The black lines represent the median values, the crosses represent the mean, and the whiskers represent the values outside the IQR. *Denotes significant difference culture media, $p < 0.05$.

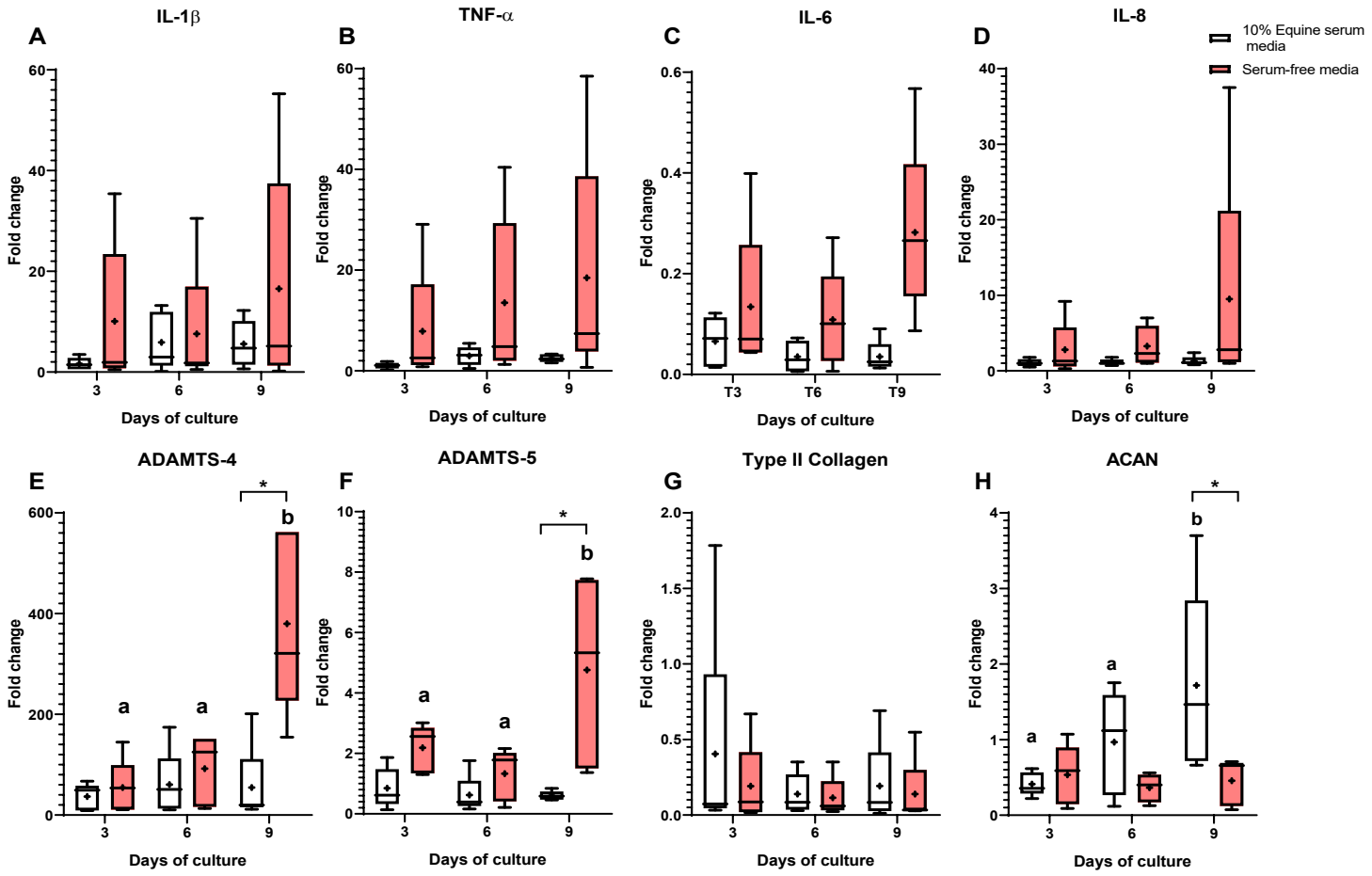

Figure 5.5. Fold change in relative gene expression comparing tissue at the harvesting time (T0) with IL-1β stimulated articular cartilage in co-cultures with 10% equine serum and serum-free media. (A) IL-1β, (B)TNF-α (C) IL-6, (D) IL-8, (E) ADAMTS-4, and (F) ADAMTS-5, (G) type II collagen, and (H) ACAN. The boxplots represent the interquartile range (IQR) of n = 5. The black lines represent the median values, the crosses represent the mean, and the whiskers represent the values outside the IQR. *Denotes significant difference culture media and the letters indicate differences between time points, $p < 0.05$.

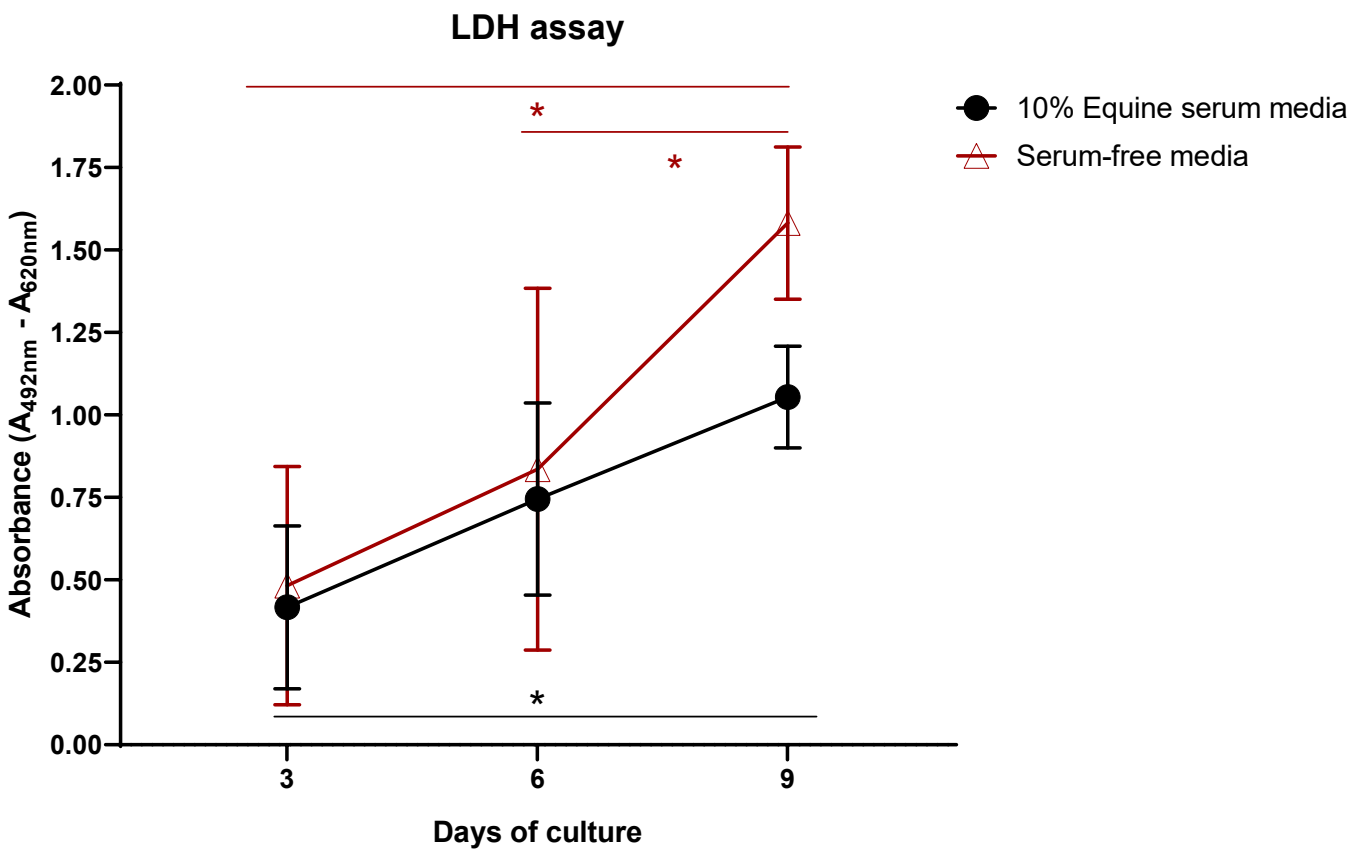

Figure 5.6. LDH concentration measured in free-serum media (red triangle) and 10% equine serum media (black circle) at 3, 6, and 9 days of culture. The symbols represent the mean and the whiskers the standard deviation. *Denotes significant difference between time points within the same group, $p < 0.05$. No significant differences between SF and ES were found at any time point.

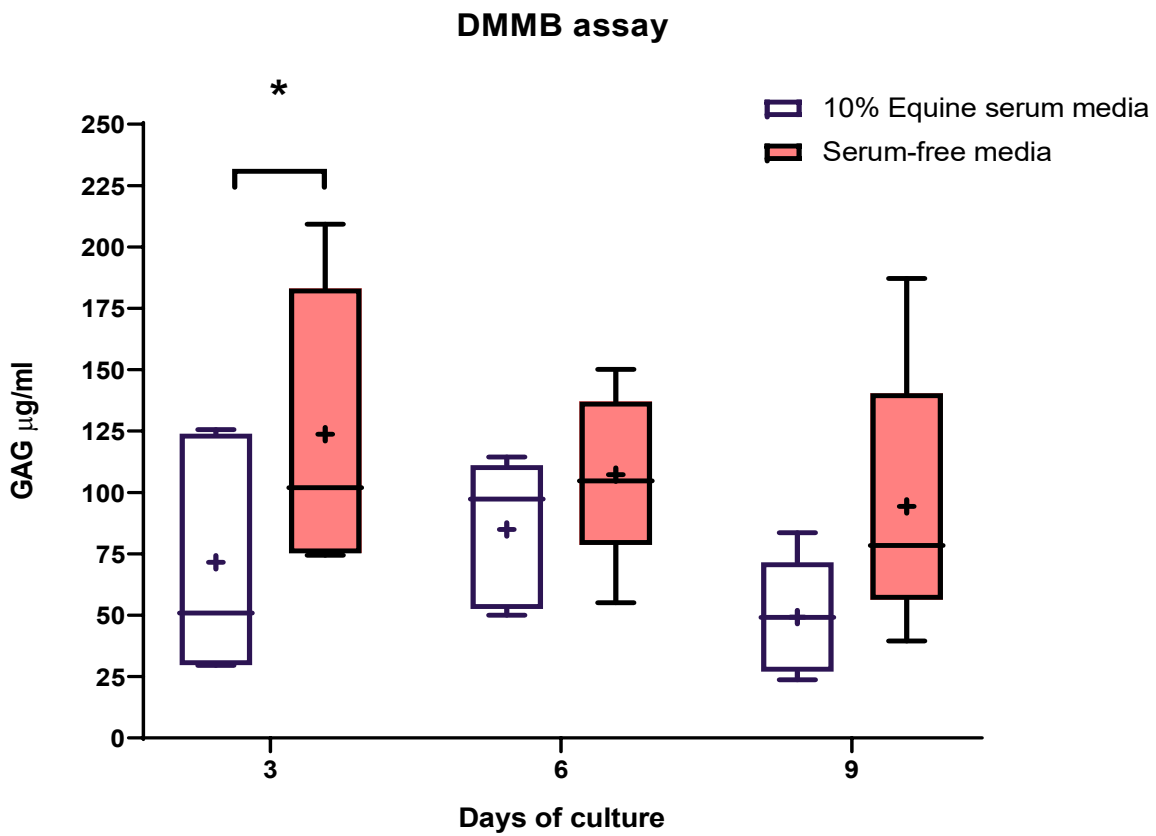

Figure 5.7. GAG concentration was measured in media for serum-free media (red) and 10% equine serum (white) at 3, 6, and 9 days of culture. The boxplots represent the interquartile range (IQR) of n = 5. The black lines represent the median values, the crosses represent the mean, and the whiskers represent the values outside the IQR. *Denotes significant difference between culture media, $p < 0.05$.

Histologic analysis

Due to processing errors, not all the samples from all the horses and time points were able to be evaluated. Each tissue (synovial membrane and articular cartilage) 4/4 samples were evaluated for every time point except for the SF group at 6 days in which 3/4 samples of synovial membrane were available for analysis. Synovial membrane and cartilage from each horse were histologically evaluated at time 0, observing a total average score of 0.4 ± 0.8 for the articular cartilage and 0.5 ± 0.86 for synovial membrane. These scores were significantly increased after stimulation at all the time points (Figure 5.8) in articular cartilage (P = 0.01) and synovial membrane (P = 0.003). No significant differences were observed between SF and ES media in any of the parameters evaluated in synovial membrane and articular cartilage (Figure 5.9 and 5.10)

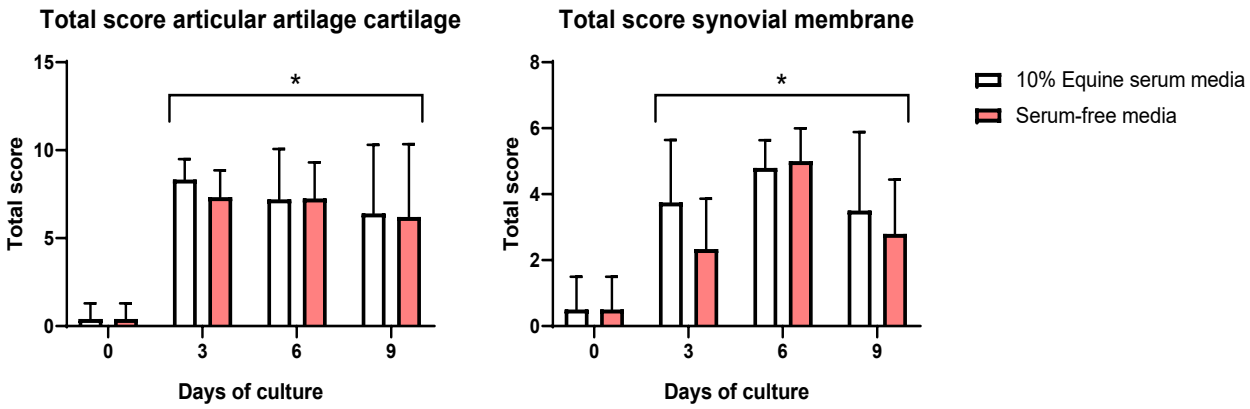

Figure 5.8 Total histologic score following the OARSI score system that was obtained in articular cartilage and synovial fluid in normal tissue (T0) and after stimulation with IL-1β. *Denotes significant difference between time 0 and stimulation with IL-β groups at different time points, $p < 0.05$.

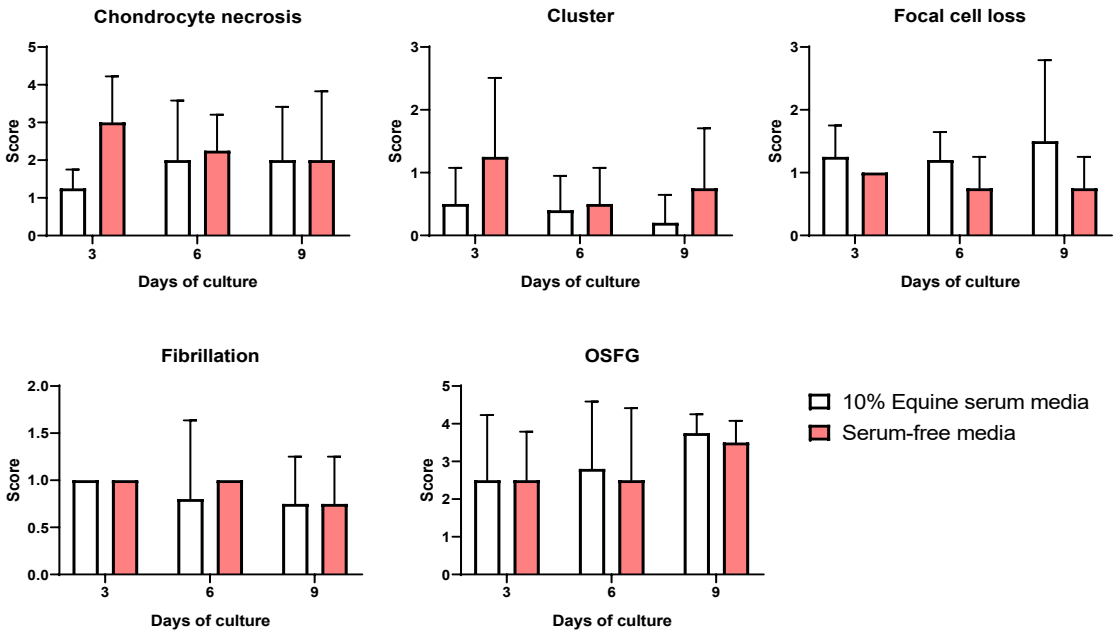

Figure 5.9 Histologic score of the parameters evaluated in articular cartilage following the OARSI recommendations.

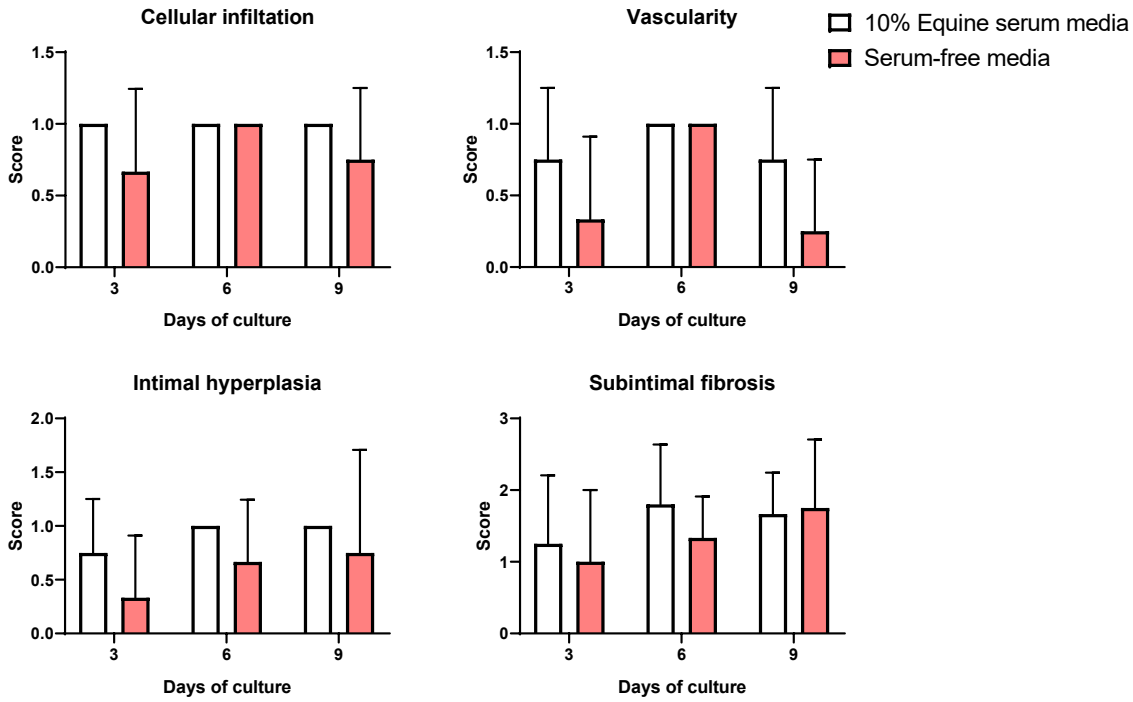

Figure 5.10 Histologic score of the parameters evaluated in synovial membrane following the OARSI recommendations.

Discussion

This study was performed to evaluate GAG concentration (matrix destruction), cellular viability, changes in gene expression, and histologic scores of tissue explants during long-term co-culture of equine articular cartilage and synovial membrane following stimulation of IL-1β, and compare differences in theses parameters after culture with SF or ES supplemented media. Inflammation was induced within the system resulting in an upregulation of inflammatory mediators and histologic changes within stimulated tissues compared to unstimulated controls. Slight differences were observed between treatment groups. The addition of ES did not improve cellular viability compared to the SF group up to 9 days of culture. Aggrecanases and TNF-α have an active role in the perpetuation of the OA inflammatory cascade and cartilage destruction, and the downregulation of these mediators and enzymes produced by the supplementation of 10% ES, could affect the establishment of OA *in vitro*. In addition, upregulation of aggrecan in the ES group represents an attempt of the tissue to enhance endogenous turnover (repair). This did not occur in the SF group. Release of GAG into the culture media was greater in the SF compared to the ES group on day 3 of culture. GAGs are release into the culture media due to destruction of the cartilage's extracellular matrix. The hypothesis was that the addition of equine serum would not improve cellular viability, but it would downregulate expression of inflammatory cytokines and decreasing GAG release from the cartilage extracellular matrix. According to the result of the study, ES did not reduce the cellular death and may dampen the inflammatory generating and matrix-destructive effects of IL-1β compared to SF media in synovial membrane and articular cartilage co-cultures.

OA has been extensively studied using animal explants *in vitro*. Explant-based models of cytokine stimulation allow one to study the cellular response simply and in a straightforward

fashion, maintaining the cells in their natural extracellular habitat.[193] In particular, the use of synovial membrane and articular cartilage co-culture systems include the cross-talk between these two tissues, better emulating the inflammatory cascade *in vivo*, given the synovial membrane is the primary source of inflammatory cytokines.[544,558,559] Animal tissue generally can be maintained in culture under well-established conditions in incubators, where the temperature is typically kept at 37°C with a controlled humidified gas mixture of 5% CO_2 and 95% O_2. To achieve good experimental reproducibility, the composition of the cell culture medium is essential. Serum provides several growth factors and hormones involved in growth promotion and specialized cell function.[545] Previous studies have found differences between cell cultures depends on media used. Schwarz et al. reported enhancing canine and equine MSC proliferation when using FBS instead of free-serum media.[560] Also, the culture of bronchial epithelial fibroblasts in the absence of serum led to decreased cell proliferation and attachment compared to FBS or ES media, and cells maintained their fibroblast morphology better in FBS than ES.[561] However, it is unknown if the addition of serum could change the cellular response when stimulating OA using cytokines such as IL-1β.

Orthobiologic therapies such as platelet-rich plasma, autologous conditioned serum or autologous protein solution have a high concentration of growth factors and cytokines/chemokines.[524] A recent study has shown that the addition of autologous conditioned serum to the culture of human MSCs enhanced their chondrogenic differentiation and increased the immunomodulatory properties of the MSCs, suggesting that coadministration of MSCs and autologous conditioned serum could have beneficial effects on the therapeutic potential of MSCs. Equine serum used for tissue culture possesses nutrients, growth factors and cytokines similar to orthobiologic products. Therefore, determining the specific cellular changes produced

by orthobiologic therapies *in vitro* could be more challenging or impossible when supplementing the culture media with equine serum. Orthobiologics have been shown to possess limited disease-modifying properties in the literature and it is crucial to use the best *in vitro* models available to further understand their benefits and cellular mechanism.

Stimulation with IL-1β triggers an inflammatory response in chondrocytes and synoviocytes, upregulating the expression and production of pro-inflammatory cytokines such as IL-1β, TNF-α, MMP-3, MMP-13, and PGE_2, as shown in previous equine OA research.[106,119,196,414] In our study, stimulation with IL-1β upregulated expression of IL-1β, IL-6, IL-8, MMP-3 and MMP-13 in synovial membrane, but expression of TNF-α was only one that was significantly upregulated at 9 days of culture, observing also significant differences between SF and ES groups. Considering that the primary production of TNF-α has been attributed to the synovial membrane,[209] differences found between culture media could have a significant biologic impact in the study of OA *in vitro.* Similarly, differences found in the expression of ADAMTS-4 and 5, the main catabolic enzymes responsible for aggrecan cleavage in OA disease,[101,102] could potentially change the course of OA development According to our results, this seems to be more critical after 9 days of culture.

Proteoglycans constitute an essential part of the extracellular matrix of articular cartilage, which can resist mechanical loading. The main proteoglycan in articular cartilage is aggrecan, accounting for around 85% of all proteoglycans.[562] The gradual upregulation in the aggrecan expression observed in the ES group during the duration of the study could be interpreted as an attempt by the articular cartilage to repair itself from the damage brought on by IL-1β, with the addition of 10% ES favoring the repair compared to SF media.

LDH quantification is utilized as a nonspecific marker of cytotoxicity and cellular death. LDH activity increased after longer culture times in both groups, suggesting cellular death after longer culture times with IL-1β, which is not unexpected. Cellular viability was not affected by the lack of serum supplementation. A study found that human chondrocytes establish protective mechanisms against reactive oxygen species via interaction with synoviocytes detecting lower LDH concentration in co-cultures than chondrocytes cultured in monoculture.[563] It is unknown if the co-culture system could produce a cellular protective effect or how supplementation with serum could affect the equine synovial membrane or articular cartilage explants individually, and further research is needed.

As GAGs are released from the extracellular matrix during cartilage catabolism, higher GAG concentrations are expected with more moderate to severe cartilage damage.[564] In a previous study, stimulation with IL-1β did not produce an increased concentration of GAG in media compared to the unstimulated group.[119] It is unknown if the increased GAG concentration in the SF group on day 3 in our study is related to the IL-1β or peripheral cellular death in the explants that occurs after harvesting, but it seems that ES media could protect the cartilage matrix at this time better than SF media.

This study had several limitations. The low (n=5) number of horses included in the study could have hidden small differences among groups. This study primarily focuses on the modification of gene expression within explants. Ideally, changes in protein expression would be correlated with protein concentration in the media to validate the importance of changes in gene expression observed. An unstimulated group should have been included to evaluate the changes in GAG and LDH in cultures without IL-1β stimulation. In our study, IL-1β was the only cytokine used to produce an inflammatory response, while other *in vitro* studies have used a

combination of IL-1β and TNF-α.[196,207] This could produce a different inflammatory response and account for differences in observed results.

In conclusion, chondroprotective effects were observed with the addition of 10% ES to culture media compared to SF media, although cellular viability was maintained equally in both conditions. This could play an important role when studying orthobiologic therapies *in vitro*. In particular, blood-derived products such as platelet-rich plasma, autologous conditioned serum, or autologous protein serum contain an increased concentration of growth factors and anti-inflammatory cytokines,[524] similar compositional characteristics of equine serum used to supplement culture media. Therefore, ES in the culture media could confound the study of the cellular response to blood-derived products in *in vitro* models of OA. According to our results, SF media was able to maintain proper tissue viability as the ES group and did not downregulate the gene expression of pro-inflammatory cytokines or aggrecanases as ES did, possibly establishing a stronger inflammatory state and OA stimulation in a long-term co-culture system. Use of SF media to study OA may produce a harsher inflammatory response and more effective OA establishment *in vitro*. Beside SF did not provide addition of growth factors and cytokines as ES could and it would avoid interferences with the study orthobiologic therapies

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Chapter 6

Effects of autologous conditioned serum, autologous protein solution, and triamcinolone on inflammatory and catabolic gene expression in equine cartilage and synovial explants treated with IL-1β in co-culture

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Abstract

Autologous conditioned serum (ACS) and autologous protein solution (APS) are newer therapeutic options for osteoarthritis (OA). Co-culture of cartilage and synovium stimulated with IL-1β produces a similar physiologic response to tissues from naturally-occurring OA. The study objective was to investigate the effects of ACS, APS, and triamcinolone (TA) on inflammatory and catabolic gene expression of inflamed joint tissues in co-culture. Blood was collected and processed for ACS and APS from six horses. Cartilage and synovial explants were harvested from the stifle, placed in co-culture, and treated as: (1) unstimulated control (2) stimulated control (3) ACS at 25% v/v (4) ACS at 50% v/v (5) APS at 25% v/v (6) APS at 50% v/v, (7) TA (10^{-6} M). Treatment groups 2-7 were stimulated with IL-1β (10 ng/ml). Cultures were maintained for 96 hours, and then both media and explants were harvested for measurement of gene expression and protein. IL-1β stimulation significantly increased IL-1β ($p = 0.029$), IL-8 ($p = 0.011$) and MMP-3 ($p = 0.043$) expression in synovium and IL-1β ($p = 0.003$) and TNF-α ($p = 0.001$) expression in cartilage. Treatment with 50% ACS and APS v/v downregulated IL-1β expression in cartilage more than TA treatment ($p = 0.001$ and $p = 0.0004$) and APS downregulated MMP-1 expression in synovial membrane ($p = 0.025$). Treatment with ACS and APS caused a trend in upregulation of IL-10 expression in synovium and type II collagen and aggrecan expression in cartilage. PGE_2 media concentrations were significantly reduced following treatment with APS (13.7-fold decrease, $p = 0.0001$) and ACS (4.13-fold decrease, $p = 0.024$); while TA did not reduce PGE_2 significantly (2.3-fold decreased $p = 0.406$). As disease-modifying therapies, ACS and APS modified the cellular response from synovial membrane and articular cartilage. ACS and APS may offer an improved strategy to improve clinical signs of horses with naturally occurring OA, compared to TA treatment.

Introduction

Lameness due to osteoarthritis (OA) is a leading cause of reduced or lost performance in horses, placing a significant economic hardship on the equine industry.[1,188] OA not only affects equine athletes, it has been shown to affect more than 80% of the equine geriatric population.[189] Currently, the mainstay of intra-articular OA therapy is modifying the symptoms of disease through temporary reduction of inflammation via administration of corticosteroids with or without viscosupplementation.[311]

The most commonly used corticosteroid by equine practitioners in high-motion joints is triamcinolone acetonide (TA).[311] TA has been shown to be chondroprotective in *in vitro* studies,[203,335,565] however, there is still concern about the effects of repeated, long term use of TA and other corticosteroids on cartilage. Therefore, intra-articular biologics may be preferred over corticosteroids when cost is not an issue or horses have become nonresponsive to corticosteroid treatment.[311] Several blood-derived orthobiologic products targeted at disease modification, such as autologous conditioned serum (ACS) and autologous protein solution (APS), are expanding the therapeutic options for clinicians treating horses with joint-related injury. Both products are obtained from the patient's blood and administered directly into the affected joint(s) for the treatment of OA. The cellular and protein profile of ACS and APS have been characterized independently in several studies.[397,398,429] Currently, only one study has compared the anti-inflammatory cytokine and growth factor concentration in ACS and APS collected from the same horse, finding APS had higher concentrations of TGF-β.[196]

Previous publications have demonstrated clinical improvement in lameness of horses treated with ACS or APS,[429,566] however, there is still little information of how they affect the cellular response in OA joints compared to corticosteroids. Synovial and cartilage explants

cultured together have physiologic responses that closely resemble OA tissues in situ.[119,207] Comparing the effect of TA to orthobiologic products (ACS and APS) using a co-culture model may provide a better understanding of their effect in clinical cases.

The study objectives were: (1) To compare the cellular composition and concentration of important cytokines and growth factors within ACS and APS from the same individual horse, and (2) to investigate the effects of ACS, APS, and TA on inflammatory and catabolic gene expression in an IL-1β stimulated cartilage and synovial membrane co-culture model of OA. Our hypotheses were: 1) ACS and APS obtained from the same horses will have a different cellular and cytokine profile, 2) IL-1β would produce an inflammatory response in the co-cultured articular cartilage and synovial tissue, 3)TA would reduce expression and production of inflammatory proteins more effectively than orthobiologics (ACS and APS), but orthobiologics would protect matrix gene expression more effectively than TA. Gaining a better understanding of how these therapies work may help veterinarians make informed decisions on the use of these products to treat joint disease in horses.

Materials and Methods

Subjects

This study was performed in accordance with Institutional and NIH guidelines for the Care and Use of Laboratory Animals, and the study was approved by the Institutional Animal Care and Use Committee (IACUC) at Auburn University. Six adult American Quarter horses (1 mare and 5 geldings, aged 14.6 ± 4.99 years) free of systemic disease and euthanized for reasons unrelated to the study were used. Horses with history of lameness related to the stifle and/or

stifle effusion were excluded from the study. Horses were deemed systemically healthy by physical examination and complete blood count.

Orthobiologic products preparation

Blood was collected aseptically and processed according to the manufacturer's instructions to produce ACS (Orthokine®; Overland Park, KS), and APS (Pro-Stride®; Owl Manor, Warsaw IN).

For ACS, 60 mL of blood was aseptically collected from the jugular vein twenty-four hours prior to euthanasia into an ACS syringe containing $CrSO_4$-treated glass beads from the jugular vein. Blood was incubated at 37°C for 24 hours then centrifuged at 3,000 RCF for 10 min and serum collected. A 3 mL aliquot of ACS was kept at 4°C after processing until use with culture media.

For APS, 104 mL of blood was aseptically collected into two syringes (52 mL of blood in each syringe) containing acid citrate dextrose (ACD-A) (Citra Labs, Baintree, MA) (8 mL in each syringe). Following collection, the blood was transferred to the APS separator and centrifuged (Owl Manor centrifuge, Owl Manor, Warsaw IN) at 3,200 RPM for 15 min. Platelet-poor plasma was removed, and the platelet–rich cell solution was transferred to the APS concentrator containing polyacrylamide beads and centrifuged at 2,000 RPM for 2 min. The volume obtained from one of the kits (3 mL) was kept at 4°C until use with culture media. The remaining product for both ACS and APS was aliquoted, snap-frozen, and stored at -80°C for further analysis.

Cellular, cytokine, and growth factor analysis of ACS and APS

The concentration of white blood cells (WBCs), red blood cells (RBCs), and platelet (PLT) counts were measured in blood as well as ACS and APS by hematologic analyzer (ADVIA® 120 Hematology System, Siemens). ELISA analysis was performed using commercially available kits (R&D Systems, Minneapolis, MN), previously validated in horses, for growth factor (TGF-β), anti-inflammatory (IL-1rap, and sTNF-R1), and pro-inflammatory (IL-1β, TNF-α, and MMP-3) cytokines.[237,371,567,568] Standards provided for the ELISA were used to prepare a standard curve following manufacturer's instructions. Samples were not diluted to measure IL-β and IL-1rap, while they were diluted at 1:40 to measure TGF-β, 1:4 to measured TNF-α and sTNF-R, and 1:10 to measure MMP-3. Cytokine measurements were performed in triplicate.

Synovial membrane and tissue harvesting

Following euthanasia, synovial membrane and articular cartilage were aseptically harvested from the femoropatellar and femorotibial joints. Tissues from horses with gross signs of osteoarthritis including cartilage erosion, score lines, discoloration, or fibrillation[553] were not used. Also, the synovial membrane was evaluated for gross signs of synovitis such as hyperemia, hypertrophy or fibrosis, and horses showing any of these changes were not included in the study. A 4 mm diameter disposable biopsy punch (Integra, Saint Priest, France) was used to obtain the cartilage and synovial membrane explants. Twenty-eight cartilage explants were obtained from the medial and lateral femoral condyles of each horse. The synovial membrane was dissected from the fibrous joint capsule, and 36 explants were obtained.

Co-culture

A co-culture was created by adding a hanging insert (WVR, Radnor, PA) containing 2 cartilage explants overtop of 3 synovial membrane explants in a 12-well culture plate. This ratio was calculated based on the ratios described for humans and mice, where synovial tissue has been shown to be 1.3x more plentiful than the articular cartilage surface in the synovial environment.[554] For each treatment group, co-cultures were plated in duplicate. Cultures were maintained for 2 hours under standard culture conditions with Dulbecco's Modified Eagle Medium (high glucose, 4500 mg/L) with L-glutamine and sodium bicarbonate, free of sodium pyruvate (BioWhittaker; Lonza, Basel, Switzerland), supplemented with streptomycin (100 µg/ml) and penicillin (100 µg/mL) with 10% equine serum to allow tissue acclimatization. This short acclimatization was chosen so that the orthobiologics were not subject to cryopreservation prior to treatment. Incubation was maintained at 37°C and 5% CO_2 room air incubator, in culture media as defined above. After 2 hours, culture media was removed, tissues were rinsed with phosphate-buffered saline, and replaced in co-culture. Media was added to the culture according to the following conditions: (1) unstimulated control, (2) stimulated control, (3) ACS at 25% v/v (4) ACS at 50% v/v, (5) APS at 25% v/v, (6) APS at 50% v/v, (7) TA (10^{-6} M) (Richardson and Dodge, 2003). Groups 2-7 were stimulated with interleukin-1β (10 ng/ml) (R&D Systems, Minneapolis, MN). Cultures were maintained at 37°C with 5% CO_2 for 96 hours. At study termination, media was snap-frozen and stored at -80°C for later analysis. Cartilage and synovial explants were removed from the culture, rinsed in phosphate-buffered saline, snap-frozen in liquid nitrogen, and stored at -80°C.

PGE_2 concentrations in culture media

A commercially available ELISA assay for PGE_2 (R&D Systems, Minneapolis, MN) was used to measure PGE_2 concentration in culture media according to manufacturer's instructions. This colorimetric assay was not equine-specific; however, it has been previously referenced and validated for cross-reactivity in equine samples.[125,237,251] Standards provided for the ELISA were used to prepare a standard curve following manufacturer's instructions. Media samples were diluted at 1:30, and PGE_2 measurements performed in triplicate.

Gene expression in cartilage and synovial membrane

Frozen cartilage samples were added to TRIzol reagent (Invitrogen, Carlsbad, CA) then pulverized with a tissue homogenizer (Polytron, Thomas Scientific, Swedesboro, NJ). Synovial membrane samples were homogenized in TRIzol using a bead homogenizer (TissueLysser LT, Qiagen, Germantown, MD) for 20 min. To isolate RNA, a chloroform extraction protocol was performed. Briefly, 200 µl of chloroform were added to the samples, mixed vigorously, and incubated at room temperature for 10 min. The mix was centrifuged at 17,000 RCF for 15 min at 4°C. The supernatant was saved (approximately 500 µl), while the remaining pellet was discarded. Equal parts of isopropanol were added and incubated at -20°C for 15 min followed by a centrifugation at 17,000 RCF for 20 minutes. The supernatant was decanted, and 1 mL of 75% cold ethanol was added and spun at same speed for 10 min. The supernatant was decanted again, and once the ethanol was completely evaporated; the pellet was re-suspended on 40 µl of nuclease free water. Nucleic acid concentrations were determined using a spectrophotometer at 260/280 nm (DeNovix, Wilmington, DE). RNA was stored at -80°C until qPCR analysis. RNA was reverse transcribed to cDNA using iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad,

Hercules, California). Relative gene expression of IL-1β, MMP-1, MMP-3, MMP-13, IL-6, IL-8, IL-10, and ADAMTS-4 (in synovial membrane) and type II collagen (COL2A1), aggrecan (ACAN), TNF-α, IL-1β, and ADAMTS-4 (in articular cartilage) was calculated. All primers were derived from the Equus caballus genome (GenBank) and designed using the NCBI-Primer-BLAST (Table 6.1).

Primer efficiencies were determined using 2-fold dilutions of cDNA and efficiencies calculated for all the primers ranged between 94-102.5%. All the qPCR experiments were performed in triplicate using SYBR Green Master Mix (PerfeCTa SYBR Green FastMix, Quantabio, Beverly, Massachusetts). The thermocycler (CFX 96 Thermocycler Bio-Rad Hercules, California) was heated at 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 55°C for 30 s, and 70°C for 30s, followed by a melting curve analysis. The relative gene expression was calculated by the comparative threshold cycle method (ΔΔCt method). Reference genes used were 18s and SCAMP3 for synovial membrane and GAPDH and SCAMP3 for articular cartilage. These genes were selected by evaluating the stability of various reference genes with equine tissue (18-S, β2M, GAPDH, SDHA, HPRT1, SCAMP-3, and β-actin). ΔΔCt values for all these genes were calculated under different stimulatory conditions in the 6 horses and the two genes for each tissue with the least amount of change in gene expression were chosen.[555] Differences in gene expression were determined as fold change of relative gene expression of the control tissues compared to the IL-1β stimulation group and IL-1β stimulation group compared to treatment groups.

Table 6.1. Equine primer sequences used for gene expression analyses.

	Primer sequence	
18 small ribonucleic acid (18S)	Forward	5′- GCCGCTAGAGGTGAAATTCT-3′
	Reverse	5′- TCGGAACTACGACGGTATCT−3′
Secretory Carrier Membrane Protein 3 (SCAMP 3)	Forward	5′-CTGTGCTGGGAATTGTGATG-3′
	Reverse	5′-ATTCTTGCTGGGCCTTCTG-3′
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Forward	5′-TGTCATCAACGGAAAGGC-3′
	Reverse	5′-GCATCAGCAGAAGGAGCA-3′
Interleukin-1 β (IL-1β)	Forward	5′-GCGGCAATGAGAATGACCTG-3′
	Reverse	5′-AGCCACAATGATTGACACGA-3′
Interleukin-6 (IL-6)	Forward	5′-AACAGCAAGGAGGTACTGGCA-3′
	Reverse	5′-CAGGTCTCCTGATTGAACCCA-3′
Interleukin-8 (IL-8)	Forward	5′-AGGGACAGCAGAGACAGAGACACAAG-3′
	Reverse	5′-TACAACCGCAGCTTCACACA-3′
Interleukin-10 (IL-10)	Forward	5′-GCCTTGTCGGAGATGATCCA-3′
	Reverse	5′-TTTTCCCCCAGGGAGTTCAC-3′
Matrix metalloproteinase (MMP-1)	Forward	5′-GGTGAAGGAAGGTCAAGTTCTGAT-3′
	Reverse	5′-AGTCTTCTACTTTGGAAAAGAGCTTCTC-3′
Matrix metalloproteinase 3 (MMP- 3)	Forward	5′-GGCAACGTAGAGCTGAGTAAAGCC-3′
	Reverse	5′-CAACGGATAGGCTGAGCACGC-3′
Matrix metalloproteinase 13 (MMP-13)	Forward	5′-GTCCCTGATGTGGGTGAATAC-3′
	Reverse	5′-ACATCAGACAAACTTTGAAGG-3′
Tumor necrosis factor (TNF-α)	Forward	5′-AAAGGACATCATGAGCACTGAAAG-3′
	Reverse	5′-GGGCCCCCTGCCTCCT-3′
ADAM metallopeptidase with thrombospondin type 1 motif 4 (ADAMTS-4)	Forward	5′-GCTGTGCTATTGTGGAGGATGATGG-3′
	Reverse	5′-CCAGGGAAAGTCACAGGCAGATG-3′
Aggrecan (ACAN)	Forward	5′-CCTTGACTCCAGTGGTCTTATC-3′
	Reverse	5′GTCGTGGACCACCTAATTCTATC-3′
Type II collagen (COL2)	Forward	5′-GCCCGTCTGCTTCTTGTAATA-3′
	Reverse	5′-CGTGACTGGGATTGGAAAGT-3′

Statistical analysis

All gene expression data were naturally log-transformed prior to analysis to equalize variances. Linear mixed models were used to analyze cytokine, growth factor, and cellular concentrations as well as gene expression. The full model for each concentration or gene expression variable included a fixed factor for condition and a random estimate for each horse. The random intercept for each horse accounted for within horse correlation. Model residuals were examined to evaluate the assumption of normality. Multiple comparisons were adjusted and analyzed using Tukey's test. Satterthwaite degrees of freedom method and restricted maximum likelihood (REML) estimation were used to evaluate significance. A Pearson correlation test was performed to evaluate the correlation between the cellular composition and cellular proteins. All analyses were performed using SAS V 9.4 (Cary, NC). Significance was set at $p < 0.05$.

Results

Cellular, cytokine, and growth factor analysis of ACS and APS

The cellular composition of peripheral blood, ACS and APS varied significantly between products (Table 6.2). ACS had significantly lower RBC (p =0.001), and lower, but no significantly lower WBC and PLT counts compared to blood (p = 0.2803 and p = 0.140). APS had significantly greater WBCs ($p < 0.001$) and PLTs (p = 0.001), but lower RBCs (p <0.001) compared to peripheral blood. APS had significantly greater WBCs ($p < 0.001$), RBCs (p = 0.0214) and PLTs ($p < 0.001$) compared to ACS.

Table 6.2. Summary of the cellular components of ACS and APS

Cellular component	Blood	ACS	ACS:Blood ratio	APS	ACS:Blood ratio
WBC count ($x10^6$/ml)	**7.57 ± 1.01**	**0.06 ± 0.04**	**0.008**	**35.37 ± 14.16 *#**	**4.9**
Platelet count ($x10^6$/ml)	**182.67 ± 56.53**	**4.5 ± 1.87**	**0.02**	**647.5 ± 257.65 *#**	**3.5**
RBC count ($x10^6$/ml)	**8.44 ± 0.83**	**0.01 ± 0.01 ***	**0.001**	**0.9 ± 0.29 *#**	**0.1**

* Significant $p < 0.005$ difference from blood values # Significant $P < 0.005$ difference from ACS

Few differences in cytokines and growth factors were observed between ACS and APS (Figure 6.1). Variability between horses in cytokines and growth factor concentrations was observed, but this variability between horses was only significantly different for TNFα (p = 0.001). No significant differences in concentrations of IL-1β, TNFα, MMP-3, and IL-1rap between products were observed. However, TGF-β (p = 0.009) and sTNF-R1 (p = 0.010) were significantly increased in ACS compared to APS. When the ratio of IL-1rap: IL-1β ratio was evaluated for each individual horse, ACS (113.31 ± 78.98) had a higher ratio compared to APS (48.22 ± 78.98), but this difference was not significant (p = 0.401). Positive and negative correlations between cytokines and cellular components were found (Table 6.3).

Table 6.3. Summary of the significant correlations between cytokines, growth factor and cellular components.

Cellular, cytokine, and growth factor components correlation	Pearson's correlation coefficient	P- value
IL-1β and MMP-3	0.5088	$p = 0.0311$
IL-1β and TGF-β	0.7018	$p = 0.0110$
WBC and TGF-β	-0.6186	$p = 0.0320$
PLTs and TGFβ	-0.6861	$p = 0.0138$
PLTs and sTNF-1R	-0.6573	$p = 0.0202$

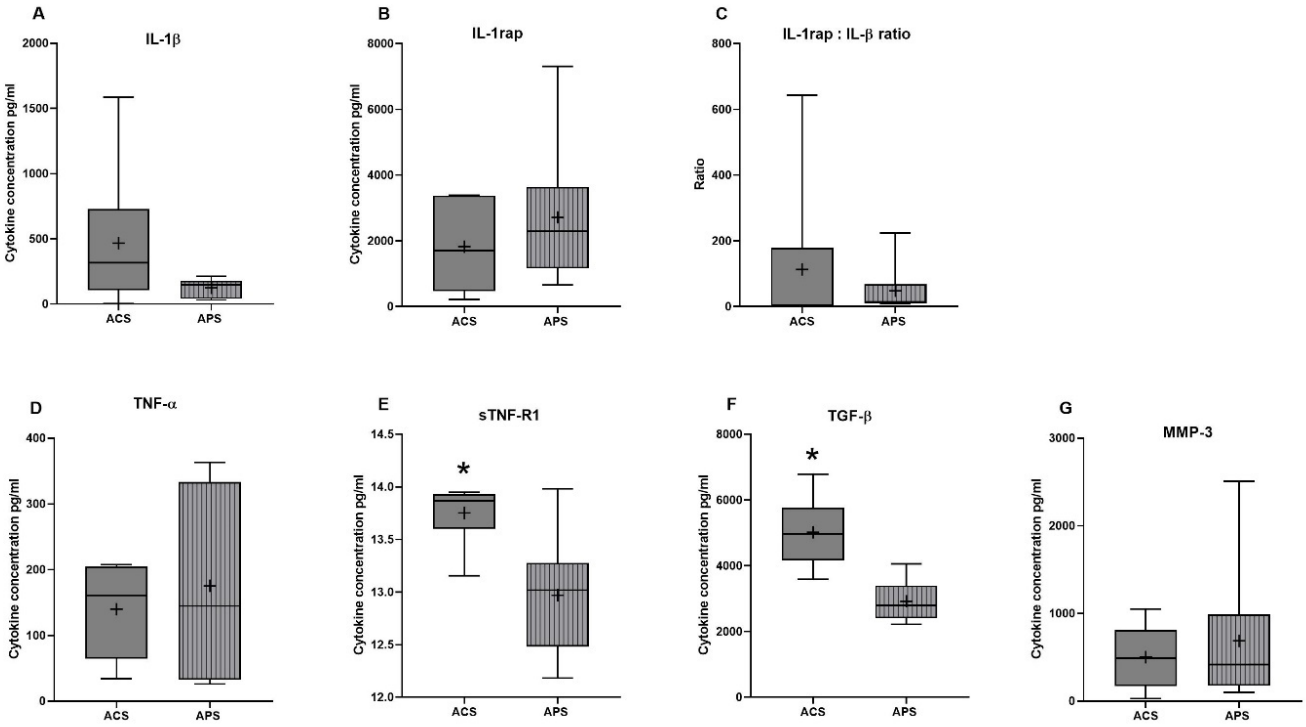

Figure 6.1. ACS and APS concentrations of (A) IL-1β (pg/ml), (B) IL-1rap (pg/ml), (C) IL-1rap: IL-1β ratio, (D) TNF-α (pg/ml), (E) sTNF-R1 (pg/ml), (F)TGF-β (pg/ml), and (G) MMP-3 (pg/ml). The boxplots represent the interquartile range (IQR) of n = 6. The black lines represent the median values, the crosses represent the mean, and the whiskers represent the values outside the IQR. *Denotes significant difference between ACS and APS, $p < 0.05$.

PGE_2 concentrations in co-culture media

All treatments reduced PGE_2 concentrations in the co-culture media compared to stimulated controls (Figure 6.2). PGE_2 concentration increased 4.7-fold ($p = 0.028$) after stimulation with IL-1β. PGE_2 concentration was reduced but not significantly changed after TA treatment ($p = 0.111$), while media with ACS at 25% v/v and 50% v/v, decreased PGE_2 concentration by 4.13-fold ($p = 0.037$ and $p = 0.038$ respectively). APS caused a dose dependent reduction in PGE_2 concentrations following IL-1β stimulation, with a 7.7-fold reduction in PGE_2 at 25% v/v ($p = 0.019$), and a 13.8-fold reduction in PGE_2 at 50% v/v ($p = 0.016$). In summary, APS 50% v/v was the most effective, while TA was the least effective at reducing PGE_2.

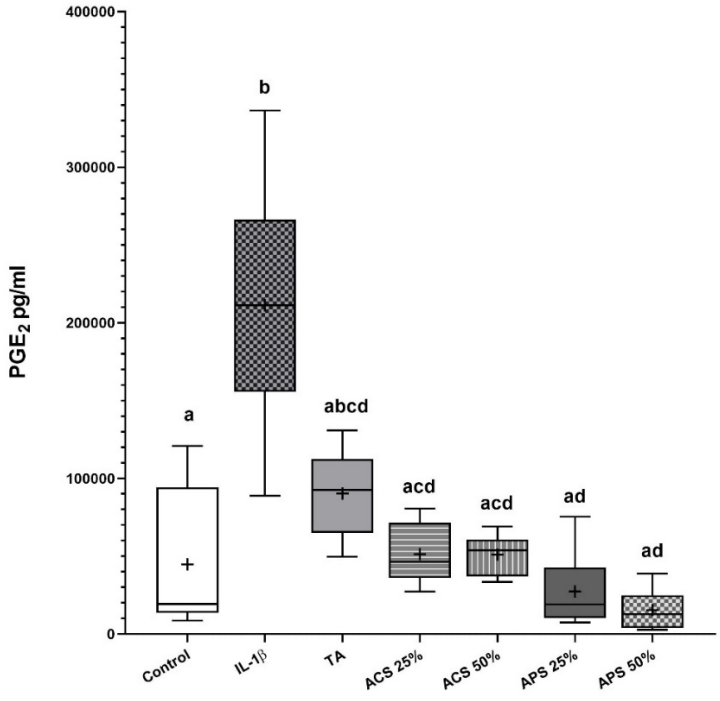

Figure 6.2. PGE2 concentrations in the co-culture media 96 hours after treatment. The boxplots represent the interquartile range (IQR) of n = 6. The black lines represent the median values, the crosses represent the mean, and the whiskers represent the values outside the IQR. Different letters denote significant differences between groups, $p < 0.05$.

Gene expression

Gene expression was assessed in synovial membrane and articular cartilage after 96 hours of co-culture. Stimulation with IL-1β upregulated IL-1β ($p = 0.029$), IL-8 ($p = 0.011$), and MMP-3 ($p = 0.043$) in synovial membrane and IL-1β ($p = 0.003$) and TNF-α ($p = 0.005$) in articular cartilage compared to the control groups (Figure 6.3).

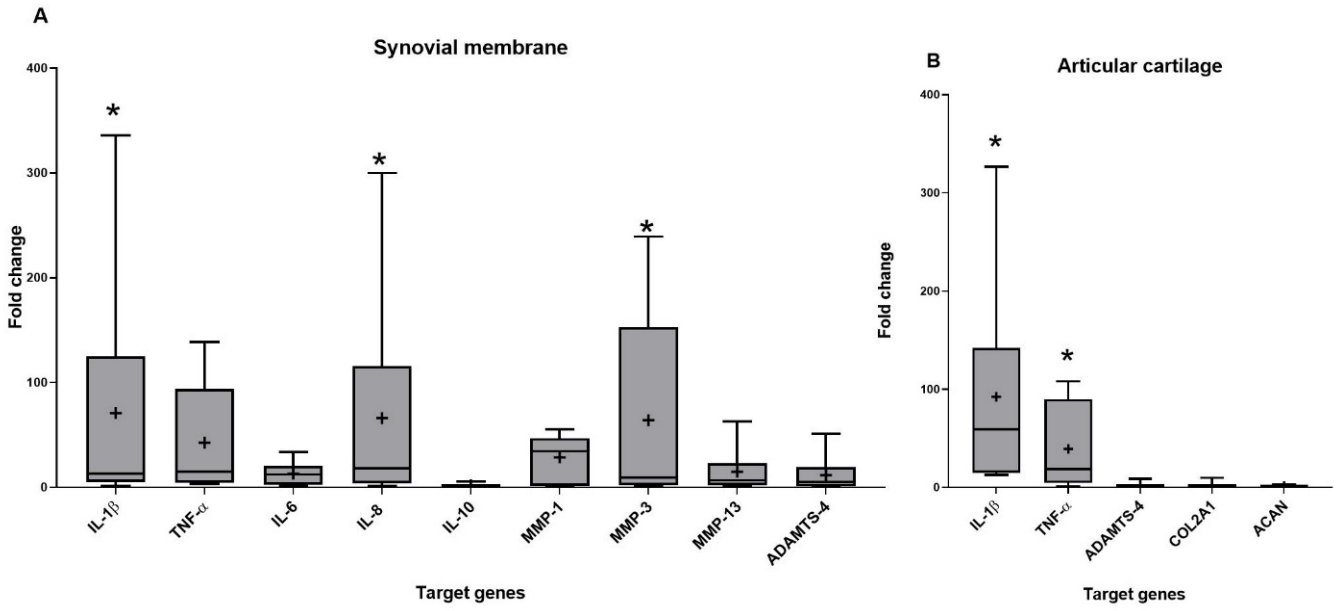

Figure 6.3. Fold change in relative gene expression comparing unstimulated tissues versus tissues stimulated with IL-1b in (A) synovial membrane tissue (B) articular cartilage tissue. The boxplots represent the interquartile range (IQR) of n = 6. The black lines represent the median values, the crosses represent the mean, and the whiskers represent the values outside the IQR. *Denotes significant difference between control group and stimulated IL-1b group gene expression, $p < 0.05$.

In synovial membrane, TA was the only treatment that reduced IL-1β expression significantly ($p = 0.011$) and trended to reduce the expression of IL-6 ($p = 0.402$) more effectively than ACS ($p = 0.780$) or APS ($p = 0.601$). APS at 50% v/v significantly reduced expression of the matrix degrading enzyme MMP-1 ($p = 0.025$), and showed a trend to reduce MMP-3 ($p = 0.275$), MMP-13 ($p = 0.140$), and ADAMTS-4 ($p = 0.158$) expression. Treatment with ACS and APS showed a trend to upregulate IL-10 gene expression more effectively than TA ($p = 0.670$ and $p = 0.452$ respectively), but did not downregulate IL-6 as effectively as TA ($p = 0.402$) (Figure 6.4).

In articular cartilage, ACS and APS treatments caused significant downregulation of IL-1β expression ($p = 0.001$), with10-fold greater downregulation than TA ($p = 0.002$) compared to the stimulated control group. In addition, treatment with 50% v/v ACS and APS downregulated TNF-α gene expression ($p = 0.014$ and $p = 0.002$, respectively). ACS and APS showed a trend toward upregulation of ACAN ($p = 0.549$ and $p = 0.529$ respectively) and COL2A1 ($p = 0.678$ and $p = 0.526$ respectively). Treatment with ACS and APS trended toward upregulation of ADAMTS-4 expression ($p = 0.309$ and $p = 0.315$ respectively). A dose-effect was observed with ACS and APS treatment, with 50% v/v treatment producing greater effect on the expression of TNF-α, ADAMTS-4, COL2A1 and ACAN versus 25% v/v (Figure 6.5).

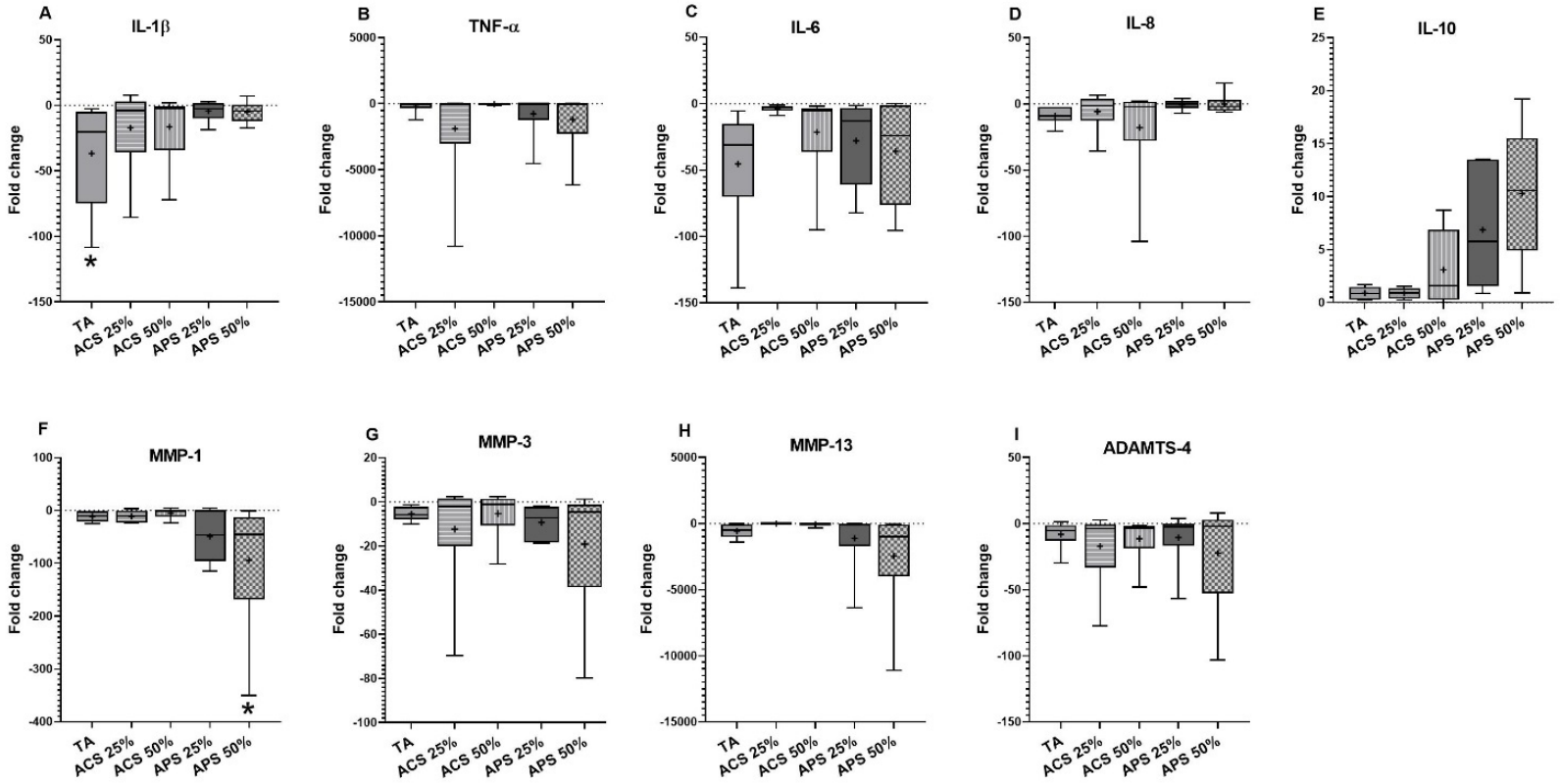

Figure 6.4. Fold change in relative gene expression comparing IL-1b stimulated synovial membrane with IL-1b stimulated tissue treated with triamcinolone (TA), autologous conditioned serum (ACS) and autologous protein solution (APS) of (A) TNF-a, (B) IL-1b, (C) IL-8, (D) IL-6, (E) IL-10, (F) MMP-1, (G) MMP-3, (H) MMP-13, and (I) ADAMTS-4. The boxplots represent the interquartile range (IQR) of n = 6. The black lines represent the median values, the crosses represent the mean, and the whiskers represent the values outside the IQR. *Denotes significant difference between stimulation IL-1b group and treatment, $p < 0.05$.

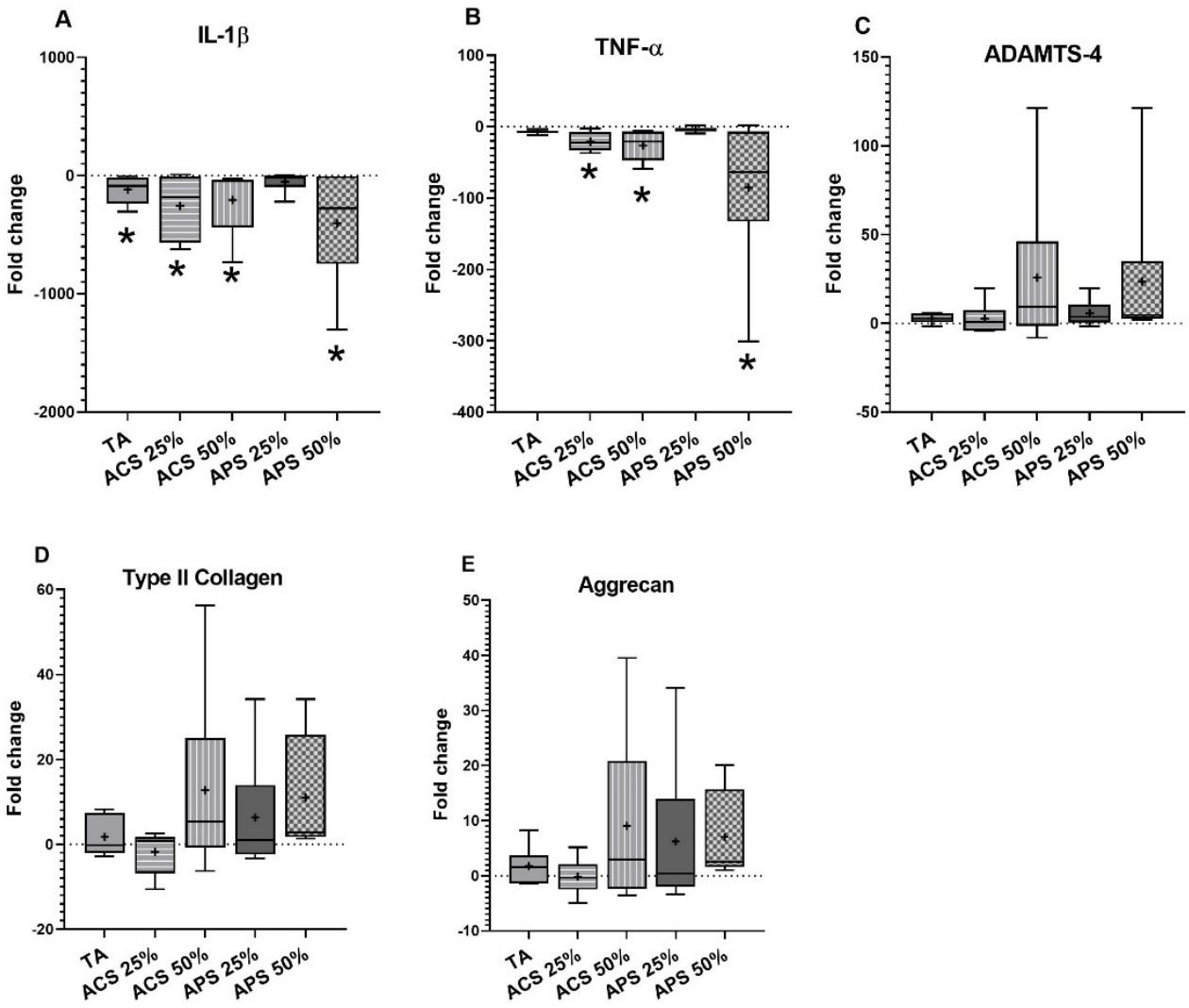

Figure 6.5. Fold change in relative gene expression comparing IL-1b stimulated articular cartilage with IL-1b stimulated tissue treated with triamcinolone (TA), autologous conditioned serum (ACS) and autologous protein solution (APS) of (A) TNF-a, (B) IL-1b, (C) ADAMTS-4, (D) COL2A1, and (E) Aggrecan. The boxplots represent the interquartile range (IQR) of n = 6. The black lines represent the median values, the cross represents the mean, and the whiskers represent the values outside the IQR. *Denotes significant difference between stimulation IL-1b group and treatment, $p < 0.05$.

Discussion

This is the first study comparing corticosteroids to orthobiologic therapies in an equine *in vitro* co-culture model of OA. As we hypothesized, TA was more efficient at downregulating IL-1β expression in the synovial membrane. Although not significant, ACS and APS produced an upregulation of important matrix proteins, COL2A1 and ACAN, and downregulation of inflammatory genes, IL-1β and TNF-α in articular cartilage, changes that might offer protection of the articular cartilage. ACS and APS also modified the inflammatory response by increasing the gene expression of the anti-inflammatory cytokine IL-10 and decreasing the concentration of PGE_2. Additionally, APS downregulated the expression of MMP-1 in synovial membrane, which is one of the main collagenases produced primarily by the synovial cells.[98]

Orthobiologic treatments, ACS and APS, aim to modify the inflammatory cascade to reduce cartilage destruction and improve endogenous repair mechanisms in OA. ACS has been shown to have disease-modifying properties in human and equine studies producing an improvement in clinical signs and modification of the cellular response.[237,414,415,569] Recently, an *in vivo* study found that treatment of ACS produced a disease-modifying effect decreasing cartilage biomarkers in horses with advanced OA.[408] Clinically, a single injection of APS improved pain scores up to one year and reduced osteophyte formation in people.[438,570,571] In horses and dogs, APS has improved pain scores and reduced lameness up to one year after treatment.[429,572] *In vitro*, limited anti-inflammatory effects have been observed following treatment of articular cells and/or tissues with APS.[88,196,433]

ACS and APS differ in their processing methods, targeting different blood components for the concentration of cells, platelets, and proteins. The current study identified significant differences in cellular composition between ACS and APS, but few significant differences in

measured cytokines and growth factors were identified. APS had higher WBCs compared to ACS and blood. The WBC, RBC, and PLT concentration of APS compared to blood has been previously reported in the horse.[429] The results of our study showed a smaller increase in WBCs (12.1 vs. 4.9-fold increase) and a greater increase in PLTs (1.6 vs. 3.5-fold increase) compared to what has been reported by Bertone et al., most likely due to individual variations in physiologic status. In our study, ACS produced a significantly higher concentration of sTNF-R1 ($p = 0.009$) and TGF-β ($p = 0.024$), compared to APS, while another publication found that APS produced a higher concentration of TGF-β compared to ACS.[196] Significant differences in cytokine concentration using different commercial kits under different physiologic conditions from the same horse has been reported with ACS,[397,398] which could explain differences between studies.

Co-culture of IL-1β stimulated cartilage and synovium has been shown to produce tissue related changes that resemble changes in tissues from joints with natural, ongoing OA compared to monoculture of synovial cells and/or tissues.[119,207] The effects of ACS and APS have been previously studied in IL-1β stimulated chondrocytes, where APS resulted in an increased concentration of chondroprotective cytokines (IL-1rap and IL-10) compared to ACS treatment. However, in that study, orthobiologics were not compared to the standard articular treatment, corticosteroids.[196]

Stimulation with IL-1β produced an inflammatory response, upregulating expression of IL-1β, TNF-α, IL-8 and MMP-3 in synovial membrane and IL-1β and TNF-α in articular cartilage as shown in other studies.[106,119,196,414] In our study, TA significantly downregulated IL-1β expression in synovial membrane and articular cartilage, as well as a trend to downregulate IL-6 in synovial membrane but did not reduce expression of other inflammatory genes such as TNFα and IL-8. ACS and APS downregulated the expression of IL-1β and TNF-α in articular

cartilage and showed a trend to upregulate COL2A1 and ACAN more effectively than TA. Additionally, APS produced a downregulation of MMP-1 and a trend for downregulation of matrix degrading enzymes in synovial membrane. Elevated MMP-1 expression has been measured in horses with OA,[573] and downregulation of this protein is essential to slow down the progress of OA disease.[574] The effect on the matrix gene expression of ACS and APS has not been evaluated previously. However, other disease-modifying effects have been reported. APS inhibited IL-1α and TNFα stimulated matrix degradation of bovine articular cartilage explants compared to direct recombinant antagonists (IL-1rap and sTNF-R1)[433] and downregulated MMP-13 in human chondrocytes stimulated with IL-1β and TNF-α.[88] In our study, both ACS and APS produced a trend to upregulate ADAMST-4 in articular cartilage, but not in synovial membrane. ADAMST-4 participates in aggrecan cleavage.[575-577] However other publications have found that ADAMTS-5 could have more significant effects on articular degradation.[578,579] Cartilage and meniscal explants cultured with double spin platelet-rich plasma (PRP) showed an upregulation of ADAMTS-4 compared to single spin PRP, suggesting that high platelet concentrations in PRP may produce a pro-inflammatory environment for cartilage.[580] In our study, ACS and APS both produced a similar trend to upregulate ADAMTS-4 expression in cartilage and downregulate it in the synovial membrane, despite the differences in platelet concentration. It is possible that cross-talk between tissues is occurring and changes in expression of this protein does not happen within these two tissues at the same time. No studies have evaluated the effect of orthobiologics on ADAMST-4 and 5 expression in long-term synovial tissue culture, further investigation in this direction is warranted.

PGE_2 is one of the primary pro-inflammatory mediators that promote catabolic destruction of articular cartilage as well as promotion of joint pain.[84] Our study showed that APS

reduced the production of PGE_2 in IL-1β stimulated tissues by 13-fold compared to the stimulated control group, while TA only reduced PGE_2 by 2.3-fold compared to the stimulated control group. *In vitro*, IL-1β stimulates PGE_2 production,[118,119] decreasing the expression of IL-1β could lead to decreased concentration of IL-1β induced PGE_2 production. Additionally, IL-10 is considered to be an essential anti-inflammatory cytokine participating in the downregulation of PGE_2.[137-139] Linardi et al. evaluated the effects of APS treatment on equine chondrocytes demonstrating enhanced IL-10, IL-1rap, and IL-6 production compared to ACS in a standard chondrocyte culture.[196] An association between a low PGE_2 concentration in culture media with upregulation of IL-10 gene expression was observed in ACS and APS. This correlation leads us to hypothesize that IL-10 upregulation produced by ACS and APS will produce a decreased production of PGE_2. In humans, PGE_2 has been shown to sensitize nociceptor neurons.[581] Therefore, downregulation of this protein may explain the clinical improvement in observed lameness in horses treated with ACS or APS.[237,408,429,582]

This study was limited by its ex vivo model as well as the inherent biologic variation in tissues and their response between horses. Variability between horses in inflammatory and anti-inflammatory mediators (cytokines and growth factors) was identified in ACS and APS, which led to unstandardized treatment between horses. However, this study was designed to reflect the clinical situation in which a horse's blood would be processed and used to treat their own joint, leading to a variable clinical response based on the composition of the biologic and degree of tissue pathology. The short-term model could be a disadvantage to fully understand how the cellular response to orthobiologic products changes with time. In humans, better clinical outcomes following treatment with APS have been observed 6-months post-treatment compared to 3 months post-treatment in patients with knee OA.[438] Therefore, a short-term model may not

fully explain the extent of modification that occurs with these products on the cellular and tissue response over time. This study primarily focuses on modification of gene expression produced. Ideally, changes in protein expression would be correlated with protein concentration in the media to validate the importance of changes in gene expression observed. In our study, IL-1β was the only cytokine used to produce an inflammatory response, while other *in vitro* studies have used a combination of IL-1β and TNF-α.[196,207] This could produce a different inflammatory response and account for differences in observed results.

In summary, TA downregulated the expression of IL-1β in synovial membrane, however, ACS and APS produced a stronger anti-inflammatory effect, modulating pro-inflammatory cytokines (IL-1β and TNF-α) involved in cartilage destruction in OA (Hedbom and Häuselmann, 2002).[583] ACS and APS, showed a chondroprotective effect by upregulating matrix gene expression, while TA treatment did not modify gene expression. Both ACS and APS significantly decreased PGE_2 in media compared to TA, which could be one of the reasons horses with naturally occurring OA show improvement in lameness after treatment with ACS or APS. Since cartilage is characterized by its poor intrinsic capacity for repair, treatments that slow down the degenerative response and increase the reparative response would be ideal in treatment of OA. Considering the results of our study, the significant PGE_2 reduction in media and the downregulation of pro-inflammatory cytokines, ACS and APS may provide important benefits in early stages of OA, slowing down the catabolic process occurring within the joint.

Acknowledgments

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Chapter 7

Clinical and biochemical effects of intra-articular autologous conditioned serum and triamcinolone in an equine model of synovitis

To be submitted to Frontiers in Veterinary Science.
Authors: Ana Velloso Alvarez, Lindsey Boone, Emily Graft, Sandra Zetterström, Bruno Menari, Fred Caldwell, John Schumacher, Anne Wooldridge.

Abstract

Synovial inflammation contributes to osteoarthritis (OA). Timely and effective treatment may result in OA modification. The study objectives were to induce synovitis *in vivo* with IL-1β within the fetlock joint and compare the effects of treatment with a corticosteroid (triamcinolone (TA)) to a blood-based orthobiologic (autologous conditioned serum (ACS)).

All four fetlocks of six healthy, adult horses were randomly assigned to treatment with the following: phosphate-buffered saline (PBS), IL-1β (100 ng), ACS, IL-1β+ACS, and IL-1β+TA. A two-week washout period was observed between treatments. Clinical signs such lameness, heat, swelling, and effusion scores were recorded for 72 hours following treatment. Synovial fluid was collected at 0, 8, 24, and 48 post-injection hours (PIH) for cytology analysis and measure PGE_2 and glycosaminoglycan concentrations. Horses injected with TA or PBS had lower heat, swelling, and effusion compared to IL-1β, ACS, and IL-1β+ACS groups ($P<0.05$). IL-1β+ACS produced a significant reduction in lameness compared to IL-1β at 24, 36, and 72 PIH ($P = 0.005$, 0.005, and 0.01 respectively) and to IL-1β+TA group at 36 and 72 hours ($P =$ 0.02 and 0.03). IL-1β+ACS had the highest total nucleated cell count (TNCC) (40,625 ± 11.01 cells/μL) and TP (3.73 ± 0.63 g/dl) within all groups. The injection of IL-1β produced an increase in PGE_2 concentration in synovial fluid at 8 hours ($P < 0.001$), but IL-1β combined with TA or ACS did not produce a peak in PGE_2 at 8 hours. The glycosaminoglycan (GAG) concentration in synovial fluid was significantly higher at 24 and 48 PIH ($P < 0.001$). Overall, TA was the most effective treatment to reduce joint effusion and swelling while ACS was most effective to reduce lameness induced by IL-1β. ACS produced the greatest increase in TNCC in the synovial fluid PIH 8, but did not produce an increase in release of GAGs observed with TA at 24 and 48 PIH. In conclusion, ACS treatment could offer benefits in early OA stages.

Introduction

Synovitis, plays a critical role in the development and progression of OA.[6,544,584] Cross-talk that occurs between the articular cartilage and synovial membrane during synovial inflammation plays an important role in promoting the production of pro-inflammatory cytokines and catabolic enzymes, which destroy the articular cartilage.[6]

Induced models of synovitis are essential to understanding of the innate immune response following injury in synovitis and ways this response could be modified to slow down disease progression. Currently, the mainstay of intra-articular therapeutics is focused on modifying the signs of disease through temporary reduction of inflammation via intra-articular administration of corticosteroids. However, there is some concern regarding the use of these medications and their potentially deleterious effects on the metabolism of the articular cartilage matrix.[585,586]

Given current understanding of the critical role that synovitis plays in the development of OA, treatment of synovitis should not only be directed to the modification of symptoms (i.e., lameness) but also be directed toward modification of the synovial environment to reduce disease progression. Synovitis presents an important condition of the synovial environment in which appropriate therapeutic intervention could have long-lasting effects on the health of the joint.

Autologous conditioned serum (ACS) is an intra-articularly administered biologic product derived from the patient's blood and used by many equine practitioners to treat OA. Incubation of blood with these activating surfaces (conditioning) results in increased production of important anti-inflammatory cytokines and growth factors [398] The conditioned serum contains increased concentrations of several anti-inflammatory cytokines and growth factors including interleukin-1 receptor antagonist protein (IL-1rap), interleukin-10 (IL-10), transforming growth

factor-β (TGF-β), and insulin-like growth factor (IGF-1).[397] These anti-inflammatory cytokines and growth factors have the potential to modulate the innate immune response of the synovium and support anabolic matrix metabolism of the articular cartilage.[6,11]

The objectives of this study were to induce a moderate but temporary model of synovitis within the metacarpal/metatarsophalangeal joints of six healthy horses using IL-1β and compare the clinical and biochemical effects of TA and ACS. The hypotheses were: 1) IL-1β would induce moderate and self-limiting synovitis in normal metacarpal/metatarsophalangeal joints (MCPJ/ MTPJ). Maximum severity was expected at 8 post-injection hours (PIH) with resolution within 24-36 PIH, and 2) ACS and TA would improve clinical signs of synovitis and protect the cartilage extracellular matrix to the same degree, but ACS would cause a stronger anti-inflammatory effect reducing the concentration of PGE_2 more efficiently than TA treatment.

Materials and Methods

Subjects

This study was performed in accordance with Institutional and NIH guidelines for the Care and Use of Laboratory Animals, and the study was approved by the Institutional Animal Care and Use Committee (IACUC) at Auburn University. Six adult male horses (3 Quarter horses, 2 Warmbloods and 1 Thoroughbred, aged 14.6 ± 4.99 years) free of systemic disease were used. Horses were not free of lameness, but lameness was not be localized to the metacarpal/metatarsophalangeal joints (MCPJ or MTPJ) based on negative response to fetlock flexion tests using an objective analysis of gait before and after flexion tests. Baseline lameness evaluations were performed and aided by an inertial sensor system (The Lameness Locator® by

Equinosis). Horses that exhibited a positive response to fetlock flexion based on subjective and objective assessment were excluded from the study.

Study Design

The study was a blinded, 5 sequence, 5 period, and 5-treatment crossover design. Each treatment period was defined as 72 hours, followed by at least a two-week washout period between study periods.[587] The first 4 randomized treatments included: phosphate-buffered saline (PBS, negative control), IL-1β induced synovitis (positive control), IL-1β induced synovitis treated with ACS, and IL-1β induced synovitis treated with triamcinolone acetonide (Fig. 1). Each fetlock received only one of the 1st 4 treatments at each treatment period. Sequence and treatment were randomly assigned using commercially available software. After analyzing raw data regarding the synovial response to treatments by an unblinded investigator (LB), a fifth treatment group consisting of 4 ml of ACS, was administered into a randomly selected fetlock from the same group of horses. This treatment was administered 3 months from the last study period. Investigators (FC, JS, AW, and LB were not blinded to treatment), while investigators that were evaluating subjects, obtaining lameness data, and evaluating synovial response (heat, effusion, circumference) were blinded to treatment (AV, SZ).

Autologous conditioned serum (ACS) preparation

ACS (Orthokine®; Overland Park, KS) was prepared according manufacturer's instructions. Briefly, blood was collected aseptically and processed according to the manufacturer's instructions to produce ACS (Orthokine®; Overland Park, KS). All the ACS was processed on each horse before starting the study to avoid any possible interference due to stress.

Briefly, 24 hours before initiation of the study (Period 1), horses were restrained within stocks. Sixty ml of blood were withdrawn aseptically into a commercially available syringe containing glass beads (Orthokine®; Overland Park, KS). Blood was incubated at 37°C for 24 hours, then centrifuged at 3,000 RCF for 10 min and serum collected. ACS was sterilely aliquoted into 6 ml syringes (4 ml ACS/syringe) and stores at -80 °C.

Intra-articular treatments:

Intra-articular injections were conducted in 4-4.5 ml volumes as follows by the unblinded investigator:

1) Negative control: 4 mL of sterile phosphate-buffered saline (PBS) in a sterile fashion.

2) Positive control: 100 ng Equine recombinant IL-1β (R&D Systems, Minneapolis, MN) diluted in 4 mL of sterile PBS to a concentration of 25ng/ml, in a sterile fashion. This dose of IL-1β has been previously used in models of temporary equine synovitis with well-documented *in vivo* response.[242]

3) ACS: 4 mL of ACS were injected intra-articular in a sterile fashion.

4) IL-1β + ACS: 100 ng of IL-1β diluted in 500 µl of PBS were administered immediately prior to injection of 4 mL of ACS in a sterile fashion.

5) IL-1β + TA: 100 ng of IL-1β diluted in 500 µl of PBS were administered immediately prior to injection of 4 mg of triamcinolone (0.4 mL) mixed with 3.6 mL of PBS in a sterile fashion.

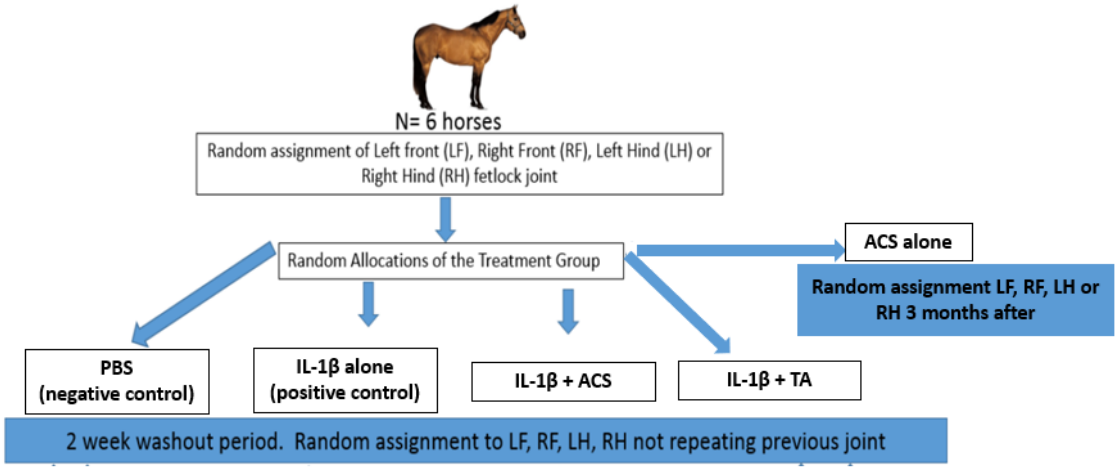

Figure 7.1 Study design. Random assignment to groups: Negative Control (NC), Positive Control (PC), Autologous Conditioned Serum (ACS) and Triamcinolone (TA).

Evaluation of Clinical Response:

During the 5 study periods, the clinical response was evaluated by two investigators that were blinded to treatment (AV, SZ).

Physical examination:

Horses were monitored throughout the study for signs of acute pain monitoring changes in temperature, pulse, and respiration. These parameters were monitored every 2 hours until PIH 8, every 4 hours until PIH 16, and then every 12 hours until PIH 72.

Metacarpo/Metatarsophalangeal (MCPJ/MTPJ) joint evaluation:

The MCPJ or MTPJ region was subjectively scored by digital palpation for heat, joint effusion, and response to flexion at PIH 0, 8, 16, 24, 36, 48 and 72 hrs. Heat was graded by palpation of the joint and graded from 0 to 3 (0 = none, 1 = minor, 2 = moderate, and 3 = severe). Joint circumference and degrees of flexion were measured at the same time points with a

standard measuring tape and a protractor, respectively. In addition, a digital infrared thermometer (ThermoPro, Toronto, ON) was used to measure the temperature of the dorsal aspect of the joint, palmar/plantar aspect of the joint and synoviocentesis site and compared to the contralateral limb (Figure 7.2).

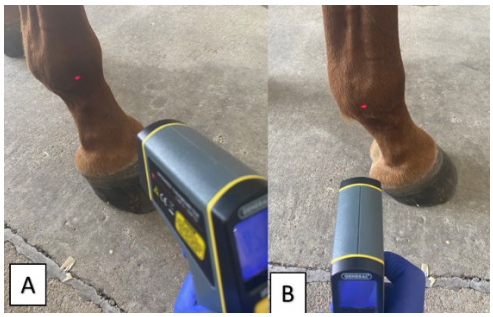

Figure 7.2. Measurement of the superficial temperature using a digital infrared thermometer of the dorsal pouch (A), and injection site (B).

Joint swelling was graded from 0 to 4 (0 = no swelling; 1 = minimal swelling localized to the injection site; 2 = mild swelling localized to the MC/MTPJ; 3 = moderate swelling extending proximally toward the carpus or tarsus; and 4 = marked swelling extending to or above the carpus or tarsus). Joint circumference (mm) was measured at the level of the proximal sesamoid bones, 2 cm proximal to the ergot,[588] using a standard cloth measuring tape. The site for circumferential measurement was marked prior to intra-articular injection by clipping the hair away on a horizontal line on the dorsal aspect of and palmar/plantar aspect of the joint. This ensured that joint circumference was consistently measured at the same site on the limb during the study period (Figure 7.3)

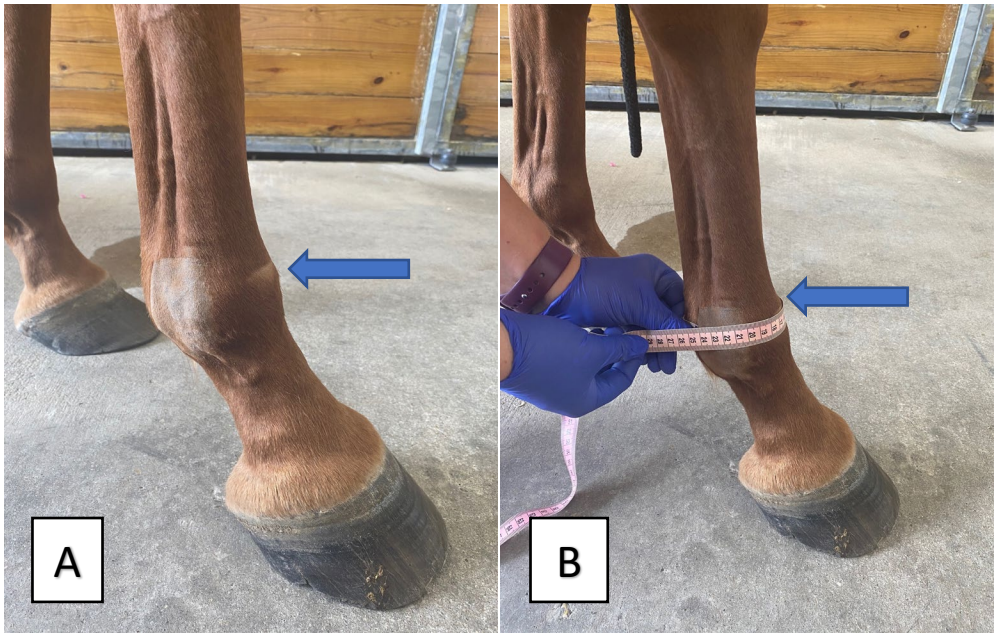

Figure 7.3. Joint circumference measurement. A) Initial landmarks (blue arrow) and B) measurement following the landmark.

Clinical evaluations were performed on each horse prior to each study period prior to synovial fluid collection.

Lameness evaluation:

Response to passive flexion was subjectively graded from 0 to 3 (0= none; 1= minor; 2= moderate; 3= severe). Additionally, a protractor was used to objectively measure the maximum range of flexion (Figure 7.4) Lameness was evaluated objectively using an inertial sensor system at 0, 8, 16, 24, 36, 48 and 72 hours PIH.

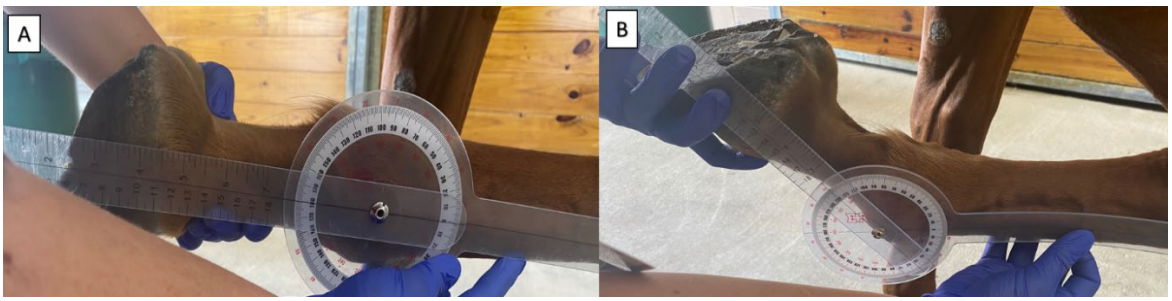

Figure 7.4. Degrees of the maximum flexion was measured objectively with a protractor. The protractor was placed over the lateral side of the joint in extension (A) and the joint was flexed until a pain response was noted and then measured (B).

Lameness evaluations were performed on each horse at each time point of the study prior to synovial fluid collection.

Synovial Fluid collection: At the start of each study period, horses were sedated with xylazine hydrochloride (0.02-0.8 mg/kg IV). The fetlock assigned for treatment during that study period was clipped and aseptically prepared. The collateral sesamoidean ligament approach to the metacarpophalangeal joint was used since synovial fluid is more readily obtained from this site.[589] Approximately 3 ml of synovial fluid were collected. Without removing the needle, the prepared treatment was injected into the fetlock by unblinded investigators (LB, FC, JS). Treatment syringes were prepared by unblinded investigators and placed in non-transparent syringes for administration. Three milliliters of synovial fluid was collected in the same manner as described at PIH 8, 24, and 48 hrs.

Synovial fluid cytology

500 µL of synovial fluid were transferred to a collection tube containing EDTA for cytological analysis: Total nucleated cell count (TNCC) and total protein (TP) were analyzed using an automated processor (Advia 2120 hematology analyzer; Siemens Healthcare Diagnostics, Erlangen, Germany). Differential cell counts were performed by a blinded board-certified veterinary clinical pathologist (EG). The remaining synovial fluid was aliquoted into

Eppendorf tubes, centrifuged (600 RMP for10 minutes at 4°C), the supernatant was removed and stored at -80°C until further analysis.

PGE_2 concentrations in synovial fluid

Thawed samples (200 µL) were hyaluronidase-digested (10 µL of 100 IU hyaluronidase/mL acetate buffer; Worthington Biochemical Corporation, Lakewood, NJ) for 30 minutes at 37°C, centrifuged (12,000 RPM for 10 min; 4°C), and the supernatant recovered. PGE_2 was quantified by ELISA (R&D Systems, Minneapolis, MN). Briefly, hyaluronidase-digested samples were solid-phase extracted (500 µL synovial fluid in 490 µL 100% ethanol and 10 µL glacial acetic acid incubated at 23°C for 5 minutes), centrifuged (600 RPM for 8 min; 4°C), and the supernatant collected.[590] Samples were diluted at 1:20, and PGE_2 measurements were performed in duplicate according to the manufacturer's instructions. This colorimetric assay was not equine-specific; however, it has been previously referenced and validated for cross-reactivity in equine samples.[125,237,251] Standards provided for the ELISA were used to prepare a standard curve following manufacturer's instructions.

1,9-dimethyl methylene blue assay (DMMB)

DMMB assay was performed to measure GAG concentration in the synovial fluid. Synovial samples were digested with hyaluronidase as previously described and the supernatant recovered. The 1,9-dimethyl methylene blue dye (Sigma-Aldrich, St. Louis, MO, USA) was prepared following the method of Farndale et al.[556] The standard curve was created by preparing solutions containing 0 to 65 µg of chondroitin sulfate (Sigma-Aldrich, St. Louis, MO, USA). Briefly, using a 96-well flat-bottom transparent plate (Stellar Scientific, Baltimore, MD, USA)

50 µL of diluted digested synovial fluid (1:10 dilution in distilled water) were mixed with 200 µL of DMMB dye (1 mN hydrochloric acid, 0.06‰ DMMB, 40 mM glycine, 27 mM NaCl pH 3) and the plate was shaken on a horizontal orbital microplate shaker (0.12" orbit) at 500 RPM for 5s. Measurement of the total GAG content was performed by a direct spectrophotometric method. Optical density was measured at 525 nm on a microplate reader (SpectraMax ID3, Molecular Devices, Sunnyvale, CA, USA).[557] Samples were measured in triplicate.

Statistical analysis

Linear or generalized linear mixed models were used to analyze each clinical or biochemical variable. Cell counts was not normally distributed, and it was natural log-transformed to obtain normality prior analysis. The generalized linear mixed models (GLMM) for heat, swelling, joint effusion and pain to flexion included fixed factors for treatment, time, and a treatment by time interaction effect. The linear mixed models (LMM) for angle, circumference, heart rate, temperature, respiratory rate, white blood cell count, total protein, red blood cell count, specific gravity, front limb lameness, hind limb push lameness and hind limb impact lameness included fixed factors for treatment, time and a treatment by time interaction effect. The LMM for temperature on limb included fixed factors for treated (yes/none), location, time and treatment and all 2-, 3- and one 4-way interaction effect. Random intercepts were included in all GLMMs and LMMs for each horse and fetlock within each horse was included to account for within horse and within fetlock correlations. Model residuals for LMMs were examined to evaluate the assumption of normality. Simple effects were tested to compare treatments at each time and multiple comparisons were adjusted for using Tukey's test. Satterthwaite degrees of freedom method was used in all models.

Results

Clinical parameters:

No differences between treatments for respiratory rate ($P = 0.431$) or temperature ($P = 0.717$) were observed for all time points. Differences in the heart rate between ACS alone and PBS, IL-1β, IL-1β + TA, and IL-1β + ACS groups were observed at 6, 8, 12 and 24 hours PIH (Figure 7.5). At 6, 8, 12 and 24 PIH, the heart rate was lower in the group receiving ACS compared to IL-1β alone ($P = 0.03$, $P = 0.001$, $P = 0.02$ and $P = 0.01$, respectively). At 8 and 12 PIH, the ACS group had a lower heart rate compared to IL-1β + TA group ($P = 0.001$ and $P = 0.03$).

Metacarpo/Metatarsophalangeal (MCPJ/MTPJ) joint evaluation:

Joints injected with IL-1β, IL-1β + TA, and IL-1β + ACS had higher temperatures than the contralateral joint at all the time points ($P < 0.05$). Differences in heat scores between treatment groups were observed at 8, 16, 36, 48, and 72 hours post-injection (Figure 7.6). Eight hours post-injection, the heat score in the ACS group was lower than the IL-1β, IL-1β + TA, and IL-1β + ACS ($P = 0.001$). At 16 hours, the IL-1β + TA group presented lower heat compared to PBS ($P = 0.04$), IL-1β ($P = 0.01$) and IL-1β + ACS ($P = 0.03$). At 36 and 48 hours post-injection, the IL-1β + TA group still had lower temperature than all treatment groups ($P = 0.001$), although 72 hours, the ACS group had a significantly lower temperature than the rest of the groups ($P = 0.001$).

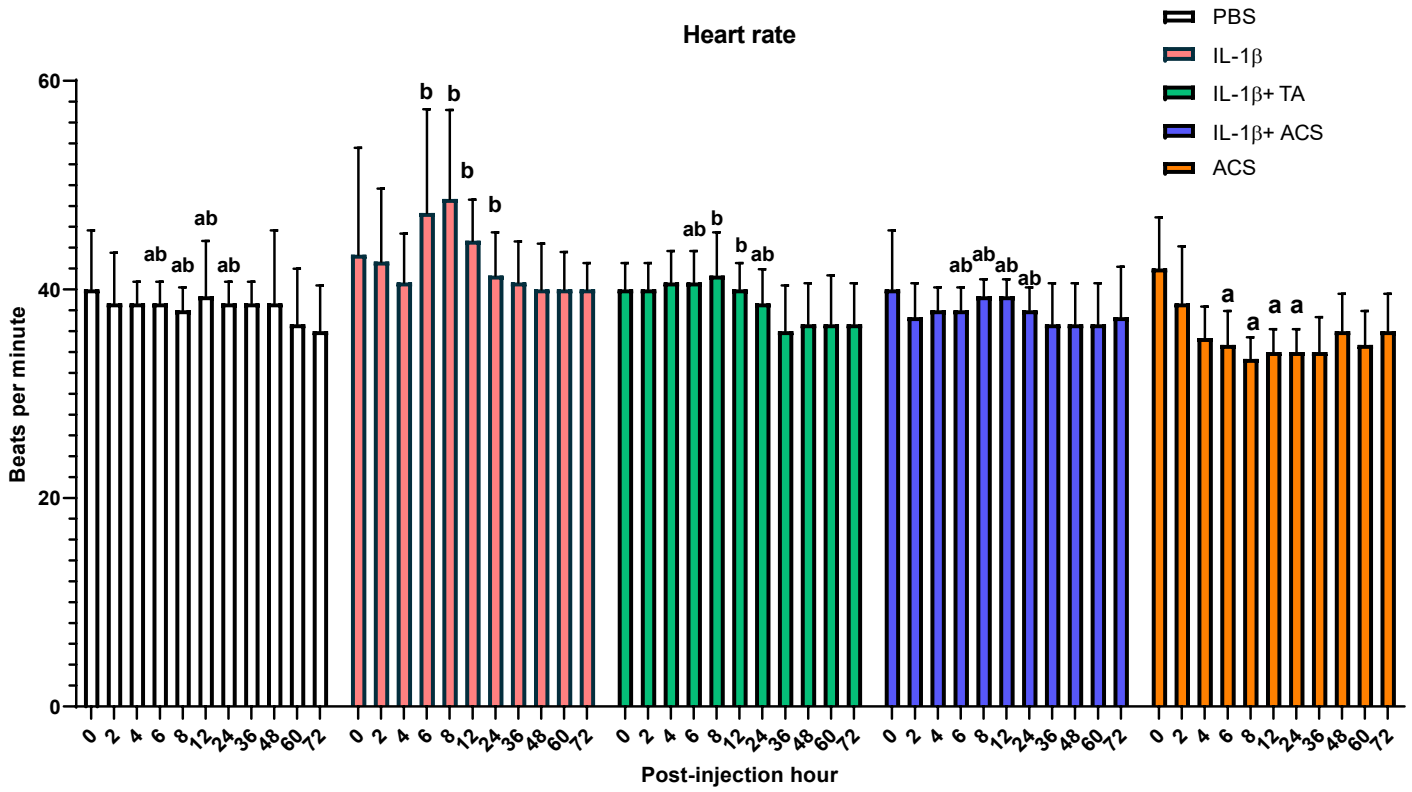

Figure 7.5. Heart rate after injection of PBS, IL-1β, IL-1β + TA, IL-1β + ACS, or ACS. Different letters indicate a significant difference ($P<0.05$) between groups at 6, 8, 12 and 24 PIH. Bar graphs indicate the mean and standard deviation.

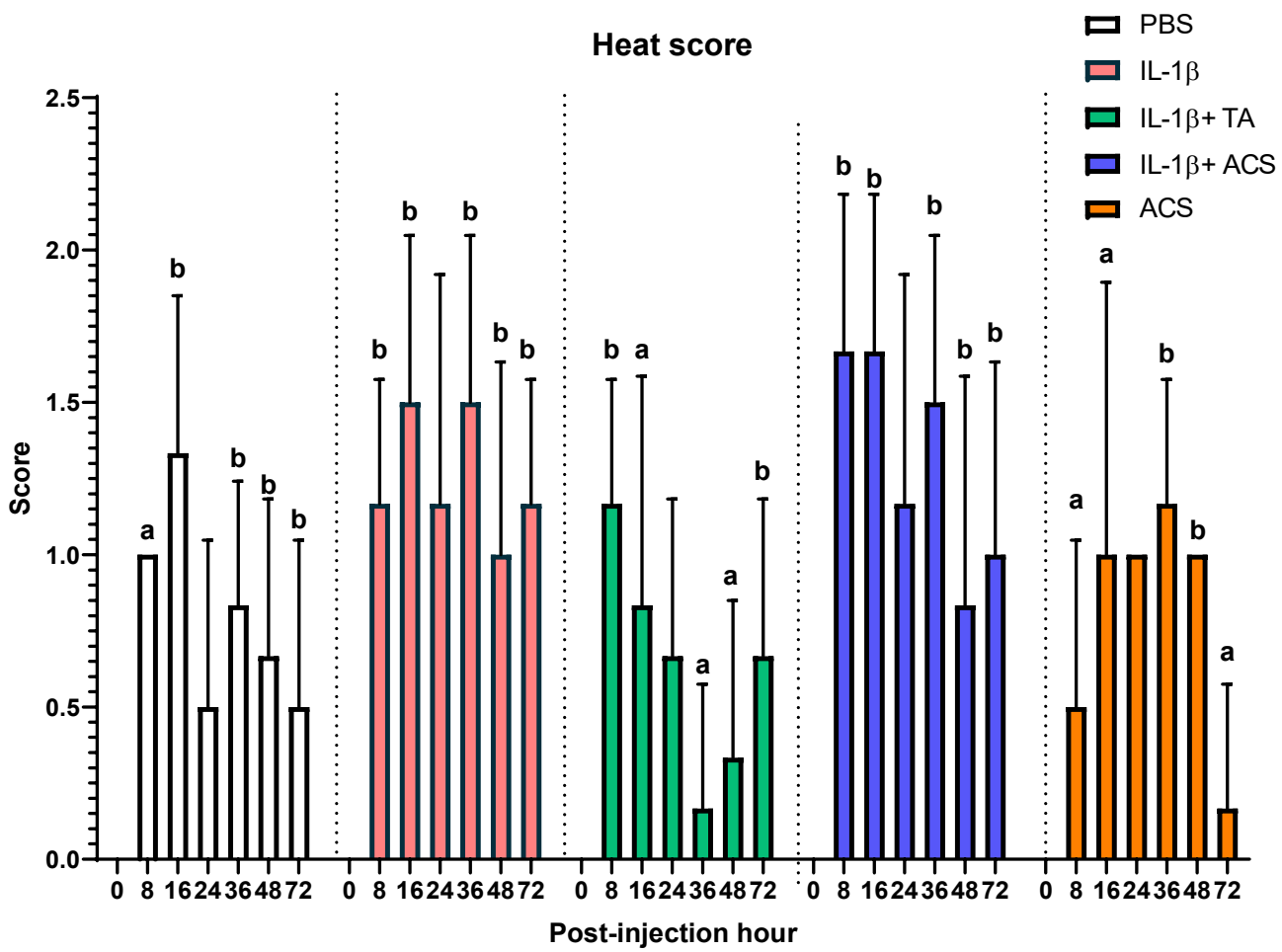

Figure 7.6. Heat score after injection of PBS, IL-1β, IL-1β + TA, IL-1β + ACS, or ACS. Different letters indicate a significant difference ($P<0.05$) between groups at 8, 16, 36, 48 and 72 PIH. Bar graphs indicate the mean and standard deviation.

Swelling was more severe at 72 hours in the PBS, IL-1β, and IL-1β + TA, and IL-1β + ACS but not in the ACS group. Significant differences in swelling scores between treatment groups were observed at 8, 16, 24, 36, 48, 72 hours post-injection (Figure 7.7). Eight hours post-injection, the swelling score in the ACS group was lowest compared to the rest of the groups ($P = 0.001$). At 16 hours, the ACS group had decreased swelling compared to IL-1β ($P = 0.03$) and IL-1β + ACS ($P = 0.04$). At 24 and 36 hours post-injection, IL-1β + TA had lower swelling scores compared to IL-1β ($P = 0.001$ and $P = 0.003$), IL-1β + ACS ($P = 0.001$ for both time points), and ACS ($P = 0.01$ and $P = 0.03$) groups. The groups IL-1β + TA and ACS group had the lower swelling scores compared to the IL-1β and IL-1β + ACS at 48 hours ($P = 0.001$) and 72 hours ($P = 0.002$).

Differences in the joint effusion scores between treatment groups were observed at 8, 16, 24, 36, 48, 72 hours post-injection (Figure 7.8). During all time points post-injection, the injection of IL-1β and IL-1β + ACS produced the most marked joint effusion than the remaining groups ($P = 0.001$). IL-1β + TA group produced lowest joint effusion scores at 36, and 48 PIH ($P = 0.001$).

In summary, when IL-1β was injected alone or in combination with TA and ACS produced an increased heat and synovial effusion at 8 PIH. However, when TA was combined after the 8 PIH this group showed the lowest scores in the following time points compared to ACS.

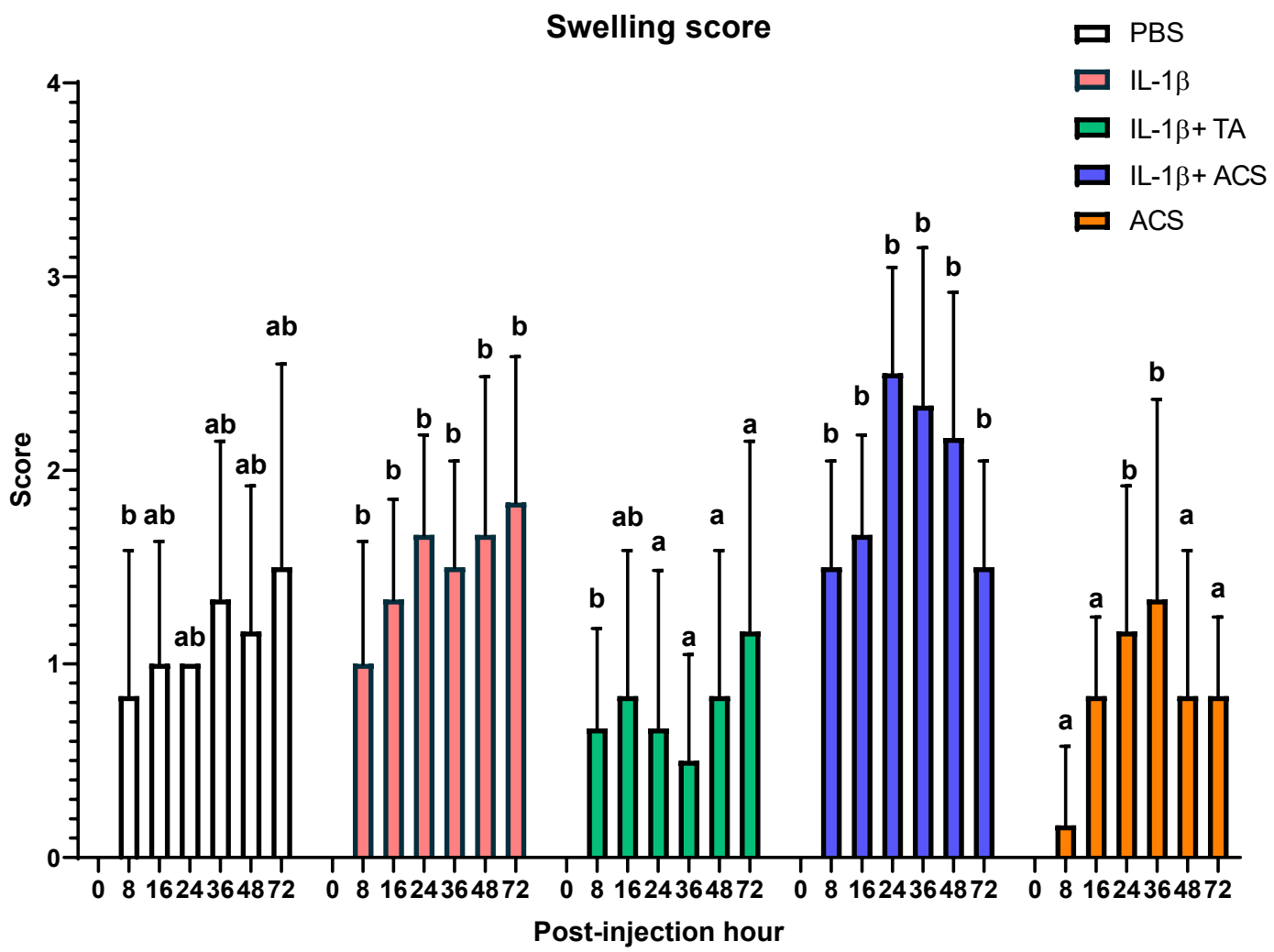

Figure 7.7. Swelling score after injection of PBS, IL-1β, IL-1β + TA, IL-1β + ACS, or ACS. Different letters indicate a significant difference ($P<0.05$) between groups at 8, 16, 24, 36, 48 and 72 PIH. Bar graphs indicate the mean and standard deviation.

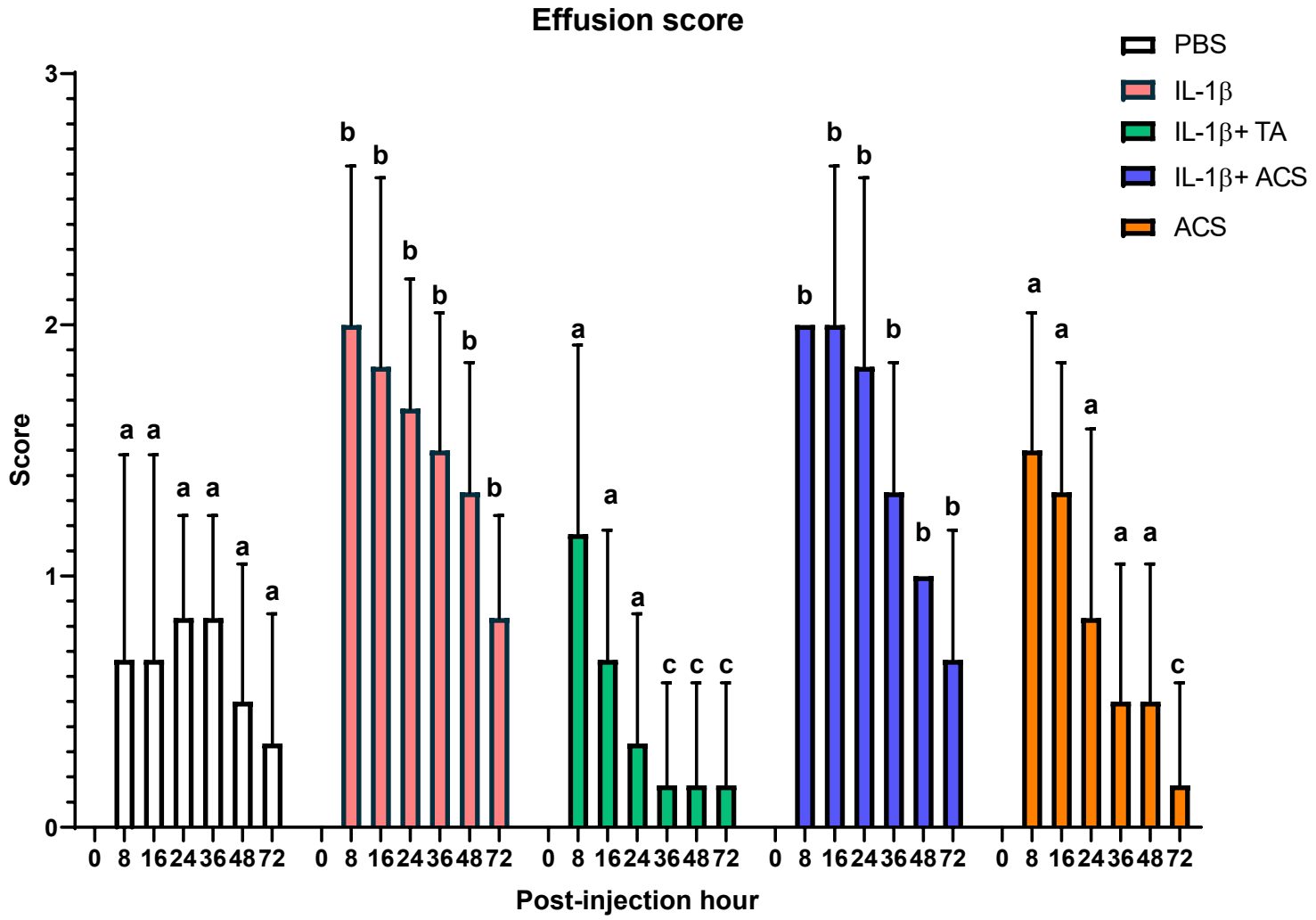

Figure 7.8. Joint effusion score after injection of PBS, IL-1β, IL-1β + TA, IL-1β + ACS, or ACS. Different letters indicate a significant difference ($P<0.05$) between groups at 8, 16, 24, 36, 48 and 72 PIH. Bar graphs indicate the mean and standard deviation.

Lameness evaluation

No differences in pain response to flexion were found between groups at any time point. When measuring the range of flexion with a protractor, the IL-1β group presented a significantly reduced range of flexion compared to IL-1β + TA (P = 0.04) and ACS (P = 0.03) at 48 hours. The vector sum value obtained for each time point was compared to baseline, and each treatment group was compared at 8, 16, 24, 36, 48 PIH. In the groups where IL-1β was injected alone or combined with TA or ACS, a mild to moderate increase in lameness was observed (higher vector sum). Injection IL-1β + ACS induced a less significant lameness compared to IL-1β alone at 24, 36, and 72 PIH (P = 0.005, 0.005, and 0.01 respectively). When injecting ACS alone compared to IL-1β + ACS, lameness was less severe at 24 and 36 PIH (P = 0.04 and 0.01). When comparing IL-1β + TA and IL-1β + ACS groups, lameness was less marked with the addition of ACS compared to TA at 36 and 72 PIH (P = 0.02 and 0.03, respectively).

Synovial fluid cytology

Synoviocentesis was performed at each time point and synovial fluid was assessed for TNCC, TP, and differential cell counts (Figure 7.6). Synovial fluid was obtained from all horses for all study periods and time points. Horses injected with IL-1β, IL-1β + ACS, and ACS alone produced a significant increase in TNCC composed mainly of neutrophils at 8 PIH (P < 0.001) that gradually decreased over time. The neutrophil percentage was lower in the PBS group at all time points than the remaining treatments (P < 0.001). The percentage of monocytes was increased in the ACS group at 24 and 48 PIH compared to IL-1β, IL-1β + TA, and IL-1β + ACS group (P = 0.001). When comparing groups, the IL-1β + ACS group produced the highest TNCC

(40,625 ± 11.01 cells/uL; P = 0.001). At 8 and 24 PIH, the TNCC was higher in all the groups compared to PBS (P = 0.01).

Similarly, IL-1β, IL-1β + ACS, and ACS alone produced a significant increase in TP in synovial fluid at 8 PIH compared to baseline (P < 0.001). This increase in TP remained in the IL-1β + ACS and ACS groups at 24 PIH (P = 0.001 and 0.01 respectively), but no differences between groups were observed at 48 PIH.

PGE_2 concentrations in synovial fluid

PGE_2 synovial fluid concentration was evaluated at 0, 8, 24, and 48 PIH (Figure 7.7). An increased PGE_2 concentration by 1.6-fold compared to baseline was measured in the IL-1β group (P < 0.001), with no significant increase produced with all other treatments at any time point. When only IL- 1β was injected, one of the horses produced an increase of PGE_2 5.7 times higher than the average of other five horses included in the study at 8 PIH.

1,9-dimethyl methylene blue assay (DMMB)

GAG concentration was measured in synovial fluid at 0, 8, 24, and 48 PIH. No differences were observed in the GAG concentration in the PBS, IL-1β, IL-1β + ACS, and ACS groups during the different time points. When comparing treatment groups, an increased concentration of GAG was measured in the IL-1β + TA group at 24 and 48 PIH (P<0.001).

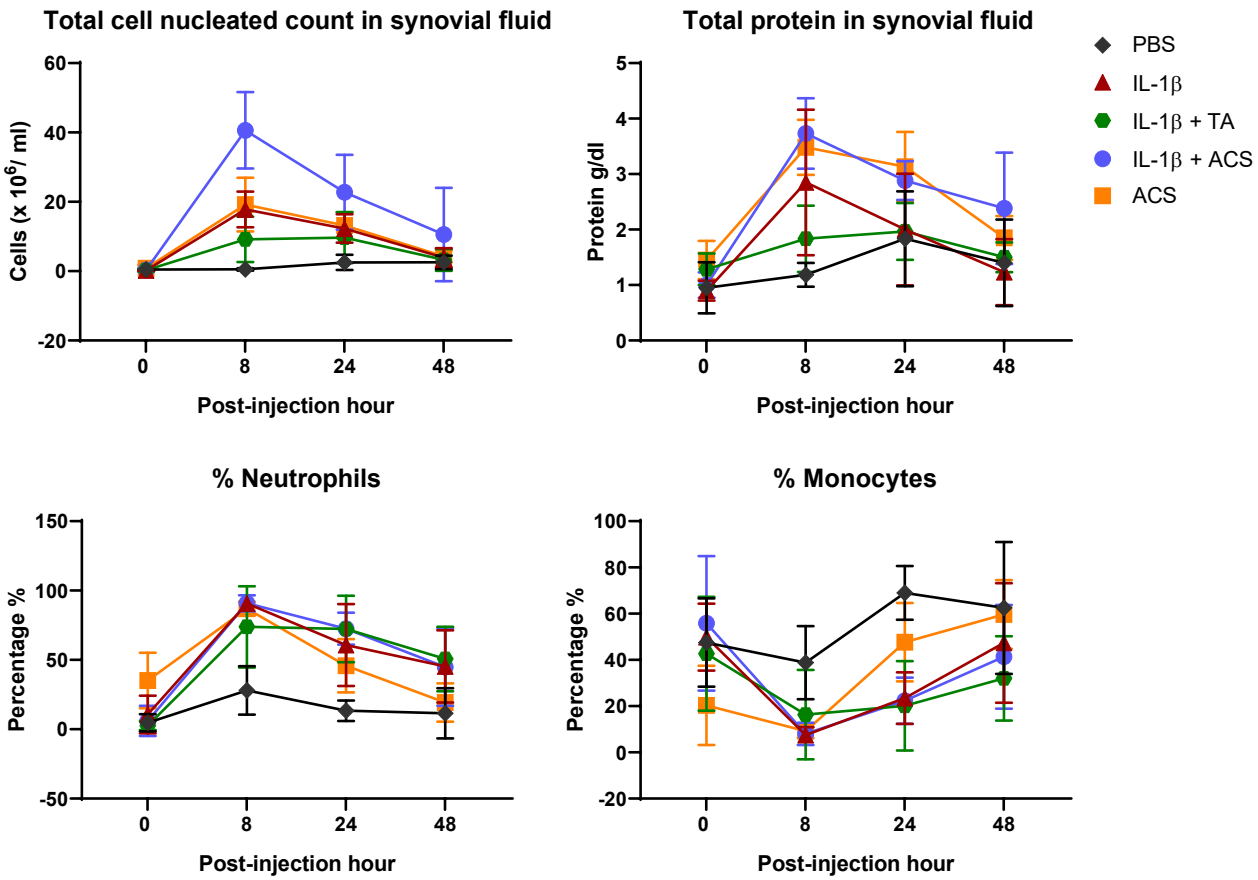

Figure 7.9. Synovial fluid analysis parameters after injection PBS, IL-1β, IL-1β + TA, IL-1β + ACS, or ACS treatment groups. The symbols represent the mean, and the error bars the standard deviation.

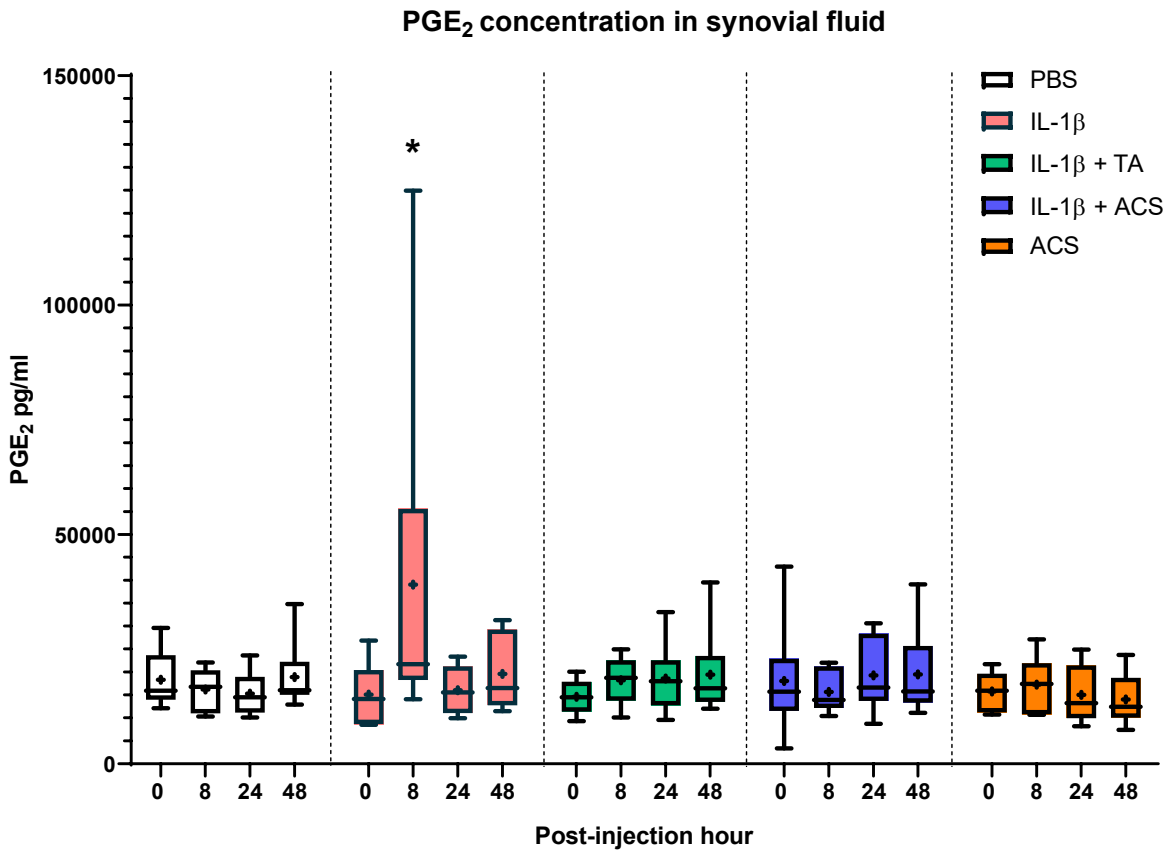

Figure 7.10. PGE_2 concentrations in the synovial fluid at 0, 8, 24, and 48 hours post-injection. The boxplots represent the interquartile range (IQR) of n = 6. The black lines represent the median values, the black dot represent the mean, and the whiskers represent the values outside the IQR. *Denotes significant difference between IL-1β group at 8 PIH compared to the rest of the groups at the same time point, $p < 0.05$.

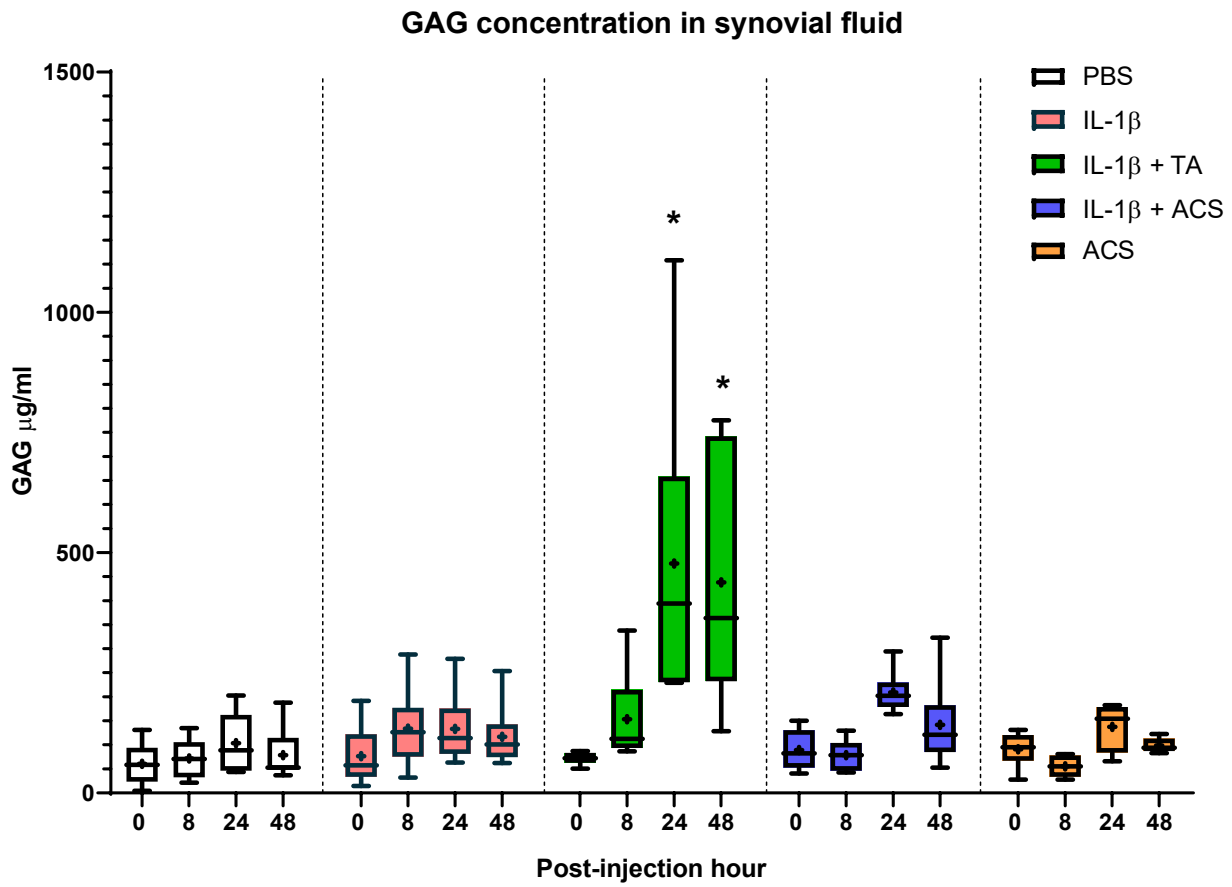

Figure 7.11. GAG concentrations in the synovial fluid at 0, 8, 24, and 48 hours post-injection. The boxplots represent the interquartile range (IQR) of n = 6. The black lines represent the median values, black dot represent the mean, and the whiskers represent the values outside the IQR. *Denotes significant difference between IL-1β + TA group at 24 and 48 PIH compared to the rest of the groups at the same time point, $p < 0.05$.

Discussion

Synovitis leads to activation of the inflammatory cytokine cascade that can produce articular cartilage matrix destruction leading to OA. The response of the synovial membrane injury is a primary mechanism for OA pathogenesis.[544,559] The effects of the intra-articular treatment with TA or ACS have been evaluated in both disease-free and OA afflicted joints in horses,[237,336,345,408] but no studies have evaluated their effect in an *in vivo* synovitis model using IL-1β for induction. Different synovitis models have been described in the horse, but IL-1β was selected for this model since is one of the most important pro-inflammatory cytokines participating in OA and it has validated in the horse in a synovitis model on the middle carpal joint.[242] The intra-articular injection of IL-1β produced a mild synovitis observed clinically, cytologically and producing a peak in the concentration of PGE_2 at 8 PIH.

As hypothesis the combination of IL-1β with ACS and TA decreased the inflammation decreasing the PGE_2 at 8 PIH, but different effects clinically and on the synovial fluid cellularity and GAG concentration. IL-1β + ACS induced the most marked cellular response, yet this treatment produced the most significant reduction in lameness, while TA combined with IL-1β did decrease heat, swelling and effusion from the fetlock joint but GAG concentration was higher at 24 and 48 hours. The increase in GAG concentration after TA treatment could present a significantly detrimental effect on the cartilage, which may indicate that ACS produces better chondroprotection.

Previous studies using an IL-1β synovitis model in the middle carpal joint achieved higher cellular concentrations at 8 hours (170.70 ± 37.58 cells/uL) than the model in the fetlock used in this study (17.80 ± 5.12 cells/uL).[242] Coltbath et al. reported that the same intra-articular dose of IL-1 β elicits a significantly different response in the middle carpal joint compared to the

tibiotarsal joint.[257] There is only one study in which IL-1β was used to stimulate synovitis in the metatarsophalangeal joint of the horse. However, in this study, the model was used to evaluate the efficacy of perineural local anesthetics, and synovial fluid was not analyzed after stimulation, and pain (lameness) was used as a positive indicator for induction of synovitis.[591] the metacarpo/ metatarsophalangeal joint may respond differently than the middle carpal and tibiotarsal joints. However, indicators of inflammation, including an increase in lameness, joint effusion, and heat, increase concentration of PGE_2 in synovial fluid and release of GAG indicated induction of mild to moderate synovitis in the present study. Studies to evaluate the effect of a higher dose in the fetlock joint are warranted.

A major goal of intra-articular therapies is to modify the inflammatory cascade to reduce cartilage destruction, but ACS also aims to enhance the reparative process of the synovial tissues involved in the pathologic process of OA. ACS has been shown to have disease-modifying properties in human and equine studies producing improved clinical signs and modification of the cellular response, increasing the concentration of IL-1rap in synovial fluid, and showing improved histologic scores primarily in the synovial membrane.[237,414,415,569] In this study, the injection of IL-1β with ACS produced the highest TNCC and total protein in synovial fluid, but ACS alone produced an increase in the TNCC and total protein similar to IL-1β alone. An increase in TNCC and total proteins have been considered as undesired for a healthy synovial environment.[7] However, previous research has shown that intra-articular injection of platelet-rich products or mesenchymal stem cells in horses produced a transient inflammatory reaction that can be seen both clinically (increase synovial effusion) and cytologically (increased the TNCC, neutrophil population and total protein in synovial fluid) without deleterious effects.[372,401,468] Injection of TA produced a less marked increase of TNCC and reduced the clinical signs such as

heat and synovial effusion the most compared to other treatments, but presented detrimental effects in the cartilage. In a previous study where lipopolysaccharide-induced synovitis was used, TA did not reduce the TNCC and total protein but significantly improved the lameness and synovial effusion.[592] Despite the induction of a mild yet temporary synovitis, PGE_2 concentration in the ACS alone group were not elevated at any time point. PGE_2 is one of the primary pro-inflammatory mediators that promote catabolic destruction of articular cartilage and the promotion of joint pain.[84] Therefore, the fact that PGE_2 did not increase after injecting IL-1β combined with ACS or TA shows beneficial effects of these intra-articular treatments.

According to a meta-analysis evaluating the effects of corticosteroids in humans and other species, TA has shown beneficial and detrimental dose-dependent effects, finding adverse effects on cartilage with an 18-mg cumulative dose per joint.[319] *In vivo* TA administration produced an increase of catabolic cartilage biomarkers and downregulation of collagen and aggrecan gene expression,[336,337] leading to the conclusion that TA could have detrimental effects when used over time, thus recommending judicious use. In our study, the combination of IL-1β with TA improved heat, swelling and joint effusion scores, without an increase PGE_2 concentration in synovial fluid. However, the increased concentration in GAG could indicate that treatment with ACS offers a safer treatment for articular cartilage since it does not cause as much release of GAG in the synovial fluid. In two human studies, where triamcinolone was compared to ACS for treatment of hip OA or lumbar radicular compression, ACS significantly improved the patient's overall pain compared to treatment with TA.[416,593] In our study, lameness was significantly improved in the IL-1β+ACS group compared to the IL-1β+TA. Although no other studies have compared TA and ACS in a synovitis model, Jostingmeir et al. reported that

lameness scores in horses with naturally occurring OA of the coffin joint presented improved lameness when injected with ACS compared to betamethasone and hyaluronic acid.[421]

PGE_2 is one of the primary pro-inflammatory mediators that promotes catabolic destruction of articular cartilage as well as stimulation of joint pain.[84] Previous synovitis studies in horses with lipopolysaccharide or IL-1β have measured PGE_2 and GAG concentration in synovial fluid as outcome measurements for synovitis.[242,257,305,471] Increased concentration of PGE_2 and GAG in synovial fluid correlates with repeated arthrocentesis in a previous study.[594] However, in the current results, the group injected with PBS received the same number of arthrocenteses, and the synovial fluid cytology, PGE_2 and GAG concentrations in synovial fluid were not affected. In a previous study performed by the authors showed that treatment of synovial and cartilage IL-1β stimulated co-cultures with ACS, significantly decreased the PGE_2 concentration in media, while TA did not.[208] An increased concentration of PGE_2 and GAG was correlated with OA changes in experimentally induced OA in horses.[208] In this study, the intra-articular injection of ACS also reduced lameness scores and PGE_2 concentration compared to the control group (PBS).[237] In the present study, it is possible that the reduced lameness could be correlated with a decreased PGE_2 concentrations.

This study had limitations that warrant further discussion. Inflammatory resolution is a novel concept described as an active process orchestrated mainly by macrophages needed to restore joint homeostasis,[595] and in particular IL-10 production by the macrophages seems to play an essential role in establishing synovial homeostasis.[596] This inflammatory process is needed to recover from an inflammatory insult, which makes researchers question if the total inhibition of the inflammatory reaction may not be beneficial. Measurement of IL-10 in synovial

fluid in the present study could have helped better determine if the inflammatory process occurring after ACS injection is beneficial rather than detrimental.

The initial experimental design did not include the use of ACS alone, but due to a marked increase in the TNCC and TP observed in the IL-1β + ACS group, unblinded investigators chose to evaluate the ACS effect when used alone. Variation in the treatment effect of ACS could have been affected by the inherent variation of ACS cytokine composition that has been described in horses.[397] As an example, surgical stress after castration has been shown to interfere with the cytokine concentration in autologous conditioned serum.[398] Therefore, ACS in the current study was processed on each horse before starting the study to avoid any possible interference due to stress. The model induced a short and mild synovitis response in the metacarpal/ metatarsophalangeal joint of the horses compared to previous studies using the same dose in the middle carpal or tarsocrural joint, making the evaluation of the outcomes measured during the study difficult. Additionally, variability in response to IL-1β between individuals and manufacturing concerns such as different lots, methods of reconstitution and storage have been reported to lead to varying activity levels.[242,257,587,597] However, in the present study, all IL-1β used was from the same lot and stored and reconstituted identically. Future studies evaluating different dosages of IL-1β to stimulate synovitis in the metacarpal/metatarsophalangeal joint are warranted. Also, measurement of more specific anabolic cartilage biomarkers such as CPII and CS-846 or different pro-inflammatory cytokines in synovial fluid could help us to better understand the effects of TA and ACS intra-articularly in a synovitis model.

In conclusion, ACS alone produces a response within synovial membrane observed by increased heat, swelling, joint effusion and TNCC, however, ACS treatment in the face of synovitis could offer benefits by decreasing PGE_2 production and limiting cartilage catabolism

compared to intra-articular corticosteroids. A deeper understanding of how biologic therapeutics modify the acutely inflamed synovial environment is needed.

Acknowledgment

The authors would like to thank Jessica Brown for her technical assistance, Dr. Deborah Keys for her collaboration in the statistical analysis, and to the American Quarter Horses Foundation Young Investigator Award for funding this project.

Chapter 8

Summary and conclusions

OA research is changing over time, mainly due to an increased understanding of the mechanisms involved in the pathophysiology of OA. This advanced knowledge leads to improved treatment options that need to be evaluated before clinical use. Articular cartilage has inadequate endogenous repair mechanisms due to the low, mostly metabolically inactive resident cell population as well as the avascular and anerual character of articular cartilage. One of the main goals of orthobiologic therapies is to try to cover this gap and induce a cellular response that will slow down the disease process and produces tissue with more similar characteristics to the healthy articular cartilage.

In horses, OA is a common, debilitating orthopedic disease that affects both performance and geriatric horses. Economic losses due to poor or lost performance in horses affect the equine industry. Horses can serve as models for human disease because of their similarities in the joint anatomy and cartilage thickness, which helps to extrapolate results to human OA disease, but study of OA in horses also has direct benefit to the horse itself.

Current approaches to treating OA focus on reducing pain and improving (or at least maintaining) mobility. Drugs currently used to treat OA fall into two main categories: symptom modifying therapies (NSAIDs or corticosteroids) and disease-modifying therapies (orthobiologics). Significant advances in our understanding of joint pathology have helped clarify the mechanisms of action of orthobiologics as a strategy to improve intrinsic repair and restore the articular surface. Unfortunately, even though the progress researchers have made, there are still seismic gaps in our knowledge and understanding.

For *in vitro* OA research, it is well recognized that all synovial tissues work together as an "organ system." The synovial membrane plays an essential role in regulating pro- and anti-inflammatory cytokines involved in OA. Therefore, the use of synovial membrane and articular cartilage co-culture models may offer an in vitro model that more accurately reflect an *in vivo system*.

There has been little work in the equine field using the co-culture model to evaluate the effects of certain orthobiologic therapies and comparing these to other more commonly used treatments such as corticosteroids. Therefore, the overall purpose of the investigation reported in this dissertation was to understand the use of orthobiologics by practitioners and to objectively evaluate treatment effects of autologous conditioned serum and autologous protein solution compared to standard treatments such as triamcinolone.

Equine practitioners widely use orthobiologics. Previous studies that evaluate the use of intra-articular therapies reported that practitioners tended to use these products more frequently in chronic pathology as an alternative when horses were unresponsive to corticosteroid injection. The results of the current survey showed that there is a shift in practitioner use of these products toward treatment during acute injury. Human studies concur that orthobiologic use appears to be more appropriate in the pre-OA stages before any advanced cartilage lesions develop. However, the current survey found that corticosteroids and hyaluronic acid are still the preferred treatment of joint disease among practitioners compared to orthobiologics. This could be potentially explained by the fact that corticosteroids produced a faster anti-inflammatory effect, they have been in the market for longer, or because orthobiologics are often more expensive and less readily available than corticosteroids. There is also a lack of blinded, controlled, and standardized objective studies evaluating orthobiologic therapies that prove their clinical effect,

making it more difficult for the practitioner to encourage clients to invest in orthobiologic treatments. Research investigating the disease-modifying effects of these products and investigations into best practices for how and when these products should be used is needed.

In the first *in vitro* study, results showed that the addition of 10% equine serum modified the inflammatory response produced by IL-1β stimulation. Serum-free media maintained cellular viability the same as the 10% equine serum-supplemented media, and no differences were observed between the histologic scores. Considering our results, the use of serum-free media when studying OA using a cytokine-induced *in vitro* OA model may be recommended. In particular, the effect of media supplementation with equine serum could make the study of orthobiologics *in vitro* more challenging due to similarities in the presence of growth factors, for example. The chemical profile of equine serum could be very similar to certain orthobiologics, particularly to autologous conditioned serum., which means that supplementation of media with equine serum could interfere or mask the real cellular effects produced by certain orthobiologics and should be considered when preparing a study design involving these products.

The study of orthobiologics is exceptionally challenging. One of the main reasons is the variability observed in the cellular and cytokine profile between individuals, pathologic conditions, or collection and preparation methods used to elaborate orthobiologic therapies. Performing standardized studies become extremely difficult, which could lead to inappropriate conclusions. It is crucial to have a good understanding of how preparation, or pathophysiologic condition could affect the concentration of cytokines and growth factors in orthobiologic therapies to elaborate a proper study design. In the second *in vitro* study, where ACS and APS were compared, it was found that despite the differences in cellular profiles, these products produced similar cytokine and growth factor compositions, yet their effects in an IL-1β

stimulated co-culture model of OA was different. One of the main differences between these two products is their concentration of WBC, in which APS is significantly higher than ACS. It is unknown the real effect of this concentration on white blood cells in the joint, but some human studies found that higher WBC counts correlated with higher IL-1rap, an important protein that blocks the inflammatory response. Recently, some research has been developed investigating the importance of macrophages participating in joint hemostasis. Therefore, further research evaluating macrophage composition and polarization of APS is warranted.

In the second *in vitro* study, it was observed that ACS and APS significantly decreased the concentration of PGE_2 in media, and both produced a trend to upregulate the gene expression of type II collagen and aggrecan compared to TA. The results proved that ACS and APS might provide important benefits in early stages of OA, slowing down the catabolic process within the joint. Additionally, it was observed that orthobiologics, particularly, APS might have a dose-dependent effect. In humans, when synthetic cytokine blockers such as IL-1rap are injected intra-articularly, rapid clearance from the synovial fluid has been documented, and researchers have questioned if the use of higher doses or multiple intra-articular injections should be considered. Unfortunately, proper dosage and dosing protocols have not been established, and further research is needed in this area.

Lastly, the synovitis model found that the addition of TA and ACS prevented PGE_2 from peaking at 8 hours following IL-1β induction of synovitis. The difference between TA and ACS was that ACS did not increase the GAG concentration at 24 and 48 hours, potentially offering more benefits than TA. The intra-articular injection of ACS with IL-1β or alone produced an inflammatory response, observed both clinically and cytologically. Recent investigations have studied the inflammatory mechanisms necessary for natural recovery after injury. This process

has been called inflammation resolution, which is required for re-establishing homeostasis. In these results, the injection of ACS alone produces minor synovitis without increasing lameness, which could mean that the induced synovitis in this group may not be ultimately a negative effect. Inflammation resolution is mainly orchestrated by macrophages, which depend on the joint environment to polarize into pro-inflammatory activated macrophages (M1) or anti-inflammatory macrophages (M2). Measurement of IL-10 concentration in synovial fluid or characterization of the macrophages is required to understand better the cellular effects of ACS or TA intra-articularly in horses.

It is essential to consider that different doors continue to open in the OA research field. In the last decade, there has been more discussion about the influence of micro RNAs (miRNAs) in the pathophysiology of OA. MicroRNAs have been studied in human OA and have shown promise as diagnostic biomarkers and in identification of novel therapeutic targets for intervention in OA. MicroRNAs are a class of endogenous non-coding small RNAs that regulate the expression of multiple genes to maintain cellular function. These miRNAs are included in exosomes, which are membrane-bound extracellular vesicles. This opens a new research space in equine OA. In the study presented in chapter six, despite the cellular differences between ACS and APS, the cytokine profile was very similar. These products may contain a different concentration of exosomes and encapsulated miRNAs, potentially producing different effects in the cartilage and synovial membrane. Therefore, the study of miRNA in OA and orthobiologics may be a future direction for research in the equine OA field.

Previous research have shown limited disease modifying effects of orthobiologic therapies. The use of co-culture of synovial tissues may be crucial to enhance our understanding the cellular mechanisms of these orthobiologic therapies. Results of the *in vitro* co-culture study showed that

ACS and APS modified the cellular response of synovial tissues by decreasing PGE_2 in the media while slightly increasing the expression of type II collagen and aggrecan compared to treatment with triamcinolone. On the other hand, the *in vivo* study showed that TA was as efficient as ACS in decreasing PGE_2 8 hours after induction of synovitis with IL-1β. However, TA produced a detrimental effect on the cartilage extracellular matrix observed by an increased GAG concentration in the synovial fluid. These results showed that orthobiologics, ACS and APS, may offer a more efficient anti-inflammatory and robust chondroprotective effect compared to triamcinolone for treatment of equine OA.

APPENDIX A- Sequence information from the genes used in the co-culture *in vitro* projects.

Before performing RT-PCR experiments for the *in vitro* projects described in this work, PCR products were run in agarose 1% gel to confirm the specificity of the reaction detecting a single band for each PCR product (Figure A.1). DNA samples were prepared by diluting 2µl of loading buffer (0.04 % bromophenol blue, 0.015 % xylene cyanol FF, 10 % glycerol in 5 x TBE) per 5µl of sample and loaded into the wells with DNA standards for determination of molecular size. The agarose gels were run at 80V for 45 min to 1 h and visualized using the longwave setting on an ultra-violet transilluminator. PCR products were extracted from the 1% agarose gel using the QIAquick gel extraction kit (QIAGEN, Germantown, MD, USA) following manufacturer's instructions and submitted to an external laboratory for sequencing. Results from sequencing were introduced in the database from Nucleotide BLAST (web BLAST, U.S. National Library of Medicine) to validate the primers created. The sequence results are shown below.

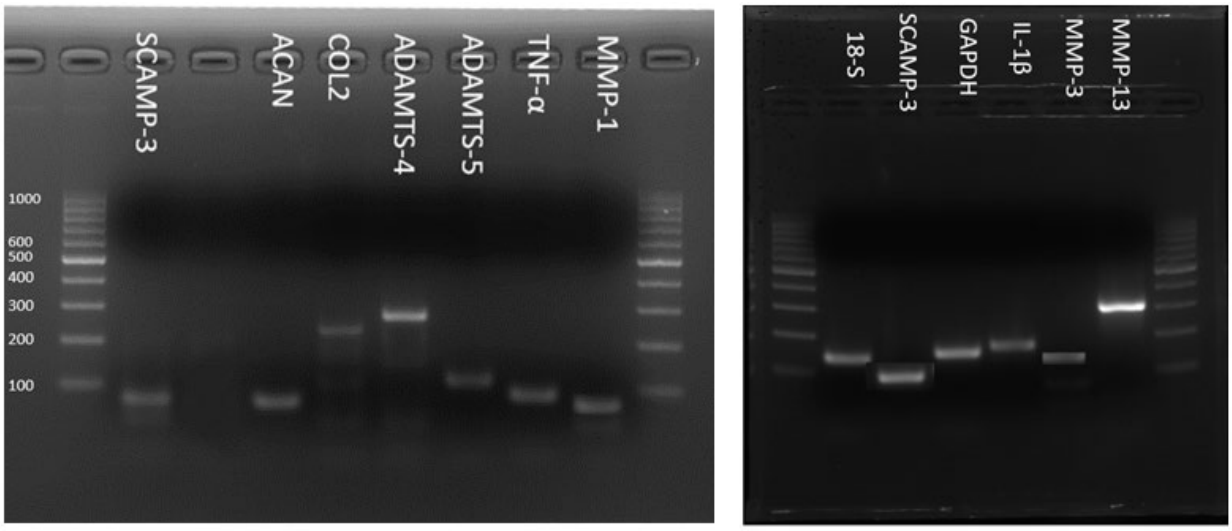

Figure A.1. Gel electrophoresis with all the genes evaluated with qPCR assays

Reference genes

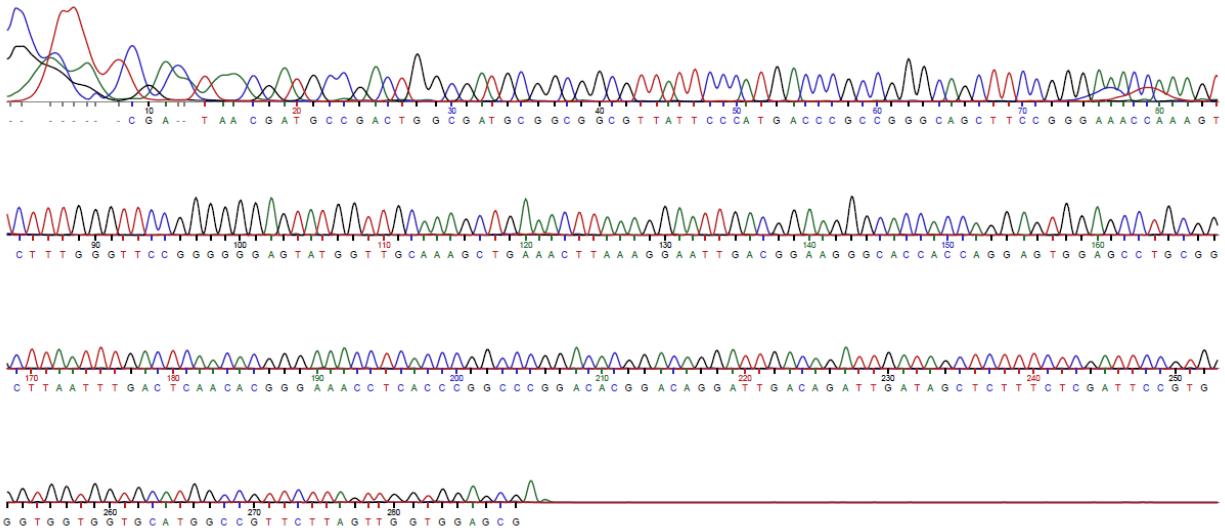

Figure A.2 18S sequence result.

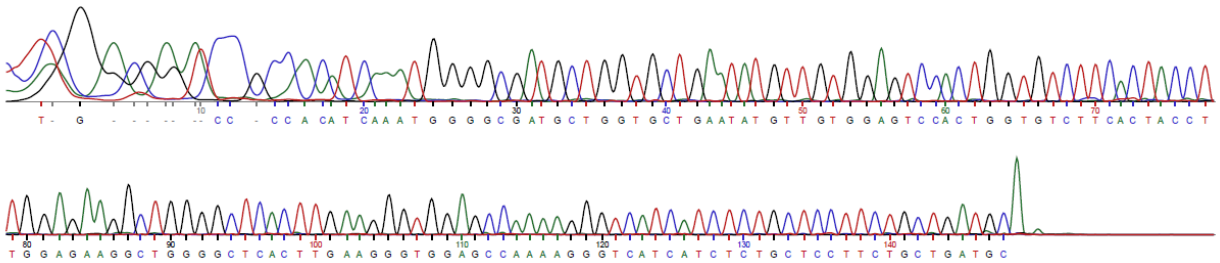

Figure A.3 GAPDH sequence result.

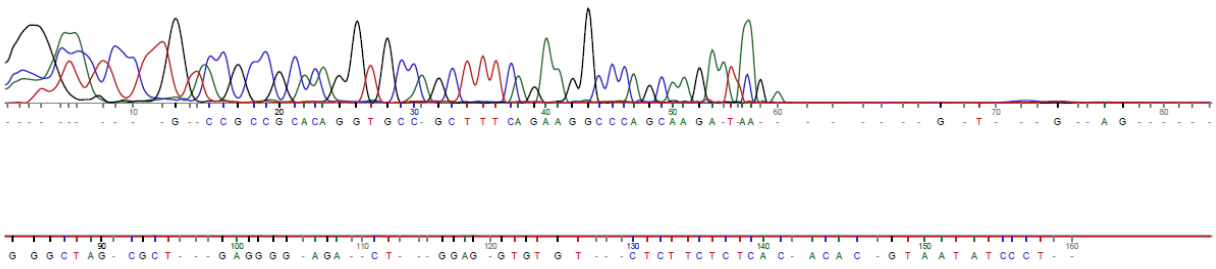

Figure A.4. SCAMP3 sequence result.

Genes evaluated during RT-PCR analysis.

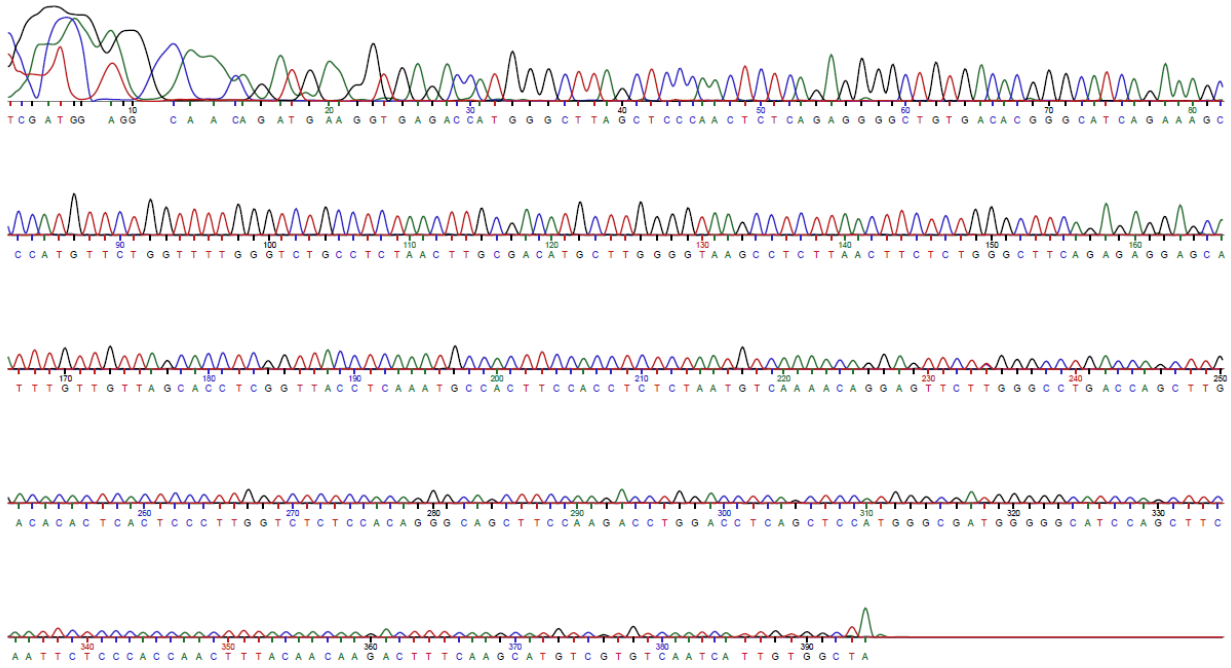

Figure A.5. IL-1β sequence result.

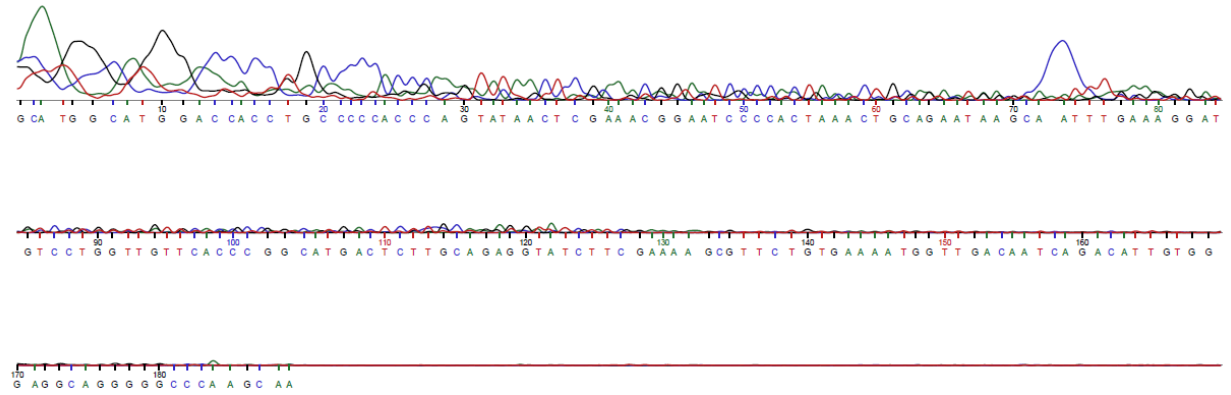

Figure A.6. TNF-α sequence result.

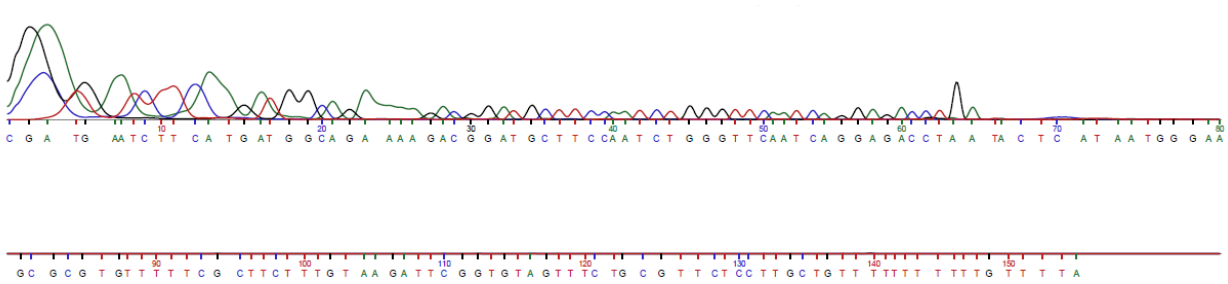

Figure A.7. IL-6 sequence result.

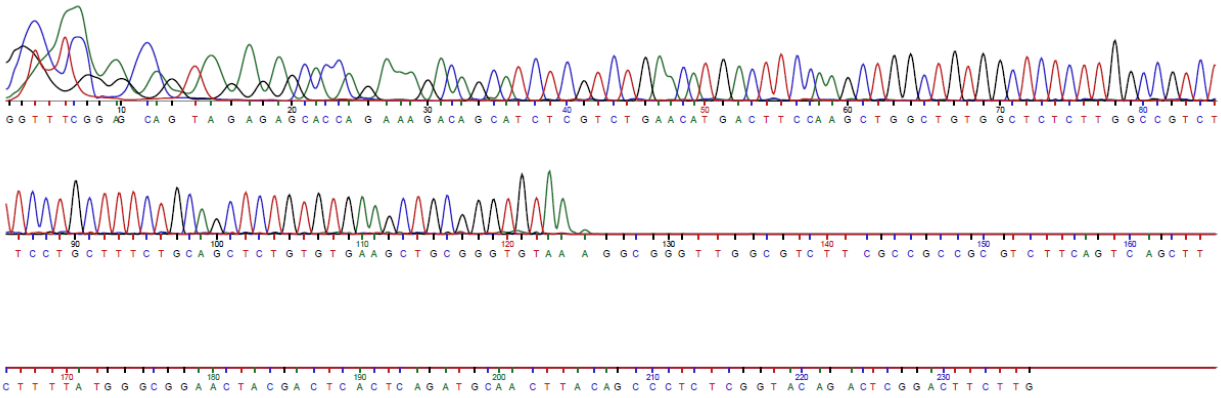

Figure A.8. IL-8 sequence result.

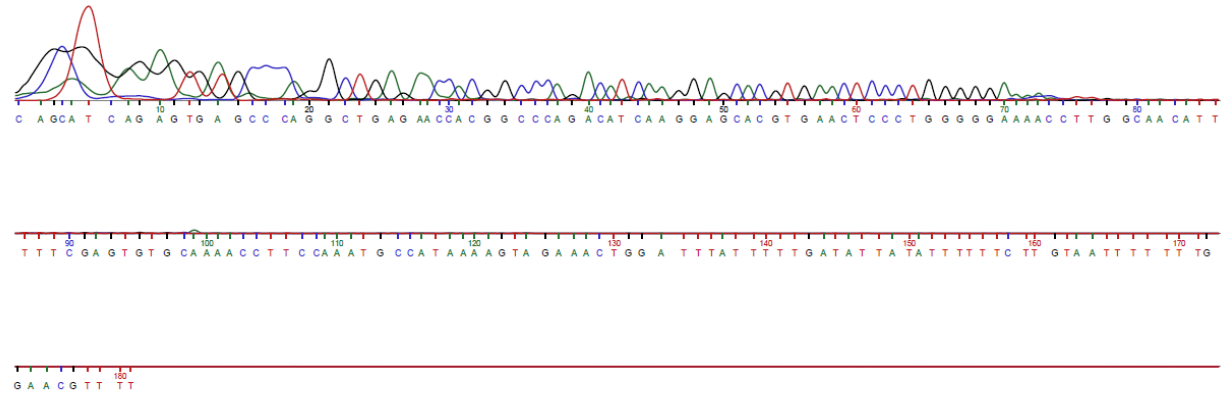

Figure A.9. IL-10 sequence result.

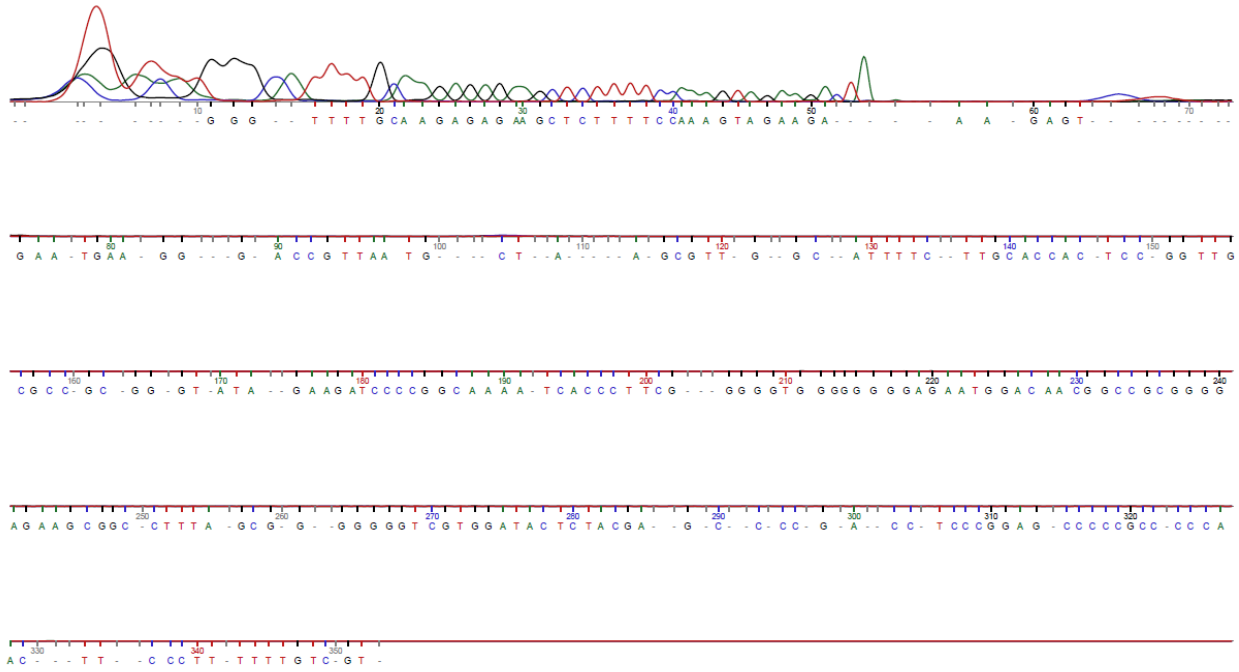

Figure A.10. MMP-1 sequence result.

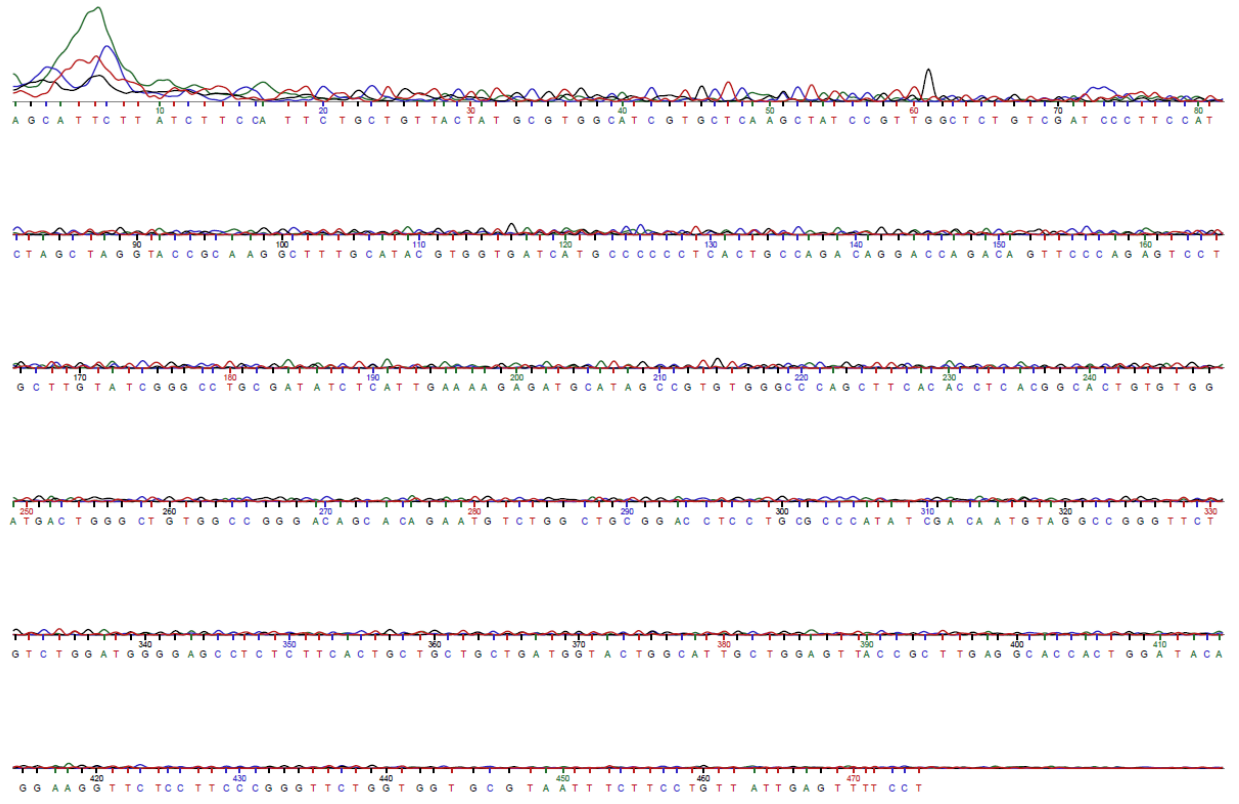

Figure A.11. MMP-3 sequence result.

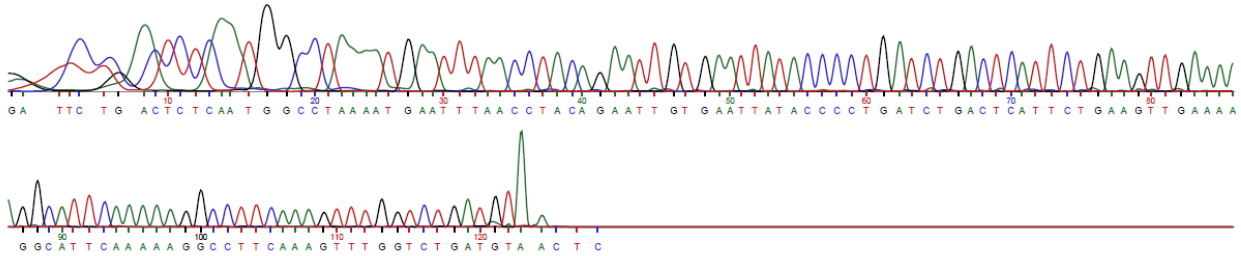

Figure A.12. MMP-13 sequence result.

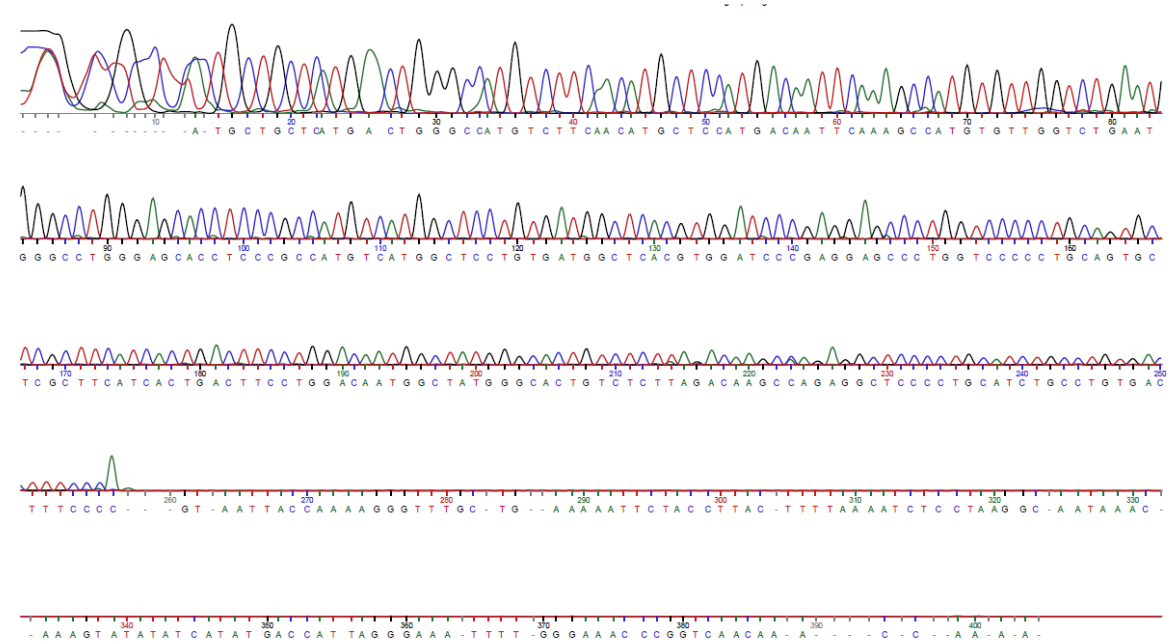

Figure A.13. ADAMTS-4 sequence result.

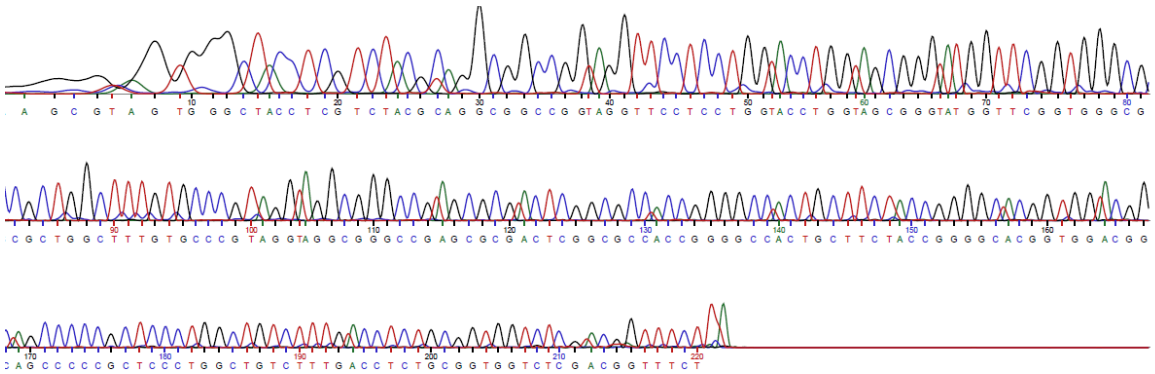

Figure A.14. ADAMTS-5 sequence result.

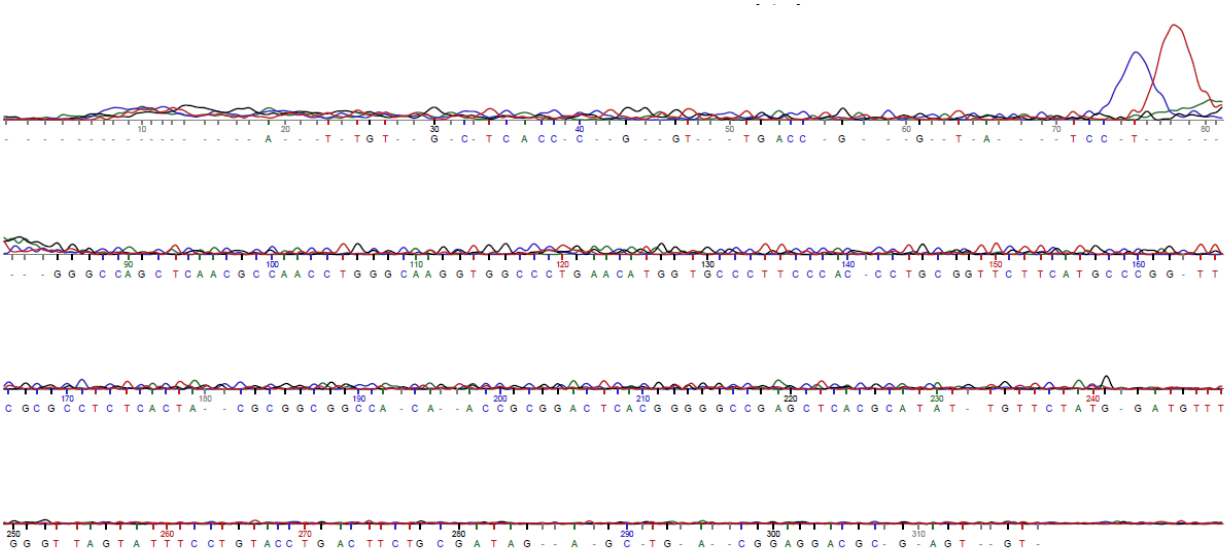

Figure A.15. Collagen type II sequence result.

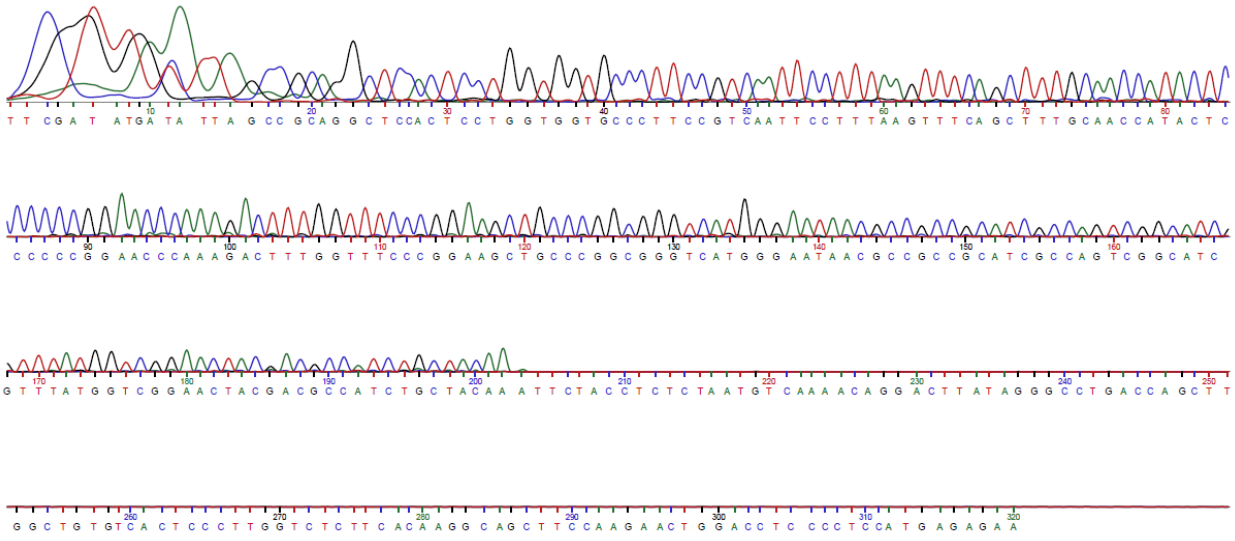

Figure A.16. Aggrecan sequence result.

APPENDIX B- Detection of genomic contamination in the RNA samples.

RNA was reverse transcribed to cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, California). The protocol was followed according to the manufacturer's instructions. During the development of the RT-PCR analysis, some of the experiments produced inconsistent results with invalid melt peak curves (Figure B.1 and 2).

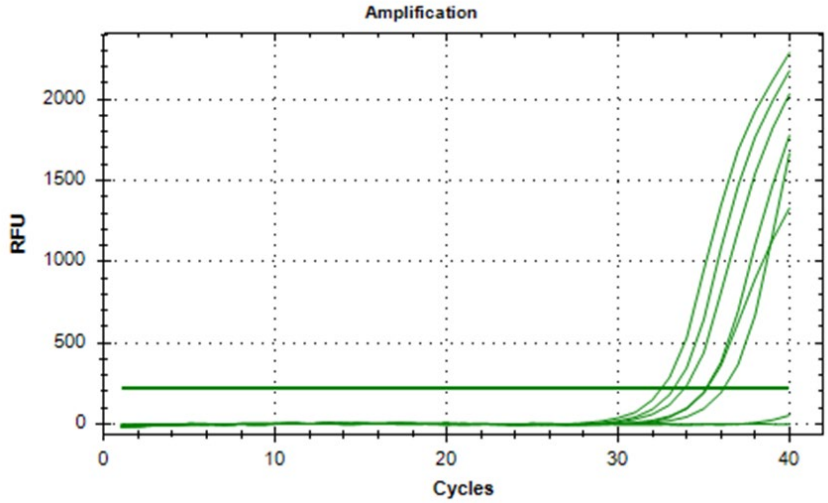

Figure B.1. Amplification results of SCAMP3 gene expression analysis.

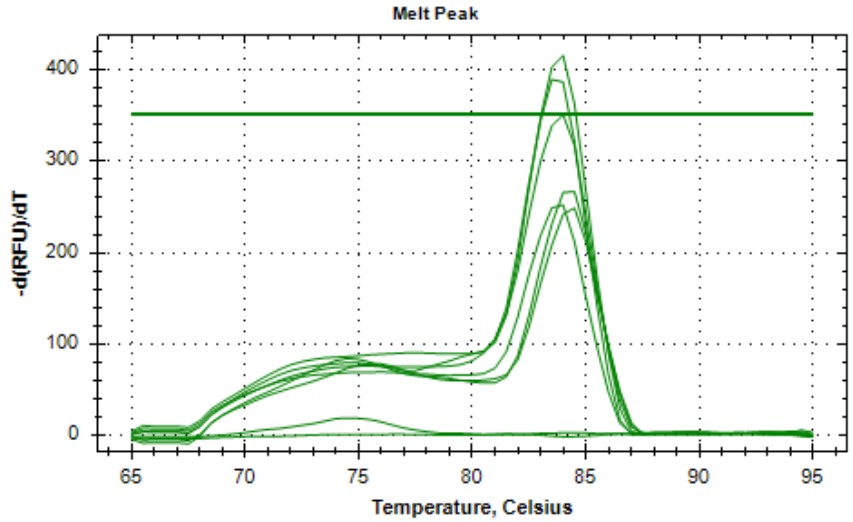

Figure B.2. Melt peak curve obtained during the SCAMP3 gene expression analysis.

Possible genomic DNA (gDNA) contamination in the RNA samples was suspected. A RT-PCR assay was carried out using GAPDH gene to evaluate contamination. The protocol was performed using a 96-well-plate that was set up using different templates for a 25µl reaction:

- 12.5 µl SYBR Green
- 5µl of water
- 1µl of forward primer
- 1µl of reverse primer
- 1µl of template: cDNA (sample), RNA (RTC, reverse transcriptase control for genomic DNA contamination) and nuclease-free water (NTC, non-template control).

The PCR products created from the cDNA, RNA and water samples were run in agarose 1% gel. Two bands of the same size where detected in the cDNA and RNA wells, while the well with samples with nuclease-free water did not produce a band. (Figure B. 3). This indicated that gDNA contamination was present in our RNA samples.

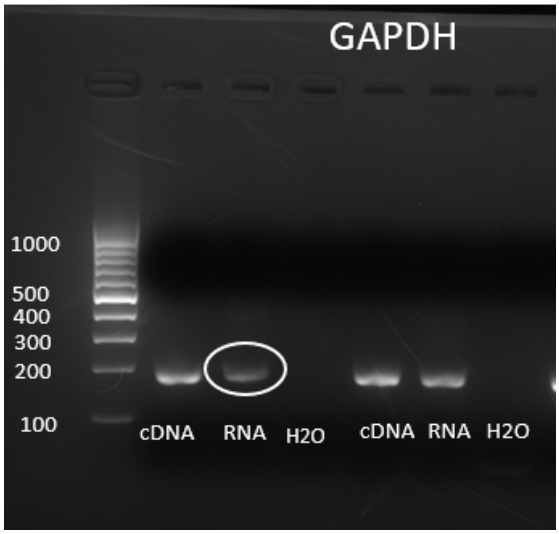

Figure B.3. Gel electrophoresis with cDNA, RNA and nuclease-free water. The white circle highlights the presence of a band in the RNA samples, which should not present a band in the absence of gDNA contamination.

After confirming the presence of gDNA contamination, RNA samples were reverse transcribed to cDNA using iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad, Hercules, California) following manufacturer's instructions. Amplification curves and melt curves improve remarkably (Figure B. 4 and 5).

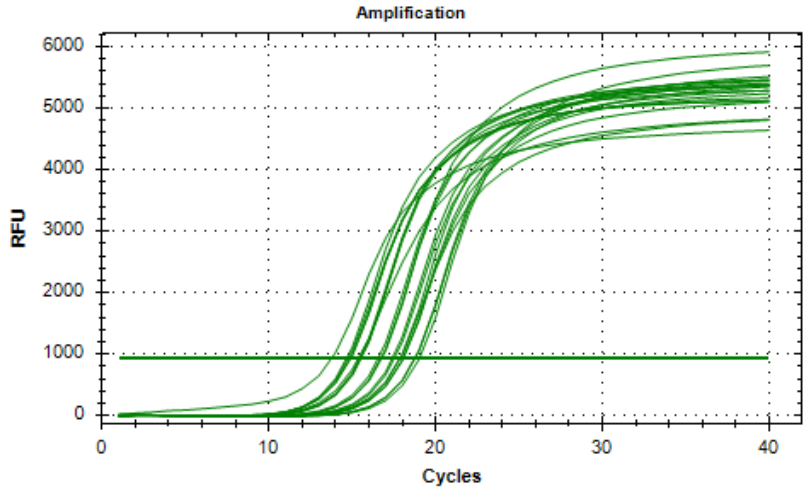

Figure B.4. Amplification results of SCAMP3 gene expression analysis after clearing gDNA contamination.

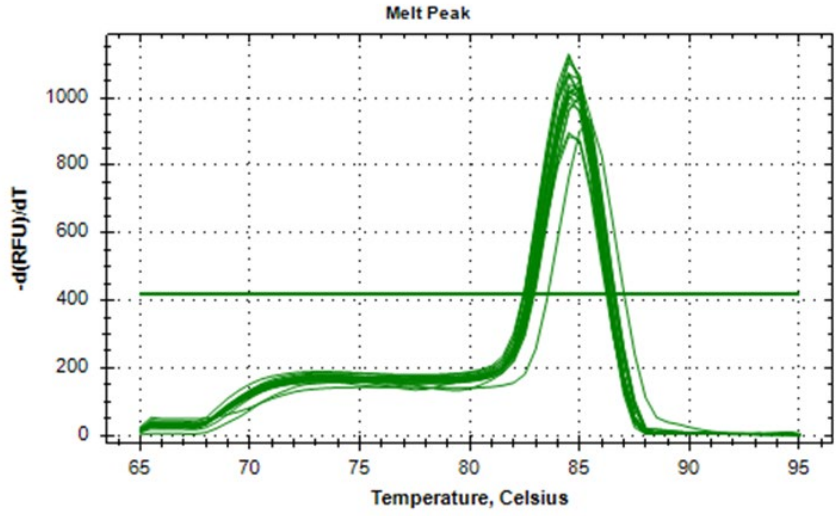

Figure B.5. Melt peak curve obtained during the SCAMP3 gene expression analysis after clearing gDNA contamination.

To confirm the clearance of gDNA contamination, the same PCR protocol as described above was performed, and the PCR products obtained were run in a 1% agarose gel (Figure B.6).

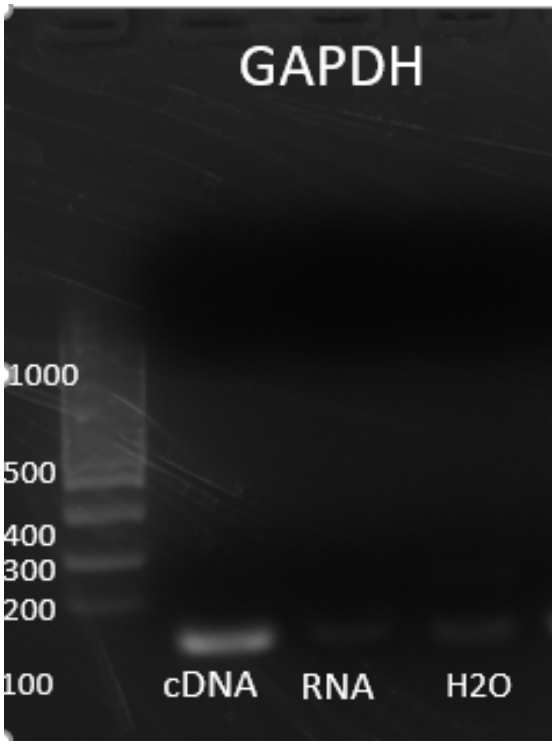

Figure B.6. Gel electrophoresis with cDNA, RNA and nuclease-free water. Only the cDNA template produced a band, which confirms that RNA samples are free of gDNA contamination.

After confirming the efficacy of the iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad, Hercules, California) to produce cDNA free of gDNA contamination, all the cDNA produced for the PCR assays during this work was performed using this kit.

APPENDIX C- Comparison of the cytokine and cellular profile of autologous protein solution, autologous conditioned serum, and serum incubated for 24 hours in healthy horses.

Ana Velloso Alvarez[1], Anne A. Wooldridge[1], Fred Caldwell[1], and Lindsey Boone[1]

[1] Department of Clinical Sciences, Auburn University, AL

Introduction:

Clinical use of autologous blood-derived intra-articular therapies such as autologous conditioned serum (ACS) or autologous protein solution (APS) has increased in horses. These products contain high concentrations of anti-inflammatory cytokines and growth factors meant to slow the catabolic degradation of articular cartilage in osteoarthritis (OA). Few studies have investigated the cytokine and growth factor composition of these products in equine blood or within horse, leaving equine practitioners' little guidance for their clinical use. The objective of this study was to measure the cellular composition and concentration of important OA modifying cytokines and growth factors in equine serum, ACS, and APS.

Methods:

Blood was obtained from 6 systemically healthy, adult horses. A complete blood count (CBC) was obtained from each horse immediately prior to blood collection. Blood was obtained and processed for each commercial product according to the manufacturer's instructions producing ACS and APS. ACS and APS were analyzed for white blood cell (WBC), red blood cell (RBC), and platelet (PLT) concentration. Additional blood was collected into plain glass vacutainers, incubated at 37°C for 24 hours, and then centrifuged to collect serum. Aliquots of all products (serum, ACS, and APS) were snap frozen and stored at -80°C until ELISA analysis. ELISA analysis was performed using commercially available kits for growth factor (TGF-β), anti-inflammatory (IL-1rap, and sTNF-R1), and pro-inflammatory (IL-1β, TNF-α, and MMP-3)

cytokines. Data was analyzed using a repeated measures one-way ANOVA and Tukey's post hoc analysis was performed. Significance was set at $p<0.05$.

Results

The WBC concentration of APS was increased compared to baseline blood ($p=0.0002$) and ACS ($p<0.0001$). The WBC concentration of ACS was decreased compared to baseline blood values ($p=0.0002$). The PLT concentration of APS was increased compared to baseline blood ($p=0.0309$) and ACS ($p<0.0001$). The PLT concentration of ACS was decreased compared to baseline blood ($p=0.0012$). The RBC concentration was increased in baseline blood compared to ACS ($p<0.0001$) and APS ($p=0.0002$) (Figure C.1).

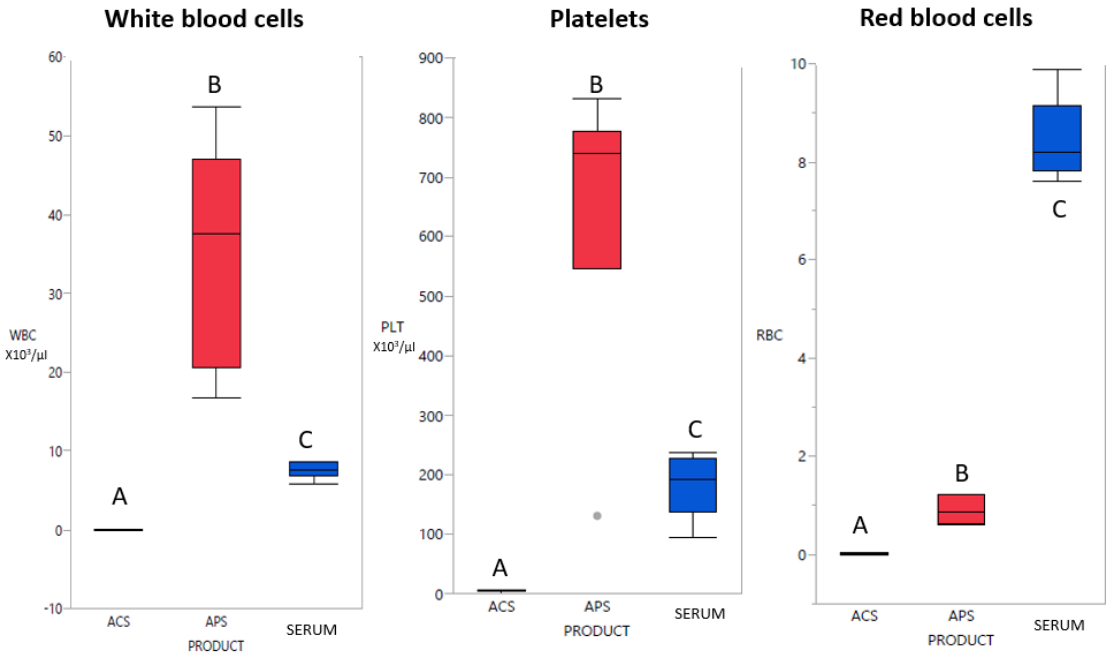

Figure C.1. Measurement of the cellular blood components in ACS, APS and serum incubated for 24 hours. The boxplots represent the interquartile range (IQR) of n = 6. The black lines represent the median values, the crosses represent the mean, and the whiskers represent the values outside the IQR. Different letters denote significant differences between products, $p<0.05$.

The concentrations of IL-1β, IL-1Ra, TNFα, IL-1β: IL-1Ra ratio, TNFα: sTNF-R1 ratio, and MMP3 were not different between incubated serum, ACS, and APS. However, sTNF-R1 was increased in incubated serum compared to APS (p=0.0289). The concentration of TGF-β was decreased in APS compared to ACS and incubated serum (p=0.0047 and p=0.0001 respectively).

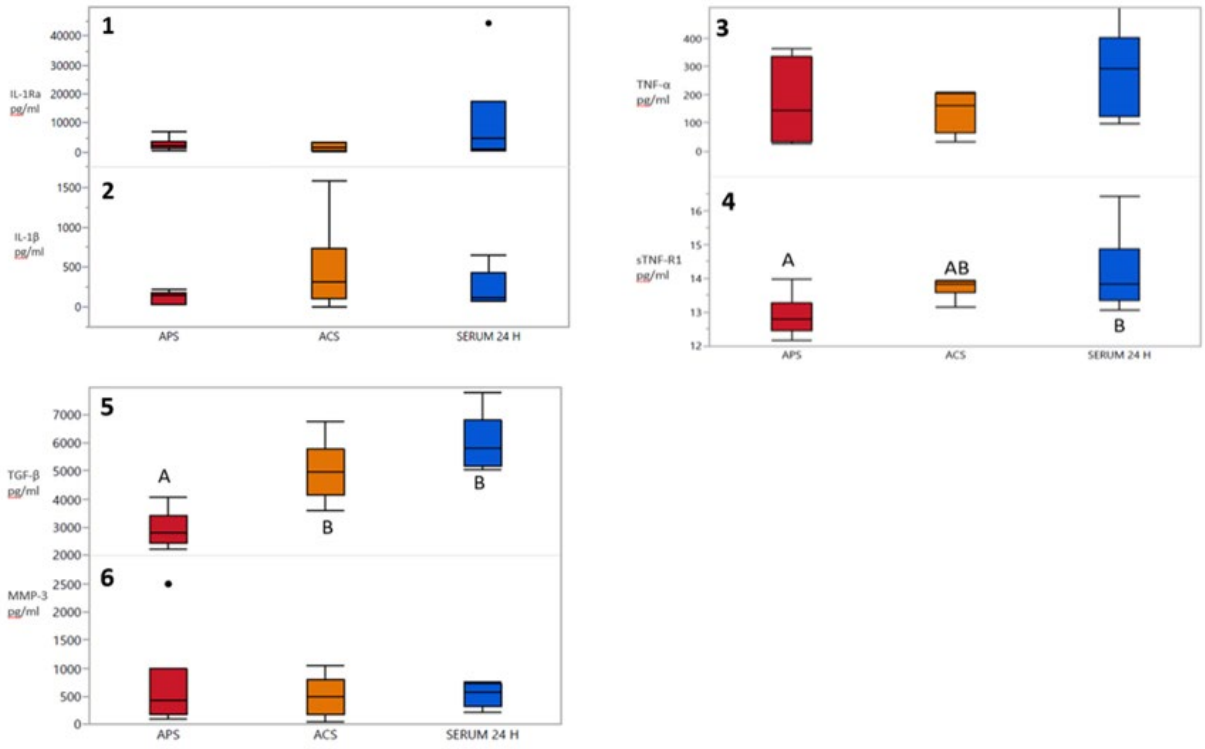

Figure C.2. Measurement of the cytokines (1) IL-1rap, (2) IL-1, (3) TNF-α, (4) sTNF-R1, (5) TGF-β and (6) MMP-3 in ACS, APS and serum incubated for 24 hours. The boxplots represent the interquartile range (IQR) of n = 6. The black lines represent the median values, the crosses represent the mean, and the whiskers represent the values outside the IQR. Different letters denote significant differences between products, $p<0.05$.

Conclusions

Despite the varied cellular composition of ACS and APS, there are few differences in cytokine concentrations despite processing methods. More importantly, whole blood incubation with processing of plain serum produces similar, if not better, cytokine and growth factor profiles compared to the commercial products. Further investigation into the use of incubated serum for treatment of joint-related injury in horses is warranted.

Acknowledgments.

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References

1. Rossdale PD, Hopes R, Digby NJ, et al. Epidemiological study of wastage among racehorses 1982 and 1983. *The Veterinary record* 1985;116:66-69.

2. Lidner A, Vonwittke P, Dingerkus A, et al. Causes and rate of training failure in Thoroughbreds. *Equine Athlete* 1992;5:1-7.

3. Jeffcott LB, Rossdale PD, Freestone J, et al. An assessment of wastage in thoroughbred racing from conception to 4 years of age. *Equine veterinary journal* 1982;14:185-198.

4. Johnson DFS. Synovial Joint Biology and Pathobiology In: Auer JA, Stick JA, Kummerle JM, et al., eds. *Equine Surgery (5th Edition)*. Saint Louis: W.B. Saunders, 2018;1326-1348.

5. Goodrich LR, Nixon AJ. Medical treatment of osteoarthritis in the horse - a review. *Veterinary journal (London, England : 1997)* 2006;171:51-69.

6. McIlwraith CW, Frisbie DD, Kawcak CE. The horse as a model of naturally occurring osteoarthritis. *Bone Joint Res* 2012;1:297-309.

7. Frisbie D, Johnson A. Synovial Joint Biology and Pathobiology In: Auer JA, Stick JA, Kummerle JM, et al., eds. *Equine Surgery (Fifth Edition)*. Saint Louis: W.B. Saunders, 2019;1326-1348.

8. van Weeren PR. General anatomy and physiology of joints. *Joint disease in the horse*: Elsevier, 2016;1-24.

9. Caron JP, Genovese RL. Principles and practices of joint disease treatment. *Diagnosis and Management of Lameness in the Horse*: Elsevier, 2003;746-764.

10. USDA L, Lameness V. Laminitis in US Horses. *USDA: APHIS: VS, CEAH, National Animal Health Monitoring System N* 2000;348.

11. McIlwraith CW. From arthroscopy to gene therapy--30 years of looking in joints. *Proceedings of the annual convention - American Association of Equine Practitioners* 2005;51:65-113.

12. Brandt KD, Radin EL, Dieppe PA, et al. Yet more evidence that osteoarthritis is not a cartilage disease. *Annals of the rheumatic diseases* 2006;65:1261-1264.

13. Lane NE, Brandt K, Hawker G, et al. OARSI-FDA initiative: defining the disease state of osteoarthritis. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2011;19:478-482.

14. McCarthy HE, Bara JJ, Brakspear K, et al. The comparison of equine articular cartilage progenitor cells and bone marrow-derived stromal cells as potential cell sources for cartilage repair in the horse. *Veterinary journal (London, England : 1997)* 2012;192:345-351.

15. Sophia Fox AJ, Bedi A, Rodeo SA. The basic science of articular cartilage: structure, composition, and function. *Sports Health* 2009;1:461-468.

16. Lorenz H, Richter W. Osteoarthritis: Cellular and molecular changes in degenerating cartilage. *Progress in Histochemistry and Cytochemistry* 2006;40:135-163.

17. van der Rest M, Garrone R. Collagen family of proteins. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 1991;5:2814-2823.

18. Vachon AM, Keeley FW, McIlwraith CW, et al. Biochemical analysis of normal articular cartilage in horses. *American journal of veterinary research* 1990;51:1905-1911.

19. McIlwraith C. Disease and problems of tendons, ligaments and related structures. *Adam's Lameness in the Horse*: Lippincott, Williams and Wilkins, 2002;594-640.

20. Ogston A. The biological functions of the glycosaminoglycans. *Chemistry and molecular biology of the intercellular matrix* 1970;3:1231-1240.

21. Heinegard D. Fell-Muir Lecture: Proteoglycans and more--from molecules to biology. *Int J Exp Pathol* 2009;90:575-586.

22. Aydelotte MB, Kuettner KE. Differences between sub-populations of cultured bovine articular chondrocytes. I. Morphology and cartilage matrix production. *Connect Tissue Res* 1988;18:205-222.

23. Henry S, Deasy B, Chu C. Articular cartilage: biology, biomechanics, and healing response. *Insall & Scott Surgery of the Knee 5th ed Philadelphia: Elsevier* 2012:146-154.

24. Mahjoub M, Berenbaum F, Houard X. Why subchondral bone in osteoarthritis? The importance of the cartilage bone interface in osteoarthritis. *Osteoporos Int* 2012;23 Suppl 8:S841-846.

25. Lyons TJ, McClure SF, Stoddart RW, et al. The normal human chondro-osseous junctional region: evidence for contact of uncalcified cartilage with subchondral bone and marrow spaces. *BMC Musculoskelet Disord* 2006;7:52.

26. Verzijl N, Bank RA, TeKoppele JM, et al. AGEing and osteoarthritis: a different perspective. *Current opinion in rheumatology* 2003;15.

27. Loeser RF, Yammani RR, Carlson CS, et al. Articular chondrocytes express the receptor for advanced glycation end products: Potential role in osteoarthritis. *Arthritis & Rheumatism* 2005;52:2376-2385.

28. Rasheed Z, Akhtar N, Haqqi TM. Advanced glycation end products induce the expression of interleukin-6 and interleukin-8 by receptor for advanced glycation end product-mediated activation of mitogen-activated protein kinases and nuclear factor-κB in human osteoarthritis chondrocytes. *Rheumatology (Oxford)* 2011;50:838-851.

29. Henderson B, Pettipher ER. The synovial lining cell: biology and pathobiology. *Semin Arthritis Rheum* 1985;15:1-32.

30. Scanzello CR, Goldring SR. The role of synovitis in osteoarthritis pathogenesis. *Bone* 2012;51:249-257.

31. Todhunter RJ. Anatomy and physiology of synovial joints. *Joint disease in the horse* 1996;1:1-28.

32. Oehler S, Neureiter D, Meyer-Scholten C, et al. Subtyping of osteoarthritic synoviopathy. *Clin Exp Rheumatol* 2002;20:633-640.

33. Tulamo RM, Heiskanen T, Salonen M. Concentration and molecular weight distribution of hyaluronate in synovial fluid from clinically normal horses and horses with diseased joints. *American journal of veterinary research* 1994;55:710-715.

34. Sellam J, Berenbaum F. The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis. *Nature reviews Rheumatology* 2010;6:625-635.

35. Berenbaum F. Osteoarthritis as an inflammatory disease (osteoarthritis is not osteoarthrosis!). *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2013;21:16-21.

36. Trumble TN, Trotter GW, Oxford JR, et al. Synovial fluid gelatinase concentrations and matrix metalloproteinase and cytokine expression in naturally occurring joint disease in horses. *American journal of veterinary research* 2001;62:1467-1477.

37. Branch M, Murray R, Dyson S, et al. Is there a characteristic distal tarsal subchondral bone plate thickness pattern in horses with no history of hindlimb lameness? *Equine veterinary journal* 2005;37:450-455.

38. Radin EL, Paul IL, Lowy M. A comparison of the dynamic force transmitting properties of subchondral bone and articular cartilage. *J Bone Joint Surg Am* 1970;52:444-456.

39. Kawcak CE, McIlwraith CW, Norrdin RW, et al. The role of subchondral bone in joint disease: a review. *Equine veterinary journal* 2001;33:120-126.

40. Sims NA, Martin TJ. Coupling the activities of bone formation and resorption: a multitude of signals within the basic multicellular unit. *Bonekey Rep* 2014;3:481.

41. Holmes JM, Mirams M, Mackie EJ, et al. Thoroughbred horses in race training have lower levels of subchondral bone remodelling in highly loaded regions of the distal metacarpus compared to horses resting from training. *Veterinary journal (London, England : 1997)* 2014;202:443-447.

42. Stewart HL, Kawcak CE. The Importance of Subchondral Bone in the Pathophysiology of Osteoarthritis. *Frontiers in veterinary science* 2018;5:178.

43. Radin EL, Rose RM. Role of subchondral bone in the initiation and progression of cartilage damage. *Clin Orthop Relat Res* 1986;213:34-40.

44. Lories RJ, Luyten FP. The bone-cartilage unit in osteoarthritis. *Nature reviews Rheumatology* 2011;7:43-49.

45. Kawcak CE, McIlwraith CW, Norrdin RW, et al. Clinical effects of exercise on subchondral bone of carpal and metacarpophalangeal joints in horses. *American journal of veterinary research* 2000;61:1252-1258.

46. Norrdin RW, Kawcak CE, Capwell BA, et al. Calcified cartilage morphometry and its relation to subchondral bone remodeling in equine arthrosis. *Bone* 1999;24:109-114.

47. Sanchez C, Deberg MA, Piccardi N, et al. Osteoblasts from the sclerotic subchondral bone downregulate aggrecan but upregulate metalloproteinases expression by chondrocytes. This effect is mimicked by interleukin-6, -1beta and oncostatin M pre-treated non-sclerotic osteoblasts. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2005;13:979-987.

48. Shibakawa A, Yudoh K, Masuko-Hongo K, et al. The role of subchondral bone resorption pits in osteoarthritis: MMP production by cells derived from bone marrow. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2005;13:679-687.

49. Walsh DA, Bonnet CS, Turner EL, et al. Angiogenesis in the synovium and at the osteochondral junction in osteoarthritis. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2007;15:743-751.

50. Pan J, Wang B, Li W, et al. Elevated cross-talk between subchondral bone and cartilage in osteoarthritic joints. *Bone* 2012;51:212-217.

51. Roudier M, Manner P, Simkin P. Tidemark Duplication In Osteoarthritis: Evidence Of Incremental Progression?: 80. *Arthritis & Rheumatism* 2013;65.

52. Zhu S, Zhu J, Zhen G, et al. Subchondral bone osteoclasts induce sensory innervation and osteoarthritis pain. *J Clin Invest* 2019;129:1076-1093.

53. Allen MR, Hock JM, Burr DB. Periosteum: biology, regulation, and response to osteoporosis therapies. *Bone* 2004;35:1003-1012.

54. McMahon SB, Koltzenburg M, Tracey I, et al. *Wall & melzack's textbook of pain e-book*: Elsevier Health Sciences, 2013.

55. Ito Y, Fitzsimmons J, Sanyal A, et al. Localization of chondrocyte precursors in periosteum. *Osteoarthritis and Cartilage* 2001;9:215-223.

56. Dubuc J, Girard C, Richard H, et al. Equine meniscal degeneration is associated with medial femorotibial osteoarthritis. *Equine veterinary journal* 2018;50:133-140.

57. Hunter W. Of the structure and disease of articulating cartilages. 1743. *Clin Orthop Relat Res* 1995;317:3-6.

58. Desjardins MR, Hurtig MB. Cartilage healing: A review with emphasis on the equine model. *The Canadian veterinary journal = La revue veterinaire canadienne* 1990;31:565-572.

59. McIlwraith CW. Traumatic arthritis and posttraumatic osteoarthritis in the horse. *Joint disease in the horse*: Elsevier, 2016;33-48.

60. Buckwalter J, Mau D. Cartilage repair in osteoarthritis In: Moskowitz RW HD, Goldberg VM, Mankin, HJ, ed. *Osteoarthritis: Diagnosis and medical/surgical management Philadelphia: Saunders*. Philadelpia PA: Saunders, 1992;71-107.

61. Hurtig MB, Fretz PB, Doige CE, et al. Effects of lesion size and location on equine articular cartilage repair. *Can J Vet Res* 1988;52:137-146.

62. Convery FR, Akeson WH, Keown GH. The repair of large osteochondral defects. An experimental study in horses. *Clin Orthop Relat Res* 1972;82:253-262.

63. Meachim G. The Effect of Scarification on Articular Cartilage in the Rabbit. *J Bone Joint Surg Br* 1963;45:150-161.

64. Armiento AR, Alini M, Stoddart MJ. Articular fibrocartilage - Why does hyaline cartilage fail to repair? *Adv Drug Deliv Rev* 2019;146:289-305.

65. Vachon AM, McIlwraith CW, Powers BE, et al. Morphologic and biochemical study of sternal cartilage autografts for resurfacing induced osteochondral defects in horses. *American journal of veterinary research* 1992;53:1038-1047.

66. Calandruccio RA, Gilmer WS. Proliferation, Regeneration, and Repair of Articular Cartilage of Immature Animals. *Journal of Bone and Joint Surgery-American Volume* 1962;44:431-455.

67. McIlwraith CW, Yovich JV, Martin GS. Arthroscopic surgery for the treatment of osteochondral chip fractures in the equine carpus. *Journal of the American Veterinary Medical Association* 1987;191:531-540.

68. McIlwraith CW, Fortier LA, Frisbie DD, et al. Equine Models of Articular Cartilage Repair. *Cartilage* 2011;2:317-326.

69. Fuerst M, Bertrand J, Lammers L, et al. Calcification of articular cartilage in human osteoarthritis. *Arthritis and rheumatism* 2009;60:2694-2703.

70. Loeser RF, Goldring SR, Scanzello CR, et al. Osteoarthritis: a disease of the joint as an organ. *Arthritis and rheumatism* 2012;64:1697-1707.

71. Goldring MB, Goldring SR. Osteoarthritis. *J Cell Physiol* 2007;213:626-634.

72. Knowlton RG, Katzenstein PL, Moskowitz RW, et al. Genetic linkage of a polymorphism in the type II procollagen gene (COL2A1) to primary osteoarthritis associated with mild chondrodysplasia. *N Engl J Med* 1990;322:526-530.

73. Caron JP. Osteoarthritis In: Ross MW,Dyson SJ, eds. *Diagnosis and Management of Lameness in the Horse*. Philadelphia: Saunders, 2011;665-668.

74. Goldring SR, Goldring MB. Changes in the osteochondral unit during osteoarthritis: structure, function and cartilage-bone crosstalk. *Nature reviews Rheumatology* 2016;12:632-644.

75. Teitelbaum SL, Bullough PG. The pathophysiology of bone and joint disease. *Am J Pathol* 1979;96:282-354.

76. Farahat MN, Yanni G, Poston R, et al. Cytokine expression in synovial membranes of patients with rheumatoid arthritis and osteoarthritis. *Annals of the rheumatic diseases* 1993;52:870-875.

77. Fernandes JC, Martel-Pelletier J, Pelletier JP. The role of cytokines in osteoarthritis pathophysiology. *Biorheology* 2002;39:237-246.

78. Hampel U, Sesselmann S, Iserovich P, et al. Chemokine and cytokine levels in osteoarthritis and rheumatoid arthritis synovial fluid. *J Immunol Methods* 2013;396:134-139.

79. Pelletier JP, DiBattista JA, Roughley P, et al. Cytokines and inflammation in cartilage degradation. *Rheum Dis Clin North Am* 1993;19:545-568.

80. Goldring MB. Osteoarthritis and cartilage: the role of cytokines. *Curr Rheumatol Rep* 2000;2:459-465.

81. Kapoor M, Martel-Pelletier J, Lajeunesse D, et al. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. *Nature reviews Rheumatology* 2011;7:33-42.

82. Jenei-Lanzl Z, Meurer A, Zaucke F. Interleukin-1beta signaling in osteoarthritis - chondrocytes in focus. *Cell Signal* 2019;53:212-223.

83. Schlueter A, Orth M. Equine osteoarthritis: a brief review of the disease and its causes. *Equine and Comparative Exercise Physiology* 2004;1:221-231.

84. Lee AS, Ellman MB, Yan D, et al. A current review of molecular mechanisms regarding osteoarthritis and pain. *Gene* 2013;527:440-447.

85. Wojdasiewicz P, Poniatowski LA, Szukiewicz D. The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of osteoarthritis. *Mediators Inflamm* 2014;2014:561459.

86. Henderson B, Pettipher ER. Arthritogenic actions of recombinant IL-1 and tumour necrosis factor alpha in the rabbit: evidence for synergistic interactions between cytokines in vivo. *Clin Exp Immunol* 1989;75.

87. Page Thomas DP, King B, Stephens T, et al. In vivo studies of cartilage regeneration after damage induced by catabolin/interleukin-1. *Annals of the rheumatic diseases* 1991;50:75-80.

88. Woodell-May J, Matuska A, Oyster M, et al. Autologous protein solution inhibits MMP-13 production by IL-1beta and TNFalpha-stimulated human articular chondrocytes. *Journal of*

orthopaedic research : official publication of the Orthopaedic Research Society 2011;29:1320-1326.

89. Goldring MB. Anticytokine therapy for osteoarthritis. *Expert Opin Biol Ther* 2001;1:817-829.

90. Joosten LA, Helsen MM, Saxne T, et al. IL-1 alpha beta blockade prevents cartilage and bone destruction in murine type II collagen-induced arthritis, whereas TNF-alpha blockade only ameliorates joint inflammation. *J Immunol* 1999;163:5049-5055.

91. Tung JT, Fenton JI, Arnold C, et al. Recombinant equine interleukin-1beta induces putative mediators of articular cartilage degradation in equine chondrocytes. *Can J Vet Res* 2002;66:19-25.

92. Boraschi D, Tagliabue A. The interleukin-1 receptor family. *Semin Immunol* 2013;25:394-407.

93. Arend WP. Interleukin-1 receptor antagonist. *Advances in immunology*: Elsevier, 1993;167-227.

94. Westacott CI, Atkins RM, Dieppe PA, et al. Tumor necrosis factor-alpha receptor expression on chondrocytes isolated from human articular cartilage. *J Rheumatol* 1994;21:1710-1715.

95. Westacott CI, Barakat AF, Wood L, et al. Tumor necrosis factor alpha can contribute to focal loss of cartilage in osteoarthritis. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2000;8:213-221.

96. Mengshol JA, Mix KS, Brinckerhoff CE. Matrix metalloproteinases as therapeutic targets in arthritic diseases - Bull's-eye or missing the mark? *Arthritis and rheumatism* 2002;46:13-20.

97. Choi MC, Jo J, Park J, et al. NF-kappaB Signaling Pathways in Osteoarthritic Cartilage Destruction. *Cells* 2019;8:734.

98. Burrage PS, Mix KS, Brinckerhoff CE. Matrix metalloproteinases: role in arthritis. *Frontiers in bioscience : a journal and virtual library* 2006;11:529-543.

99. Yoshihara Y, Nakamura H, Obata K, et al. Matrix metalloproteinases and tissue inhibitors of metalloproteinases in synovial fluids from patients with rheumatoid arthritis or osteoarthritis. *Annals of the rheumatic diseases* 2000;59:455-461.

100. Konttinen YT, Ainola M, Valleala H, et al. Analysis of 16 different matrix metalloproteinases (MMP-1 to MMP-20) in the synovial membrane: different profiles in trauma and rheumatoid arthritis. *Annals of the rheumatic diseases* 1999;58:691-697.

101. Verma P, Dalal K. ADAMTS-4 and ADAMTS-5: key enzymes in osteoarthritis. *J Cell Biochem* 2011;112:3507-3514.

102. Huang K, Wu LD. Aggrecanase and aggrecan degradation in osteoarthritis: a review. *J Int Med Res* 2008;36:1149-1160.

103. Tortorella MD, Malfait AM, Deccico C, et al. The role of ADAM-TS4 (aggrecanase-1) and ADAM-TS5 (aggrecanase-2) in a model of cartilage degradation. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2001;9:539-552.

104. Bau B, Gebhard PM, Haag J, et al. Relative messenger RNA expression profiling of collagenases and aggrecanases in human articular chondrocytes in vivo and in vitro. *Arthritis and rheumatism* 2002;46:2648-2657.

105. Pratta MA, Scherle PA, Yang G, et al. Induction of aggrecanase 1 (ADAM-TS4) by interleukin-1 occurs through activation of constitutively produced protein. *Arthritis and rheumatism* 2003;48:119-133.

106. David F, Farley J, Huang H, et al. Cytokine and chemokine gene expression of IL-1beta stimulated equine articular chondrocytes. *Veterinary surgery : VS* 2007;36:221-227.

107. Goldring SR, Goldring MB. The role of cytokines in cartilage matrix degeneration in osteoarthritis. *Clin Orthop Relat Res*, 2004.

108. Ma TW, Li Y, Wang GY, et al. Changes in Synovial Fluid Biomarkers after Experimental Equine Osteoarthritis. *J Vet Res* 2017;61:503-508.

109. Westacott CI, Sharif M. Cytokines in osteoarthritis: mediators or markers of joint destruction? *Semin Arthritis Rheum* 1996;25:254-272.

110. Takahashi A, de Andrés MC, Hashimoto K, et al. Epigenetic regulation of interleukin-8, an inflammatory chemokine, in osteoarthritis. *Osteoarthritis and cartilage* 2015;23:1946-1954.

111. Nietfeld JJ, Wilbrink B, Helle M, et al. Interleukin-1-induced interleukin-6 is required for the inhibition of proteoglycan synthesis by interleukin-1 in human articular cartilage. *Arthritis and rheumatism* 1990;33:1695-1701.

112. Suzuki M, Hashizume M, Yoshida H, et al. IL-6 and IL-1 synergistically enhanced the production of MMPs from synovial cells by up-regulating IL-6 production and IL-1 receptor I expression. *Cytokine* 2010;51:178-183.

113. Porée B, Kypriotou M, Chadjichristos C, et al. Interleukin-6 (IL-6) and/or soluble IL-6 receptor down-regulation of human type II collagen gene expression in articular chondrocytes requires a decrease of Sp1· Sp3 ratio and of the binding activity of both factors to the COL2A1 promoter. *Journal of Biological Chemistry* 2008;283:4850-4865.

114. Lotz M, Guerne PA. Interleukin-6 induces the synthesis of tissue inhibitor of metalloproteinases-1/erythroid potentiating activity (TIMP-1/EPA). *J Biol Chem* 1991;266:2017-2020.

115. Park JY, Pillinger MH, Abramson SB. Prostaglandin E2 synthesis and secretion: the role of PGE2 synthases. *Clin Immunol* 2006;119:229-240.

116. Bertone AL, Palmer JL, Jones J. Synovial fluid cytokines and eicosanoids as markers of joint disease in horses. *Veterinary surgery : VS* 2001;30:528-538.

117. Todhunter RJ, Lust G. Pathophysiology of Synovitis - Clinical Signs and Examination in Horses. *Compendium on Continuing Education for the Practicing Veterinarian* 1990;12:980-&.

118. Byron CR, Stewart MC, Stewart AA, et al. Effects of clinically relevant concentrations of glucosamine on equine chondrocytes and synoviocytes in vitro. *American journal of veterinary research* 2008;69:1129-1134.

119. Byron CR, Trahan RA. Comparison of the Effects of Interleukin-1 on Equine Articular Cartilage Explants and Cocultures of Osteochondral and Synovial Explants. *Frontiers in veterinary science* 2017;4:152.

120. Chubinskaya S, Wimmer MA. Key Pathways to Prevent Posttraumatic Arthritis for Future Molecule-Based Therapy. *Cartilage* 2013;4:13S-21S.

121. Goldring MB. The role of cytokines as inflammatory mediators in osteoarthritis: lessons from animal models. *Connect Tissue Res* 1999;40:1-11.

122. Goldring MB, Berenbaum F. The regulation of chondrocyte function by proinflammatory mediators: prostaglandins and nitric oxide. *Clin Orthop Relat Res* 2004;427:S37-46.

123. Goldring MB, Fukuo K, Birkhead JR, et al. Transcriptional suppression by interleukin-1 and interferon-gamma of type II collagen gene expression in human chondrocytes. *J Cell Biochem* 1994;54:85-99.

124. Riquet FB, Lai WF, Birkhead JR, et al. Suppression of type I collagen gene expression by prostaglandins in fibroblasts is mediated at the transcriptional level. *Mol Med* 2000;6:705-719.

125. Carrade Holt DD, Wood JA, Granick JL, et al. Equine mesenchymal stem cells inhibit T cell proliferation through different mechanisms depending on tissue source. *Stem cells and development* 2014;23:1258-1265.

126. Aigner T, Gebhard PM, Schmid E, et al. SOX9 expression does not correlate with type II collagen expression in adult articular chondrocytes. *Matrix Biology* 2003;22:363-372.

127. Saklatvala J. Tumour necrosis factor alpha stimulates resorption and inhibits synthesis of proteoglycan in cartilage. *Nature* 1986;322:547-549.

128. Alaaeddine N, Di Battista JA, Pelletier JP, et al. Inhibition of tumor necrosis factor alpha-induced prostaglandin E2 production by the antiinflammatory cytokines interleukin-4, interleukin-10, and interleukin-13 in osteoarthritic synovial fibroblasts: distinct targeting in the signaling pathways. *Arthritis and rheumatism* 1999;42:710-718.

129. Schlaak JF, Pfers I, Meyer Zum Buschenfelde KH, et al. Different cytokine profiles in the synovial fluid of patients with osteoarthritis, rheumatoid arthritis and seronegative spondylarthropathies. *Clin Exp Rheumatol* 1996;14:155-162.

130. Ishii H, Tanaka H, Katoh K, et al. Characterization of infiltrating T cells and Th1/Th2-type cytokines in the synovium of patients with osteoarthritis. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2002;10:277-281.

131. Yeh LA, Augustine AJ, Lee P, et al. Interleukin-4, an inhibitor of cartilage breakdown in bovine articular cartilage explants. *J Rheumatol* 1995;22:1740-1746.

132. van Lent PL, Holthuysen AE, Sloetjes A, et al. Local overexpression of adeno-viral IL-4 protects cartilage from metallo proteinase-induced destruction during immune complex-mediated arthritis by preventing activation of pro-MMPs. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2002;10:234-243.

133. van Meegeren ME, Roosendaal G, Jansen NW, et al. IL-4 alone and in combination with IL-10 protects against blood-induced cartilage damage. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2012;20:764-772.

134. Doi H, Nishida K, Yorimitsu M, et al. Interleukin-4 downregulates the cyclic tensile stress-induced matrix metalloproteinases-13 and cathepsin B expression by rat normal chondrocytes. *Acta Med Okayama* 2008;62:119-126.

135. Silvestri T, Pulsatelli L, Dolzani P, et al. Elevated serum levels of soluble interleukin-4 receptor in osteoarthritis. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2006;14:717-719.

136. Hart PH, Ahern MJ, Smith MD, et al. Comparison of the suppressive effects of interleukin-10 and interleukin-4 on synovial fluid macrophages and blood monocytes from patients with inflammatory arthritis. *Immunology* 1995;84:536-542.

137. Strassmann G, Patil-Koota V, Finkelman F, et al. Evidence for the involvement of interleukin 10 in the differential deactivation of murine peritoneal macrophages by prostaglandin E2. *J Exp Med* 1994;180:2365-2370.

138. Berg DJ, Zhang J, Lauricella DM, et al. Il-10 is a central regulator of cyclooxygenase-2 expression and prostaglandin production. *J Immunol* 2001;166:2674-2680.

139. Harizi H, Gualde N. Pivotal role of PGE2 and IL-10 in the cross-regulation of dendritic cell-derived inflammatory mediators. *Cellular & molecular immunology* 2006;3:271-277.

140. Jung YK, Kim GW, Park HR, et al. Role of interleukin-10 in endochondral bone formation in mice: anabolic effect via the bone morphogenetic protein/Smad pathway. *Arthritis and rheumatism* 2013;65:3153-3164.

141. van Meegeren ME, Roosendaal G, Coeleveld K, et al. A single intra-articular injection with IL-4 plus IL-10 ameliorates blood-induced cartilage degeneration in haemophilic mice. *Br J Haematol* 2013;160:515-520.

142. de Waal Malefyt R, Figdor CG, Huijbens R, et al. Effects of IL-13 on phenotype, cytokine production, and cytotoxic function of human monocytes. Comparison with IL-4 and modulation by IFN-gamma or IL-10. *J Immunol* 1993;151.

143. Arend WP, Welgus HG, Thompson RC, et al. Biological properties of recombinant human monocyte-derived interleukin 1 receptor antagonist. *J Clin Invest* 1990;85.

144. Wehling P, Moser C, Frisbie D, et al. Autologous conditioned serum in the treatment of orthopedic diseases: the orthokine therapy. *BioDrugs : clinical immunotherapeutics, biopharmaceuticals and gene therapy* 2007;21:323-332.

145. Lantz M, Gullberg U, Nilsson E, et al. Characterization in vitro of a human tumor necrosis factor-binding protein. A soluble form of a tumor necrosis factor receptor. *J Clin Invest* 1990;86:1396-1402.

146. Porteu F, Nathan C. Shedding of tumor necrosis factor receptors by activated human neutrophils. *J Exp Med* 1990;172:599-607.

147. Leeuwenberg JF, Jeunhomme TM, Buurman WA. Slow release of soluble TNF receptors by monocytes in vitro. *J Immunol* 1994;152:4036-4043.

148. Iannone F, Lapadula G. The pathophysiology of osteoarthritis. *Aging Clin Exp Res* 2003;15:364-372.

149. Van Zee KJ, Kohno T, Fischer E, et al. Tumor necrosis factor soluble receptors circulate during experimental and clinical inflammation and can protect against excessive tumor necrosis factor alpha in vitro and in vivo. *Proc Natl Acad Sci U S A* 1992;89:4845-4849.

150. Fioravanti A, Fabbroni M, Cerase A, et al. Treatment of erosive osteoarthritis of the hands by intra-articular infliximab injections: a pilot study. *Rheumatol Int* 2009;29:961-965.

151. Grunke M, Schulze-Koops H. Successful treatment of inflammatory knee osteoarthritis with tumour necrosis factor blockade. *Annals of the rheumatic diseases* 2006;65:555-556.

152. Su S, Grover J, Roughley PJ, et al. Expression of the tissue inhibitor of metalloproteinases (TIMP) gene family in normal and osteoarthritic joints. *Rheumatol Int* 1999;18:183-191.

153. Clutterbuck AL, Harris P, Allaway D, et al. Matrix metalloproteinases in inflammatory pathologies of the horse. *Veterinary journal (London, England : 1997)* 2010;183:27-38.

154. Baker AH, Edwards DR, Murphy G. Metalloproteinase inhibitors: biological actions and therapeutic opportunities. *J Cell Sci* 2002;115:3719-3727.

155. van der Kraan PM. Differential Role of Transforming Growth Factor-beta in an Osteoarthritic or a Healthy Joint. *J Bone Metab* 2018;25:65-72.

156. Blaney Davidson EN, van der Kraan PM, van den Berg WB. TGF-beta and osteoarthritis. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2007;15:597-604.

157. Freyria AM, Mallein-Gerin F. Chondrocytes or adult stem cells for cartilage repair: the indisputable role of growth factors. *Injury* 2012;43:259-265.

158. Darling EM, Athanasiou KA. Growth factor impact on articular cartilage subpopulations. *Cell Tissue Res* 2005;322:463-473.

159. Sun Y, Feng Y, Zhang CQ, et al. The regenerative effect of platelet-rich plasma on healing in large osteochondral defects. *Int Orthop* 2010;34:589-597.

160. van Beuningen HM, van der Kraan PM, Arntz OJ, et al. Transforming growth factor-beta 1 stimulates articular chondrocyte proteoglycan synthesis and induces osteophyte formation in the murine knee joint. *Lab Invest* 1994;71:279-290.

161. van Beuningen HM, van der Kraan PM, Arntz OJ, et al. In vivo protection against interleukin-1-induced articular cartilage damage by transforming growth factor-beta 1: age-related differences. *Annals of the rheumatic diseases* 1994;53:593-600.

162. Mueller MB, Tuan RS. Anabolic/Catabolic balance in pathogenesis of osteoarthritis: identifying molecular targets. *PM & R : the journal of injury, function, and rehabilitation* 2011;3:S3-11.

163. Ongchai S, Somnoo O, Kongdang P, et al. TGF-β1 upregulates the expression of hyaluronan synthase 2 and hyaluronan synthesis in culture models of equine articular chondrocytes. *J Vet Sci* 2018;19:735-743.

164. Bakker AC, van de Loo FA, van Beuningen HM, et al. Overexpression of active TGF-beta-1 in the murine knee joint: evidence for synovial-layer-dependent chondro-osteophyte formation. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2001;9:128-136.

165. Wei FY, Lee JK, Wei L, et al. Correlation of insulin-like growth factor 1 and osteoarthritic cartilage degradation: a spontaneous osteoarthritis in guinea-pig. *Eur Rev Med Pharmacol Sci* 2017;21:4493-4500.

166. Studer RK, Levicoff E, Georgescu H, et al. Nitric oxide inhibits chondrocyte response to IGF-I: inhibition of IGF-IRbeta tyrosine phosphorylation. *Am J Physiol Cell Physiol* 2000;279:C961-969.

167. Luyten FP, Hascall VC, Nissley SP, et al. Insulin-like growth factors maintain steady-state metabolism of proteoglycans in bovine articular cartilage explants. *Arch Biochem Biophys* 1988;267:416-425.

168. Morisset S, Frisbie DD, Robbins PD, et al. IL-1ra/IGF-1 gene therapy modulates repair of microfractured chondral defects. *Clin Orthop Relat Res* 2007;462:221-228.

169. Fukui N, Zhu Y, Maloney WJ, et al. Stimulation of BMP-2 expression by pro-inflammatory cytokines IL-1 and TNF-alpha in normal and osteoarthritic chondrocytes. *J Bone Joint Surg Am* 2003;85-A Suppl 3:59-66.

170. Fortier LA, Barker JU, Strauss EJ, et al. The role of growth factors in cartilage repair. *Clin Orthop Relat Res* 2011;469:2706-2715.

171. Loeuille D, Chary-Valckenaere I, Champigneulle J, et al. Macroscopic and microscopic features of synovial membrane inflammation in the osteoarthritic knee: correlating magnetic resonance imaging findings with disease severity. *Arthritis and rheumatism* 2005;52:3492-3501.

172. Iagnocco A, Filippucci E, Ossandon A, et al. High resolution ultrasonography in detection of bone erosions in patients with hand osteoarthritis. *J Rheumatol* 2005;32:2381-2383.

173. McIlwraith CW, Frisbie DD, Kawcak CE, et al. The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the horse. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2010;18 Suppl 3:S93-105.

174. Takano S, Uchida K, Miyagi M, et al. Synovial macrophage-derived IL-1beta regulates the calcitonin receptor in osteoarthritic mice. *Clin Exp Immunol* 2016;183:143-149.

175. Sun AR, Friis T, Sekar S, et al. Is synovial macrophage activation the inflammatory link between obesity and osteoarthritis? *Current rheumatology reports* 2016;18:57.

176. Chen Y, Jiang W, Yong H, et al. Macrophages in osteoarthritis: pathophysiology and therapeutics. *Am J Transl Res* 2020;12:261-268.

177. Bondeson J, Blom AB, Wainwright S, et al. The role of synovial macrophages and macrophage-produced mediators in driving inflammatory and destructive responses in osteoarthritis. *Arthritis and rheumatism* 2010;62:647-657.

178. Bondeson J, Wainwright SD, Lauder S, et al. The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other destructive and inflammatory responses in osteoarthritis. *Arthritis Res Ther* 2006;8:R187.

179. Manferdini C, Paolella F, Gabusi E, et al. From osteoarthritic synovium to synovial-derived cells characterization: synovial macrophages are key effector cells. *Arthritis Res Ther* 2016;18:83.

180. Shapouri-Moghaddam A, Mohammadian S, Vazini H, et al. Macrophage plasticity, polarization, and function in health and disease. *J Cell Physiol* 2018;233:6425-6440.

181. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 2008;8:958-969.

182. Kraus VB, McDaniel G, Huebner JL, et al. Direct in vivo evidence of activated macrophages in human osteoarthritis. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2016;24:1613-1621.

183. Utomo L, van Osch GJ, Bayon Y, et al. Guiding synovial inflammation by macrophage phenotype modulation: an in vitro study towards a therapy for osteoarthritis. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2016;24:1629-1638.

184. Neogi T. The epidemiology and impact of pain in osteoarthritis. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2013;21:1145-1153.

185. Wallace IJ, Worthington S, Felson DT, et al. Knee osteoarthritis has doubled in prevalence since the mid-20th century. *Proceedings of the National Academy of Sciences* 2017;114:9332-9336.

186. Nguyen US, Zhang Y, Zhu Y, et al. Increasing prevalence of knee pain and symptomatic knee osteoarthritis: survey and cohort data. *Ann Intern Med* 2011;155:725-732.

187. Murray CJ, Atkinson C, Bhalla K, et al. The state of US health, 1990-2010: burden of diseases, injuries, and risk factors. *JAMA* 2013;310:591-608.

188. Neundorf RH, Lowerison MB, Cruz AM, et al. Determination of the prevalence and severity of metacarpophalangeal joint osteoarthritis in Thoroughbred racehorses via quantitative macroscopic evaluation. *American journal of veterinary research* 2010;71:1284-1293.

189. Ireland JL, Clegg PD, McGowan CM, et al. Disease prevalence in geriatric horses in the United Kingdom: veterinary clinical assessment of 200 cases. *Equine veterinary journal* 2012;44:101-106.

190. Ahern BJ, Parvizi J, Boston R, et al. Preclinical animal models in single site cartilage defect testing: a systematic review. *Osteoarthritis and Cartilage* 2009;17:705-713.

191. Frisbie D, Cross M, McIlwraith C. A comparative study of articular cartilage thickness in the stifle of animal species used in human pre-clinical studies compared to articular cartilage thickness in the human knee. *Veterinary and comparative orthopaedics and traumatology* 2006;19:142-146.

192. Malda J, Benders KE, Klein TJ, et al. Comparative study of depth-dependent characteristics of equine and human osteochondral tissue from the medial and lateral femoral condyles. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2012;20:1147-1151.

193. Johnson CI, Argyle DJ, Clements DN. In vitro models for the study of osteoarthritis. *Veterinary journal (London, England : 1997)* 2016;209:40-49.

194. Madden JC, Hewitt M, Przybylak K, et al. Strategies for the optimisation of in vivo experiments in accordance with the 3Rs philosophy. *Regulatory Toxicology and Pharmacology* 2012;63:140-154.

195. Von Der Mark K, Gauss V, Von Der Mark H, et al. Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. *Nature* 1977;267:531-532.

196. Linardi RL, Dodson ME, Moss KL, et al. The Effect of Autologous Protein Solution on the Inflammatory Cascade in Stimulated Equine Chondrocytes. *Frontiers in veterinary science* 2019;6:64.

197. Gilbertie JM, Long JM, Schubert AG, et al. Pooled Platelet-Rich Plasma Lysate Therapy Increases Synoviocyte Proliferation and Hyaluronic Acid Production While Protecting Chondrocytes From Synoviocyte-Derived Inflammatory Mediators. *Frontiers in veterinary science* 2018;5.

198. Hemphill DD, McIlwraith CW, Slayden RA, et al. Adeno-associated virus gene therapy vector scAAVIGF-I for transduction of equine articular chondrocytes and RNA-seq analysis. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2016;24:902-911.

199. Martins MC, Peffers MJ, Lee K, et al. Effects of stanozolol on normal and IL-1β-stimulated equine chondrocytes in vitro. *BMC veterinary research* 2018;14:1-7.

200. Caron JP, Gandy JC, Brown JL, et al. Omega-3 fatty acids and docosahexaenoic acid oxymetabolites modulate the inflammatory response of equine recombinant interleukin1β-stimulated equine synoviocytes. *Prostaglandins & Other Lipid Mediators* 2019;142:1-8.

201. Pezzanite L, Chow L, Piquini G, et al. Use of in vitro assays to identify antibiotics that are cytotoxic to normal equine chondrocytes and synovial cells. *Equine veterinary journal* 2020;n/a.

202. Dechant JE, Baxter G, Frisbie D, et al. Effects of glucosamine hydrochloride and chondroitin sulphate, alone and in combination, on normal and interleukin-1 conditioned equine articular cartilage explant metabolism. *Equine veterinary journal* 2005;37:227-231.

203. Dechant JE, Baxter GM, Frisbie DD, et al. Effects of dosage titration of methylprednisolone acetate and triamcinolone acetonide on interleukin-1-conditioned equine articular cartilage explants in vitro. *Equine veterinary journal* 2003;35:444-450.

204. Moses VS, Hardy J, Bertone AL, et al. Effects of anti-inflammatory drugs on lipopolysaccharide-challenged and-unchallenged equine synovial explants. *American journal of veterinary research* 2001;62:54-60.

205. Von Rechenberg B, McIlwraith C, Akens M, et al. Spontaneous production of nitric oxide (NO), prostaglandin (PGE2) and neutral metalloproteinases (NMPs) in media of explant cultures of equine synovial membrane and articular cartilage from normal and osteoarthritic joints. *Equine veterinary journal* 2000;32:140-150.

206. Bertuglia A, Lacourt M, Girard C, et al. Osteoclasts are recruited to the subchondral bone in naturally occurring post-traumatic equine carpal osteoarthritis and may contribute to cartilage degradation. *Osteoarthritis and Cartilage* 2016;24:555-566.

207. Haltmayer E, Ribitsch I, Gabner S, et al. Co-culture of osteochondral explants and synovial membrane as in vitro model for osteoarthritis. *PloS one* 2019;14:e0214709.

208. Velloso Alvarez A, Boone LH, Pondugula SR, et al. Effects of Autologous Conditioned Serum, Autologous Protein Solution, and Triamcinolone on Inflammatory and Catabolic Gene Expression in Equine Cartilage and Synovial Explants Treated With IL-1β in Co-culture. *Frontiers in veterinary science* 2020;7.

209. Kjelgaard-Petersen C, Bay-Jensen A-C, Christiansen T, et al. Establishment of a Human Synovium and Cartilage Co-culture. *Osteoarthritis and Cartilage* 2017;25:S276-S277.

210. Beekhuizen M, Bastiaansen-Jenniskens YM, Koevoet W, et al. Osteoarthritic synovial tissue inhibition of proteoglycan production in human osteoarthritic knee cartilage: establishment and

characterization of a long-term cartilage-synovium coculture. *Arthritis and rheumatism* 2011;63:1918-1927.

211. Mehta S, Akhtar S, Porter RM, et al. Interleukin-1 receptor antagonist (IL-1Ra) is more effective in suppressing cytokine-induced catabolism in cartilage-synovium co-culture than in cartilage monoculture. *Arthritis Research & Therapy* 2019;21:238.

212. Haupt JL, Frisbie DD, McIlwraith CW, et al. Dual transduction of insulin-like growth factor-I and interleukin-l receptor antagonist protein controls cartilage degradation in an osteoarthritic culture model. *Journal of Orthopaedic Research* 2005;23:118-126.

213. Tallheden T, Karlsson C, Brunner A, et al. Gene expression during redifferentiation of human articular chondrocytes. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2004;12:525-535.

214. Rosser J, Bachmann B, Jordan C, et al. Microfluidic nutrient gradient–based three-dimensional chondrocyte culture-on-a-chip as an in vitro equine arthritis model. *Materials Today Bio* 2019;4:100023.

215. Sun HB. Mechanical loading, cartilage degradation, and arthritis. *Annals of the New York Academy of Sciences* 2010;1211:37-50.

216. Henrotin Y, Pesesse L, Sanchez C. Identification of Mechanosensitive Genes in Chondrocytes and Osteoblasts and Their Role in OA Pathogenesis In: Kamkin A,Kiseleva I, eds. *Mechanical Stretch and Cytokines*. Dordrecht: Springer Netherlands, 2012;223-233.

217. Fitzgerald JB, Jin M, Chai DH, et al. Shear-and compression-induced chondrocyte transcription requires MAPK activation in cartilage explants. *Journal of Biological Chemistry* 2008;283:6735-6743.

218. Sanchez C, Gabay O, Salvat C, et al. Mechanical loading highly increases IL-6 production and decreases OPG expression by osteoblasts. *Osteoarthritis and Cartilage* 2009;17:473-481.

219. Liu XH, Kirschenbaum A, Yao S, et al. Interactive effect of interleukin-6 and prostaglandin E2 on osteoclastogenesis via the OPG/RANKL/RANK system. *Annals of the New York Academy of Sciences* 2006;1068:225-233.

220. Bakker A, Klein-Nulend J, Tanck E, et al. Different responsiveness to mechanical stress of bone cells from osteoporotic versus osteoarthritic donors. *Osteoporosis international* 2006;17:827-833.

221. Gregg AJ, Fortier LA, Mohammed HO, et al. Assessment of the catabolic effects of interleukin-1beta on proteoglycan metabolism in equine cartilage cocultured with synoviocytes. *American journal of veterinary research* 2006;67:957-962.

222. Takafuji VA, McIlwraith CW, Howard RD. Effects of equine recombinant interleukin-1α and interleukin-1β on proteoglycan metabolism and prostaglandin E2 synthesis in equine articular cartilage explants. *American journal of veterinary research* 2002;63:551-558.

223. Byron CR, Barger AM, Stewart AA, et al. In vitro expression of receptor activator of nuclear factor-κB ligand and osteoprotegerin in cultured equine articular cells. *American journal of veterinary research* 2010;71:615-622.

224. Anderson JR, Phelan MM, Foddy L, et al. Ex-Vivo Equine Cartilage Explant Osteoarthritis Model - A Metabolomics and Proteomics Study. *bioRxiv* 2020:2020.2003.2003.974501.

225. Little CB, Flannery CR, Hughes CE, et al. Cytokine induced metalloproteinase expression and activity does not correlate with focal susceptibility of articular cartilage to degeneration. *Osteoarthritis and Cartilage* 2005;13:162-170.

226. Hoff P, Buttgereit F, Burmester G-R, et al. Osteoarthritis synovial fluid activates pro-inflammatory cytokines in primary human chondrocytes. *International Orthopaedics* 2013;37:145-151.

227. Cook JL, Kuroki K, Stoker A, et al. Review of in vitro models and development and initial validation of a novel co-culture model for the study of osteoarthritis. *Current Rheumatology Reviews* 2007;3:172-182.

228. Cope PJ, Ourradi K, Li Y, et al. Models of osteoarthritis: the good, the bad and the promising. *Osteoarthritis and Cartilage* 2019;27:230-239.

229. Kuyinu EL, Narayanan G, Nair LS, et al. Animal models of osteoarthritis: classification, update, and measurement of outcomes. *Journal of orthopaedic surgery and research* 2016;11:1-27.

230. Teeple E, Jay GD, Elsaid KA, et al. Animal Models of Osteoarthritis: Challenges of Model Selection and Analysis. *The AAPS journal* 2013;15:438-446.

231. Gregory MH, Capito N, Kuroki K, et al. A review of translational animal models for knee osteoarthritis. *Arthritis* 2012;2012.

232. Strauss EJ, Goodrich LR, Chen C-T, et al. Biochemical and Biomechanical Properties of Lesion and Adjacent Articular Cartilage after Chondral Defect Repair in an Equine Model. *The American journal of sports medicine* 2005;33:1647-1653.

233. Pritzker KP, Gay S, Jimenez SA, et al. Osteoarthritis cartilage histopathology: grading and staging. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2006;14:13-29.

234. Little CB, Smith MM. Animal models of osteoarthritis. *Current Rheumatology Reviews* 2008;4:175-182.

235. Frisbie DD, Al-Sobayil F, Billinghurst RC, et al. Changes in synovial fluid and serum biomarkers with exercise and early osteoarthritis in horses. *Osteoarthritis and Cartilage* 2008;16:1196-1204.

236. Frisbie DD, Ghivizzani SC, Robbins PD, et al. Treatment of experimental equine osteoarthritis by in vivo delivery of the equine interleukin-1 receptor antagonist gene. *Gene Ther* 2002;9:12-20.

237. Frisbie DD, Kawcak CE, Werpy NM, et al. Clinical, biochemical, and histologic effects of intra-articular administration of autologous conditioned serum in horses with experimentally induced osteoarthritis. *American journal of veterinary research* 2007;68:290-296.

238. Lampropoulou-Adamidou K, Lelovas P, Karadimas EV, et al. Useful animal models for the research of osteoarthritis. *European Journal of Orthopaedic Surgery & Traumatology* 2014;24:263-271.

239. McIlwraith CW, Van Sickle DC. Experimentally induced arthritis of the equine carpus: histologic and histochemical changes in the articular cartilage. *American journal of veterinary research* 1981;42:209-217.

240. Gustafson S, Trotter G, Norrdin R, et al. Evaluation of intra-articularly administered sodium monoiodoacetate-induced chemical injury to articular cartilage of horses. *American journal of veterinary research* 1992;53:1193.

241. Trotter GW, Yovich JV, McIlwraith CW, et al. Effects of intramuscular polysulfated glycosaminoglycan on chemical and physical defects in equine articular cartilage. *Can J Vet Res* 1989;53:224-230.

242. Ross TN, Kisiday JD, Hess T, et al. Evaluation of the inflammatory response in experimentally induced synovitis in the horse: a comparison of recombinant equine interleukin 1

beta and lipopolysaccharide. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2012;20:1583-1590.

243. Auer JA, Fackelman GE, Gingerich DA, et al. Effect of hyaluronic acid in naturally occurring and experimentally induced osteoarthritis. *American journal of veterinary research* 1980;41:568-574.

244. Harreveld PDv, Lillich JD, Kawcak CE, et al. Clinical evaluation of the effects of immobilization followed by remobilization and exercise on the metacarpophalangeal joint in horses. *Journal of the American Veterinary Medical Association* 2002;63:282-288.

245. Bolam CJ, Hurtig MB, Cruz A, et al. Characterization of experimentally induced post-traumatic osteoarthritis in the medial femorotibial joint of horses. *American journal of veterinary research* 2006;67:433-447.

246. Boyce MK, Trumble TN, Carlson CS, et al. Non-terminal animal model of post-traumatic osteoarthritis induced by acute joint injury. *Osteoarthritis and Cartilage* 2013;21:746-755.

247. Kawcak CE, Norrdin RW, Frisbie DD, et al. Effects of osteochondral fragmentation and intra-articular triamcinolone acetonide treatment on subchondral bone in the equine carpus. *Equine veterinary journal* 1998;30:66-71.

248. Frisbie DD, Kawcak CE, Baxter GM, et al. Effects of 6alpha-methylprednisolone acetate on an equine osteochondral fragment exercise model. *American journal of veterinary research* 1998;59:1619-1628.

249. Frisbie DD, Kawcak CE, McIlwraith CW. Evaluation of autologous conditioned serum using an experimental model of equine osteoarthritis. Proceedings of the 51st Annual Convention of the American Association of Equine Practitioners, Seattle, Washington, USA, 3-7 December, 2005 2005;374-375.

250. Simmons EJ, Bertone AL, Weisbrode SE. Instability-induced osteoarthritis in the metacarpophalangeal joint of horses. *American journal of veterinary research* 1999;60:7-13.

251. Maninchedda U, Lepage OM, Gangl M, et al. Development of an equine groove model to induce metacarpophalangeal osteoarthritis: a pilot study on 6 horses. *PloS one* 2015;10:e0115089.

252. Broeckx SY, Pille F, Buntinx S, et al. Evaluation of an osteochondral fragment–groove procedure for induction of metacarpophalangeal joint osteoarthritis in horses. *American journal of veterinary research* 2019;80:246-258.

253. Peloso J, Stick J, Caron J, et al. Effects of hylan on amphotericin-induced carpal lameness in equids. *American journal of veterinary research* 1993;54:1527-1534.

254. Palmer JL, Bertone AL. Experimentally-induced synovitis as a model for acute synovitis in the horse. *Equine veterinary journal* 1994;26:492-495.

255. Palmer JL, Bertone AL, Malemud CJ, et al. Biochemical and biomechanical alterations in equine articular cartilage following an experimentally-induced synovitis. *Osteoarthritis and Cartilage* 1996;4:127-137.

256. Hardy J, Bertone AL, Weisbrode SE, et al. Cell trafficking, mediator release, and articular metabolism in acute inflammation of innervated or denervated isolated equine joints. *American journal of veterinary research* 1998;59:88-100.

257. Colbath AC, Dow SW, Hopkins LS, et al. Induction of Synovitis Using Interleukin-1 Beta: Are There Differences in the Response of Middle Carpal Joint Compared to the Tibiotarsal Joint? *Frontiers in veterinary science* 2018;5:208.

258. Cornelissen BP, Rijkenhuizen AB, van den Hoogen BM, et al. Experimental model of synovitis/capsulitis in the equine metacarpophalangeal joint. *American journal of veterinary research* 1998;59:978-985.

259. Owens JG, Kamerling SG, Stanton SR, et al. Effects of pretreatment with ketoprofen and phenylbutazone on experimentally induced synovitis in horses. *American journal of veterinary research* 1996;57:866-874.

260. Turley SM, Thambyah A, Riggs CM, et al. Microstructural changes in cartilage and bone related to repetitive overloading in an equine athlete model. *Journal of Anatomy* 2014;224:647-658.

261. Norrdin RW, Kawcak CE, Capwell BA, et al. Subchondral Bone Failure in an Equine Model of Overload Arthrosis. *Bone* 1998;22:133-139.

262. Little CB, Hunter DJ. Post-traumatic osteoarthritis: from mouse models to clinical trials. *Nature reviews Rheumatology* 2013;9:485-497.

263. Felson DT. The sources of pain in knee osteoarthritis. *Current opinion in rheumatology* 2005;17:624-628.

264. Keegan KG, Dent EV, Wilson DA, et al. Repeatability of subjective evaluation of lameness in horses. *Equine veterinary journal* 2010;42:92-97.

265. Fuller CJ, Bladon BM, Driver AJ, et al. The intra- and inter-assessor reliability of measurement of functional outcome by lameness scoring in horses. *Veterinary journal (London, England : 1997)* 2006;171:281-286.

266. Arkell M, Archer RM, Guitian FJ, et al. Evidence of bias affecting the interpretation of the results of local anaesthetic nerve blocks when assessing lameness in horses. *The Veterinary record* 2006;159:346-349.

267. Keegan KG, MacAllister CG, Wilson DA, et al. Comparison of an inertial sensor system with a stationary force plate for evaluation of horses with bilateral forelimb lameness. *American journal of veterinary research* 2012;73:368-374.

268. Donnell JR, Frisbie DD, King MR, et al. Comparison of subjective lameness evaluation, force platforms and an inertial-sensor system to identify mild lameness in an equine osteoarthritis model. *The Veterinary Journal* 2015;206:136-142.

269. Kawcak CE, Frisbie DD, Werpy NM, et al. Effects of exercise vs experimental osteoarthritis on imaging outcomes. *Osteoarthritis and Cartilage* 2008;16:1519-1525.

270. Denoix J-M, Audigie; AgF. Ultrasonographic examination of joints in horses. American Association of Equine Practitioners (AAEP) Convention 2001;366-375.

271. Dyson S. Subjective and quantitative scintigraphic assessment of the equine foot and its relationship with foot pain. *Equine veterinary journal* 2002;34:164-170.

272. Bailey RE, Dyson SJ, Parkin TD. Focal increased radiopharmaceutical uptake in the dorsoproximal diaphyseal region of the equine proximal phalanx. *Veterinary Radiology & Ultrasound* 2007;48:460-466.

273. Spriet M, Espinosa-Mur P, Cissell DD, et al. 18F-sodium fluoride positron emission tomography of the racing Thoroughbred fetlock: Validation and comparison with other imaging modalities in nine horses. *Equine veterinary journal* 2019;51:375-383.

274. Li Q, Amano K, Link TM, et al. Advanced Imaging in Osteoarthritis. *Sports health* 2016;8:418-428.

275. Tranquille C, Murray R, Parkin T. Can we use subchondral bone thickness on high-field magnetic resonance images to identify Thoroughbred racehorses at risk of catastrophic lateral condylar fracture? *Equine veterinary journal* 2017;49:167-171.

276. Tranquille CA, Parkin TD, Murray RC. Magnetic resonance imaging-detected adaptation and pathology in the distal condyles of the third metacarpus, associated with lateral condylar fracture in Thoroughbred racehorses. *Equine veterinary journal* 2012;44:699-706.

277. Nelson BB, Kawcak CE, Goodrich LR, et al. Comparison between computed tomographic arthrography, radiography, ultrasonography, and arthroscopy for the diagnosis of femorotibial joint disease in western performance horses. *Veterinary Radiology & Ultrasound* 2016;57:387-402.

278. Murray RC, Branch MV, Tranquille C, et al. Validation of magnetic resonance imaging for measurement of equine articular cartilage and subchondral bone thickness. *American journal of veterinary research* 2005;66:1999-2005.

279. Woertler K, Strothmann M, Tombach B, et al. Detection of articular cartilage lesions: Experimental evaluation of low- and high-field-strength MR imaging at 0.18 and 1.0 T. *Journal of Magnetic Resonance Imaging* 2000;11:678-685.

280. Rovel T, Audigié F, Coudry V, et al. Evaluation of standing low-field magnetic resonance imaging for diagnosis of advanced distal interphalangeal primary degenerative joint disease in horses: 12 cases (2010–2014). *Journal of the American Veterinary Medical Association* 2019;254:257-265.

281. Young BD, Samii VF, Mattoon JS, et al. Subchondral bone density and cartilage degeneration patterns in osteoarthritic metacarpal condyles of horses. *American journal of veterinary research* 2007;68:841-849.

282. Vanderperren K, Ghaye B, Snaps FR, et al. Evaluation of computed tomographic anatomy of the equine metacarpophalangeal joint. *American journal of veterinary research* 2008;69:631-638.

283. Hadj-Salah M, Mourali S, Abdelali M, et al. Spiral computed tomographic (CT) arthrography versus arthroscopy in internal derangements of the knee. *La Tunisie medicale* 2006;84:734-737.

284. Crijns CP, Gielen IM, van Bree HJ, et al. The use of CT and CT arthrography in diagnosing equine stifle injury in a Rheinlander gelding. *Equine veterinary journal* 2010;42:367-371.

285. Suarez Sanchez-Andrade J, Richter H, Kuhn K, et al. Comparison between magnetic resonance imaging, computed tomography, and arthrography to identify artificially induced cartilage defects of the equine carpal joints. *Veterinary Radiology & Ultrasound* 2018;59:312-325.

286. Nixon AJ, Grol MW, Lang HM, et al. Disease-Modifying Osteoarthritis Treatment With Interleukin-1 Receptor Antagonist Gene Therapy in Small and Large Animal Models. *Arthritis & Rheumatology* 2018;70:1757-1768.

287. Nelson BB, Mäkelä JTA, Lawson TB, et al. Evaluation of equine articular cartilage degeneration after mechanical impact injury using cationic contrast-enhanced computed tomography. *Osteoarthritis and Cartilage* 2019;27:1219-1228.

288. Frisbie DD, Barrett MF, McIlwraith CW, et al. Diagnostic stifle joint arthroscopy using a needle arthroscope in standing horses. *Veterinary surgery : VS* 2014;43:12-18.

289. Kadic DT, Miagkoff L, Bonilla AG. Needle arthroscopy of the radiocarpal and middle carpal joints in standing sedated horses. *Veterinary Surgery* 2020.

290. Adrian A, Barrett M, Werpy N, et al. A comparison of arthroscopy to ultrasonography for identification of pathology of the equine stifle. *Equine veterinary journal* 2017;49:314-321.

291. McIlwraith CW, Billinghurst RC, Frisbie DD. Current and future diagnostic means to better characterize osteoarthritis in the horse—routine synovial fluid analysis and synovial fluid and serum markers. AAEP Proceedings 2001;171-179.

292. Tamer TM. Hyaluronan and synovial joint: function, distribution and healing. *Interdiscip Toxicol* 2013;6:111-125.

293. Naylor S. Biomarkers: current perspectives and future prospects. *Expert Rev Mol Diagn* 2003;3:525-529.

294. Frisbie DD, McIlwraith CW, de Grauw JC. Synovial Fluid and Serum Biomarkers In: McIlwraith CW, Frisbie DD, Kawcak CE, et al., eds. *Joint Disease in the Horse (Second Edition)*. Edinburgh: W.B. Saunders, 2016;179-191.

295. Hurter K, Spreng D, Rytz U, et al. Measurements of C-reactive protein in serum and lactate dehydrogenase in serum and synovial fluid of patients with osteoarthritis. *The Veterinary Journal* 2005;169:281-285.

296. Walter EL, Spreng D, Schmöckel H, et al. Distribution of lactate dehydrogenase in healthy and degenerative canine stifle joint cartilage. *Histochemistry and cell biology* 2007;128:7-18.

297. Kulkarni P, Deshpande S, Koppikar S, et al. Glycosaminoglycan measured from synovial fluid serves as a useful indicator for progression of Osteoarthritis and complements Kellgren-Lawrence Score. *BBA Clin* 2016;6:1-4.

298. de Grauw JC, van de Lest CH, van Weeren PR. A targeted lipidomics approach to the study of eicosanoid release in synovial joints. *Arthritis research & therapy* 2011;13:R123.

299. De Grauw J, Van de Lest C, van Weeren R, et al. Arthrogenic lameness of the fetlock: synovial fluid markers of inflammation and cartilage turnover in relation to clinical joint pain. *Equine veterinary journal* 2006;38:305-311.

300. De Grauw J, Van de Lest C, Brama P, et al. In vivo effects of meloxicam on inflammatory mediators, MMP activity and cartilage biomarkers in equine joints with acute synovitis. *Equine veterinary journal* 2009;41:693-699.

301. de Grauw JC, van Loon JP, van de Lest CH, et al. In vivo effects of phenylbutazone on inflammation and cartilage-derived biomarkers in equine joints with acute synovitis. *Veterinary journal (London, England : 1997)* 2014;201:51-56.

302. Van Loon J, De Grauw J, van Dierendonck M, et al. Intra-articular opioid analgesia is effective in reducing pain and inflammation in an equine LPS induced synovitis model. *Equine veterinary journal* 2010;42:412-419.

303. Frisbie D, Ray C, Ionescu M, et al. Measurement of synovial fluid and serum concentrations of the 846 epitope of chondroitin sulfate and of carboxy propeptides of type II procollagen for diagnosis of osteochondral fragmentation in horses. *American journal of veterinary research* 1999;60:306-309.

304. de Grauw JC, van de Lest CH, van Weeren PR. Inflammatory mediators and cartilage biomarkers in synovial fluid after a single inflammatory insult: a longitudinal experimental study. *Arthritis Research & Therapy* 2009;11:R35.

305. Lucia JL, Coverdale JA, Arnold CE, et al. Influence of an intra-articular lipopolysaccharide challenge on markers of inflammation and cartilage metabolism in young horses. *Journal of animal science* 2013;91:2693-2699.

306. Cleary OB, Trumble TN, Merritt KA, et al. Effect of exercise and osteochondral injury on synovial fluid and serum concentrations of carboxy-terminal telopeptide fragments of type II collagen in racehorses. *American journal of veterinary research* 2010;71:33-40.

307. Verwilghen DR, Enzerink E, Martens A, et al. Relationship between arthroscopic joint evaluation and the levels of Coll2-1, Coll2-1NO(2), and myeloperoxidase in the blood and synovial fluid of horses affected with osteochondrosis of the tarsocrural joint. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2011;19:1323-1329.

308. Peffers MJ, Thornton DJ, Clegg PD. Characterization of neopeptides in equine articular cartilage degradation. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2016;34:106-120.

309. Skiöldebrand E, Ekman S, Mattsson Hultén L, et al. Cartilage oligomeric matrix protein neoepitope in the synovial fluid of horses with acute lameness: A new biomarker for the early stages of osteoarthritis. *Equine veterinary journal* 2017;49:662-667.

310. Caron JP. Intra-articular injections for joint disease in horses. *The Veterinary clinics of North America Equine practice* 2005;21:559-573, v.

311. Ferris DJ, Frisbie DD, McIlwraith CW, et al. Current joint therapy usage in equine practice: a survey of veterinarians 2009. *Equine veterinary journal* 2011;43:530-535.

312. Monteiro SO, Bettencourt EV, Lepage OM. Biologic Strategies for Intra-articular Treatment and Cartilage Repair. *Journal of Equine Veterinary Science* 2015;35:175-190.

313. Rhen T, Cidlowski JA. Antiinflammatory action of glucocorticoids--new mechanisms for old drugs. *N Engl J Med* 2005;353:1711-1723.

314. Cuming RS, Groover ES, Wooldridge AA, et al. Review of glucocorticoid therapy in horses. Part 1: Pharmacology. *Equine Veterinary Education* 2018;30:141-150.

315. Harkins JD, Carney JM, Tobin T. Clinical use and characteristics of the corticosteroids. *The Veterinary clinics of North America Equine practice* 1993;9:543-562.

316. Ferguson D, Dirikolu L, Hoenig M. Glucocorticoids, mineralocorticoids and adrenolytic drugs. *Veterinary pharmacology and therapeutics* 2009;771:802.

317. Stahn C, Löwenberg M, Hommes DW, et al. Molecular mechanisms of glucocorticoid action and selective glucocorticoid receptor agonists. *Mol Cell Endocrinol* 2007;275:71-78.

318. Ericson-Neilsen W, Kaye AD. Steroids: pharmacology, complications, and practice delivery issues. *Ochsner J* 2014;14:203-207.

319. Wernecke C, Braun HJ, Dragoo JL. The Effect of Intra-articular Corticosteroids on Articular Cartilage: A Systematic Review. *Orthop J Sports Med* 2015;3:2325967115581163-2325967115581163.

320. Garvican ER, Vaughan-Thomas A, Redmond C, et al. MMP-mediated collagen breakdown induced by activated protein C in equine cartilage is reduced by corticosteroids. *Journal of Orthopaedic Research* 2010;28:370-378.

321. McIlwraith CW. The use of intra-articular corticosteroids in the horse: what is known on a scientific basis? *Equine veterinary journal* 2010;42:563-571.

322. McIlwraith CW. Intraarticular corticosteroids. *Joint disease in the horse*: Elsevier, 2016;202-214.

323. Smith L, Wylie C, Palmer L, et al. A longitudinal study of fractures in 1488 Thoroughbred racehorses receiving intrasynovial medication: 2006–2011. *Equine veterinary journal* 2018;50:774-780.

324. Wheat J. The use of hydrocortisone in the treatment of joint and tendon disorders in large animals. *Journal of the American Veterinary Medical Association* 1955;127:64-67.

325. Frisbie DD, Kawcak CE, Trotter GW, et al. Effects of triamcinolone acetonide on an in vivo equine osteochondral fragment exercise model. *Equine veterinary journal* 1997;29:349-359.

326. Foland J, McIlwraith C, Trotter G, et al. Effect of betamethasone and exercise on equine carpal joints with osteochondral fragments. *Veterinary Surgery* 1994;23:369-376.

327. Frisbie D, Johnson S. Medical Treatment of Joint Disease, 2019;1348-1363.

328. Murphy DJ, Todhunter RJ, Fubini SL, et al. The effects of methylprednisolone on normal and monocyte-conditioned medium-treated articular cartilage from dogs and horses. *Veterinary surgery : VS* 2000;29:546-557.

329. Trotter GW, McIlwraith CW, Yovich JV, et al. Effects of intra-articular administration of methylprednisolone acetate on normal equine articular cartilage. *American journal of veterinary research* 1991;52:83-87.

330. Byron CR, Benson BM, Stewart AA, et al. Effects of methylprednisolone acetate and glucosamine on proteoglycan production by equine chondrocytes in vitro. *American journal of veterinary research* 2008;69:1123-1128.

331. Murray RC, DeBowes RM, Gaughan E, et al. The effects of intra-articular methylprednisolone and exercise on the mechanical properties of articular cartilage in the horse. *Osteoarthritis and cartilage* 1998;6:106-114.

332. Frean SP, Cambridge H, Lees P. Effects of anti-arthritic drugs on proteoglycan synthesis by equine cartilage. *Journal of veterinary pharmacology and therapeutics* 2002;25:289-298.

333. Caron JP, Gandy JC, Schmidt M, et al. Influence of corticosteroids on interleukin-1beta-stimulated equine chondrocyte gene expression. *Veterinary surgery : VS* 2013;42:231-237.

334. Bolt DM, Ishihara A, Weisbrode SE, et al. Effects of triamcinolone acetonide, sodium hyaluronate, amikacin sulfate, and mepivacaine hydrochloride, alone and in combination, on morphology and matrix composition of lipopolysaccharide-challenged and unchallenged equine articular cartilage explants. *American journal of veterinary research* 2008;69:861-867.

335. Schaefer EC, Stewart AA, Durgam SS, et al. Effects of sodium hyaluronate and triamcinolone acetonide on glucosaminoglycan metabolism in equine articular chondrocytes treated with interleukin-1. *American journal of veterinary research* 2009;70:1494-1501.

336. Céleste C, Ionescu M, Poole AR, et al. Repeated intraarticular injections of triamcinolone acetonide alter cartilage matrix metabolism measured by biomarkers in synovial fluid. *Journal of Orthopaedic Research* 2005;23:602-610.

337. Knych HK, Vidal M, Chouicha N, et al. Cytokine, catabolic enzyme and structural matrix gene expression in synovial fluid following intra-articular administration of triamcinolone acetonide in exercised horses. *Equine veterinary journal* 2017;49:107-115.

338. Bessonnat A, Picotte K, Lavoie J. Intra-articular triamcinolone acetonide improves lung function in horses with severe asthma. *Equine veterinary journal* 2020;52:131-135.

339. Bailey S, Elliott J. The corticosteroid laminitis story: 2. Science of if, when and how. *Equine veterinary journal* 2007;39:7-11.

340. Bathe A. The corticosteroid laminitis story. 3. The clinician's viewpoint. 2007.

341. Ozturk C, Atamaz F, Hepguler S, et al. The safety and efficacy of intraarticular hyaluronan with/without corticosteroid in knee osteoarthritis: 1-year, single-blind, randomized study. *Rheumatology International* 2006;26:314-319.

342. Wang SZ, Wu DY, Chang Q, et al. Intra-articular, single-shot co-injection of hyaluronic acid and corticosteroids in knee osteoarthritis: A randomized controlled trial. *Exp Ther Med* 2018;16:1928-1934.

343. Doyle AJ, Stewart AA, Constable PD, et al. Effects of sodium hyaluronate and methylprednisolone acetate on proteoglycan synthesis in equine articular cartilage explants. *American journal of veterinary research* 2005;66:48-53.

344. Roneus B, Lindblad A, Lindholm A, et al. Effects of intraarticular corticosteroid and sodium hyaluronate injections on synovial fluid production and synovial fluid content of sodium hyaluronate and proteoglycans in normal equine joints. *Zentralbl Veterinarmed A* 1993;40:10-16.

345. de Grauw Jd, Visser-Meijer M, Lashley F, et al. Intra-articular treatment with triamcinolone compared with triamcinolone with hyaluronate: A randomised open-label multicentre clinical trial in 80 lame horses. *Equine veterinary journal* 2016;48:152-158.

346. Smith MM, Ghosh P. The synthesis of hyaluronic acid by human synovial fibroblasts is influenced by the nature of the hyaluronate in the extracellular environment. *Rheumatology international* 1987;7:113-122.

347. Hilbert B, Rowley G, Antonass K, et al. Changes in the synovia after the intra-articular injection of sodium hyaluronate into normal horse joints and after arthrotomy and experimental cartilage damage. *Australian veterinary journal* 1985;62:182-184.

348. Frisbie DD. Hyaluronan In: McIlwraith CW, Frisbie DD, Kawcak CE, et al., eds. *Joint Disease in the Horse (Second Edition)*. Edinburgh: W.B. Saunders, 2016;215-219.

349. Navarro-Sarabia F, Coronel P, Collantes E, et al. A 40-month multicentre, randomised placebo-controlled study to assess the efficacy and carry-over effect of repeated intra-articular injections of hyaluronic acid in knee osteoarthritis: the AMELIA project. *Annals of the rheumatic diseases* 2011;70:1957-1962.

350. Wang Y, Hall S, Hanna F, et al. Effects of Hylan GF 20 supplementation on cartilage preservation detected by magnetic resonance imaging in osteoarthritis of the knee: a two-year single-blind clinical trial. *BMC Musculoskeletal Disorders* 2011;12:195.

351. Nelson AE, Allen KD, Golightly YM, et al. A systematic review of recommendations and guidelines for the management of osteoarthritis: The chronic osteoarthritis management initiative of the U.S. bone and joint initiative. *Semin Arthritis Rheum* 2014;43:701-712.

352. Frisbie DD, Kawcak CE, McIlwraith CW, et al. Evaluation of polysulfated glycosaminoglycan or sodium hyaluronan administered intra-articularly for treatment of horses with experimentally induced osteoarthritis. *American journal of veterinary research* 2009;70:203-209.

353. Kawcak C, Frisbie D, Trotter G, et al. Effects of intravenous administration of sodium hyaluronate on carpal joints in exercising horses after arthroscopic surgery and osteochondral fragmentation. *American journal of veterinary research* 1997;58:1132-1140.

354. Machin J, Duer W, Maylin G, et al. Variability in plasma concentrations of methylprednisolone 6 days after intrasynovial injection of methylprednisolone acetate in racing horses: A field study. *Equine veterinary journal* 2019;51:343-348.

355. May SA, Hooke RE, Lees P. The effect of drugs used in the treatment of osteoarthrosis on stromelysin (proteoglycanase) of equine synovial cell origin. *Equine veterinary journal Supplement* 1988;20:28-32.

356. Todhunter RJ, Lust G. Polysulfated glycosaminoglycan in the treatment of osteoarthritis. *Journal of the American Veterinary Medical Association* 1994;204:1245-1251.

357. McIlwraith CW. Polysulfated glycosaminoglycan (Adequan®). *Joint disease in the horse*: Elsevier, 2016;220-223.

358. Glade M. Polysulfated glycosaminoglycan accelerates net synthesis of collagen and glycosaminoglycans by arthritic equine cartilage tissues and chondrocytes. *American journal of veterinary research* 1990;51:779-785.

359. Caron J, Eberhart S, Nachreiner R. Influence of polysulfated glycosaminoglycan on equine articular cartilage in explant culture. *American journal of veterinary research* 1991;52:1622-1625.

360. Gustafson S, McIlwraith C, Jones R. Comparison of the effect of polysulfated glycosaminoglycan, corticosteroids, and sodium hyaluronate in the potentiation of a subinfective dose of Staphylococcus aureus in the midcarpal joint of horses. *American journal of veterinary research* 1989;50:2014.

361. Gustafson SB, McIlwraith CW, Jones RL, et al. Further investigations into the potentiation of infection by intra-articular injection of polysulfated glycosaminoglycan and the effect of filtration and intra-articular injection of amikacin. *American journal of veterinary research* 1989;50:2018-2022.

362. McIlwraith CW, Goodman NL, Frisbie DD. Prospective study on the prophylactic value of intravenous hyaluronan in 2-year old racing Quarter horses. Proceedings 44th Annual Convention of the AAEP 1998;269-271.

363. Gaustad G, Larsen S. Comparison of polysulphated glycosaminoglycan and sodium hyaluronate with placebo in treatment of traumatic arthritis in horses. *Equine veterinary journal* 1995;27:356-362.

364. Todhunter R, Minor R, Wootton J, et al. Effects of exercise and polysulfated glycosaminoglycan on repair of articular cartilage defects in the equine carpus. *Journal of orthopaedic research* 1993;11:782-795.

365. Yovich JV, Trotter GW, McIlwraith CW, et al. Effects of polysulfated glycosaminoglycan on chemical and physical defects in equine articular cartilage. *American journal of veterinary research* 1987;48:1407-1414.

366. Dhillon MS, Behera P, Patel S, et al. Orthobiologics and platelet rich plasma. *Indian J Orthop* 2014;48:1-9.

367. Schnabel L, Boone L, Peroni J. Regenerative Medicine, 2019;104-122.

368. Filardo G, Kon E, Buda R, et al. Platelet-rich plasma intra-articular knee injections for the treatment of degenerative cartilage lesions and osteoarthritis. *Knee surgery, sports traumatology, arthroscopy : official journal of the ESSKA* 2010;19.

369. Textor J. Autologous biologic treatment for equine musculoskeletal injuries: platelet-rich plasma and IL-1 receptor antagonist protein. *The Veterinary clinics of North America Equine practice* 2011;27:275-298.

370. Textor JA, Willits NH, Tablin F. Synovial fluid growth factor and cytokine concentrations after intra-articular injection of a platelet-rich product in horses. *The Veterinary Journal* 2013;198:217-223.

371. Rios DL, Lopez C, Carmona JU. Platelet-Rich Gel Supernatants Stimulate the Release of Anti-Inflammatory Proteins on Culture Media of Normal Equine Synovial Membrane Explants. *Veterinary medicine international* 2015;2015:547052.

372. Textor JA, Tablin F. Intra-Articular Use of a Platelet-Rich Product in Normal Horses: Clinical Signs and Cytologic Responses. *Veterinary Surgery* 2013;42:499-510.

373. Hessel LN, Bosch G, van Weeren PR, et al. Equine autologous platelet concentrates: A comparative study between different available systems. *Equine veterinary journal* 2015;47:319-325.

374. Franklin SP, Garner BC, Cook JL. Characteristics of canine platelet-rich plasma prepared with five commercially available systems. *American journal of veterinary research* 2015;76:822-827.

375. Roh YH, Kim W, Park KU, et al. Cytokine-release kinetics of platelet-rich plasma according to various activation protocols. *Bone & joint research* 2016;5:37-45.

376. Giraldo CE, Lopez C, Alvarez ME, et al. Effects of the breed, sex and age on cellular content and growth factor release from equine pure-platelet rich plasma and pure-platelet rich gel. *BMC Vet Res* 2013;9:29.

377. Weibrich G, Kleis WK, Hafner G, et al. Comparison of platelet, leukocyte, and growth factor levels in point-of-care platelet-enriched plasma, prepared using a modified Curasan kit, with preparations received from a local blood bank. *Clin Oral Implants Res* 2003;14.

378. Boswell SG, Schnabel LV, Mohammed HO, et al. Increasing platelet concentrations in leukocyte-reduced platelet-rich plasma decrease collagen gene synthesis in tendons. *The American journal of sports medicine* 2014;42:42-49.

379. Dohan Ehrenfest DM, Rasmusson L, Albrektsson T. Classification of platelet concentrates: from pure platelet-rich plasma (P-PRP) to leucocyte-and platelet-rich fibrin (L-PRF). *Trends Biotechnol* 2009;27.

380. McCarrel TM, Mall NA, Lee AS, et al. Considerations for the use of platelet-rich plasma in orthopedics. *Sports Med* 2014;44:1025-1036.

381. Gilbertie JM, Schaer TP, Schubert AG, et al. Platelet-rich plasma lysate displays antibiofilm properties and restores antimicrobial activity against synovial fluid biofilms in vitro. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2020;38:1365-1374.

382. Perrone G, Lastra Y, González C, et al. Treatment With Platelet Lysate Inhibits Proteases of Synovial Fluid in Equines With Osteoarthritis. *Journal of Equine Veterinary Science* 2020;88:102952.

383. Sundman EA, Cole BJ, Karas V, et al. The anti-inflammatory and matrix restorative mechanisms of platelet-rich plasma in osteoarthritis. *The American journal of sports medicine* 2014;42:35-41.

384. Yang J, Lu Y, Guo A. Platelet-rich plasma protects rat chondrocytes from interleukin-1beta-induced apoptosis. *Molecular medicine reports* 2016.

385. Bendinelli P, Matteucci E, Dogliotti G, et al. Molecular basis of anti-inflammatory action of platelet-rich plasma on human chondrocytes: Mechanisms of NF-κB inhibition via HGF. *Journal of cellular physiology* 2010;225:757-766.

386. Akeda K, An HS, Okuma M, et al. Platelet-rich plasma stimulates porcine articular chondrocyte proliferation and matrix biosynthesis. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2006;14:1272-1280.

387. Mariani E, Canella V, Cattini L, et al. Leukocyte-Rich Platelet-Rich Plasma Injections Do Not Up-Modulate Intra-Articular Pro-Inflammatory Cytokines in the Osteoarthritic Knee. *PloS one* 2016;11:e0156137.

388. Cavallo C, Filardo G, Mariani E, et al. Comparison of platelet-rich plasma formulations for cartilage healing: an in vitro study. *JBJS* 2014;96:423-429.

389. Rios DL, Lopez C, Carmona JU. Evaluation of the anti-inflammatory effects of two platelet-rich gel supernatants in an in vitro system of cartilage inflammation. *Cytokine* 2015;76:505-513.

390. Lai LP, Stitik TP, Foye PM, et al. Use of Platelet-Rich Plasma in Intra-Articular Knee Injections for Osteoarthritis: A Systematic Review. *PM & R : the journal of injury, function, and rehabilitation* 2015;7:637-648.

391. Brossi PM, Moreira JJ, Machado TSL, et al. Platelet-rich plasma in orthopedic therapy: a comparative systematic review of clinical and experimental data in equine and human musculoskeletal lesions. *BMC Veterinary Research* 2015;11:98.

392. Seo JP, Kambayashi Y, Itho M, et al. Effects of a synovial flap and gelatin/beta-tricalcium phosphate sponges loaded with mesenchymal stem cells, bone morphogenetic protein-2, and platelet rich plasma on equine osteochondral defects. *Research in veterinary science* 2015;101:140-143.

393. Fortier LA, Chapman HS, Pownder SL, et al. BioCartilage Improves Cartilage Repair Compared With Microfracture Alone in an Equine Model of Full-Thickness Cartilage Loss. *The American journal of sports medicine* 2016;44:2366-2374.

394. Tsuzuki N, Seo JP, Yamada K, et al. The effect of a gelatin beta-tricalcium phosphate sponge loaded with mesenchymal stem cells (MSC), bone morphogenic protein-2, and platelet-rich plasma (PRP) on equine articular cartilage defect. *The Canadian veterinary journal = La revue veterinaire canadienne* 2013;54:573-580.

395. Broeckx S, Zimmerman M, Crocetti S, et al. Regenerative Therapies for Equine Degenerative Joint Disease: A Preliminary Study. *PloS one* 2014;9:e85917.

396. Bembo F, Eraud J, Philandrianos C, et al. Combined use of platelet rich plasma & micro-fat in sport and race horses with degenerative joint disease: preliminary clinical study in eight horses. *Muscles Ligaments Tendons J* 2016;6:198-204.

397. Hraha TH, Doremus KM, McIlwraith CW, et al. Autologous conditioned serum: the comparative cytokine profiles of two commercial methods (IRAP and IRAP II) using equine blood. *Equine veterinary journal* 2011;43:516-521.

398. Fjordbakk CT, Johansen GM, Lovas AC, et al. Surgical stress influences cytokine content in autologous conditioned serum. *Equine veterinary journal* 2015;47:212-217.

399. Evans CH, Chevalier X, Wehling P. Autologous Conditioned Serum. *Physical medicine and rehabilitation clinics of North America* 2016;27:893-908.

400. Marques-Smith P, Kallerud AS, Johansen GM, et al. Is clinical effect of autologous conditioned serum in spontaneously occurring equine articular lameness related to ACS cytokine profile? *BMC Veterinary Research* 2020;16:181.

401. Smit Y, Marais HJ, Thompson PN, et al. Clinical findings, synovial fluid cytology and growth factor concentrations after intra-articular use of a platelet-rich product in horses with osteoarthritis. *Journal of the South African Veterinary Association* 2019;90:1-9.

402. Mirza MH, Bommala P, Richbourg HA, et al. Gait Changes Vary among Horses with Naturally Occurring Osteoarthritis Following Intra-articular Administration of Autologous Platelet-Rich Plasma. *Frontiers in veterinary science* 2016;3:29.

403. Moraes AP, Moreira JJ, Brossi PM, et al. Short- and long-term effects of platelet-rich plasma upon healthy equine joints: Clinical and laboratory aspects. *The Canadian veterinary journal = La revue veterinaire canadienne* 2015;56:831-838.

404. Pichereau F, Décory M, Cuevas Ramos G. Autologous Platelet Concentrate as a Treatment for Horses with Refractory Fetlock Osteoarthritis. *Journal of Equine Veterinary Science* 2014;34:489-493.

405. Abellanet I, Prades M. Intra-articular platelet rich plasma (PRP) therapy: evaluation in 42 sport horses with OA. Proceedings of the International Congress of World Equine Veterinary Association: September 2009.

406. Carmona J, López C, Prades M. Uso de concentrados autólogos de plaquetas obtenidos mediante el método del tubo como tratamiento de artropatías en caballos. *Archivos de medicina veterinaria* 2009;41:175-179.

407. Carmona JU, Argüelles D, Climent F, et al. Autologous Platelet Concentrates as a Treatment of Horses with Osteoarthritis: A Preliminary Pilot Clinical Study. *Journal of Equine Veterinary Science* 2007;27:167-170.

408. Lasarzik J, Bondzio A, Rettig M, et al. Evaluation of Two Protocols Using Autologous Conditioned Serum for Intra-articular Therapy of Equine Osteoarthritis-A Pilot Study Monitoring

Cytokines and Cartilage-Specific Biomarkers. *Journal of Equine Veterinary Science* 2018;60:35-+.

409. Lasarzik de Ascurra J, Ehrle A, Einspanier R, et al. Influence of Incubation Time and Incubation Tube on the Cytokine and Growth Factor Concentrations of Autologous Conditioned Serum in Horses. *Journal of Equine Veterinary Science* 2019;75:30-34.

410. Meijer H, Reinecke J, Becker C, et al. The production of anti-inflammatory cytokines in whole blood by physico-chemical induction. *Inflammation research : official journal of the European Histamine Research Society [et al]* 2003;52.

411. Magalon J, Bausset O, Veran J, et al. Physico-chemical factors influencing autologous conditioned serum purification. *BioResearch open access* 2014;3:35-38.

412. Ionita CR, Troillet AR, Vahlenkamp TW, et al. Comparison of humoral insulin-like growth factor-1, platelet-derived growth factor-BB, transforming growth factor-beta1, and interleukin-1 receptor antagonist concentrations among equine autologous blood-derived preparations. *American journal of veterinary research* 2016;77:898-905.

413. Rutgers M, Saris DB, Dhert WJ, et al. Cytokine profile of autologous conditioned serum for treatment of osteoarthritis, in vitroeffects on cartilage metabolism and intra-articular levels after injection. *Arthritis Research & Therapy* 2010;12:R114.

414. Carlson ER, Stewart AA, Carlson KL, et al. Effects of serum and autologous conditioned serum on equine articular chondrocytes treated with interleukin-1beta. *American journal of veterinary research* 2013;74:700-705.

415. Baltzer AW, Moser C, Jansen SA, et al. Autologous conditioned serum (Orthokine) is an effective treatment for knee osteoarthritis. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2009;17:152-160.

416. Baltzer AWA, Ostapczuk MS, Stosch D, et al. A New Treatment for Hip Osteoarthritis: Clinical Evidence for the Efficacy of Autologous Conditioned Serum. *Orthopedic Reviews* 2013;5:e13.

417. Damjanov N, Barac B, Colic J, et al. The efficacy and safety of autologous conditioned serum (ACS) injections compared with betamethasone and placebo injections in the treatment of chronic shoulder joint pain due to supraspinatus tendinopathy: a prospective, randomized, double-blind, controlled study. *Medical ultrasonography* 2018;20:335-341.

418. Shirokova K, Noskov S, Shirokova L. Comparison of clinical efficacy of platelet-rich plasma and autologous conditioned serum treatment in patients with osteoarthritis of the knee. *Osteoarthritis and Cartilage* 2017;25:S438.

419. Ajrawat P, Dwyer T, Chahal J. Autologous Interleukin 1 Receptor Antagonist Blood-Derived Products for Knee Osteoarthritis: A Systematic Review. *Arthroscopy: The Journal of Arthroscopic & Related Surgery* 2019;35:2211-2221.

420. Zarringam D, Bekkers JEJ, Saris DBF. Long-term Effect of Injection Treatment for Osteoarthritis in the Knee by Orthokin Autologous Conditioned Serum. *Cartilage* 2018;9:140-145.

421. Jostingmeier U, Reinecke J, Hertsch B. Comparison of intraarticular injection of autologous conditioned serum (ACS, irap) vs sodium hyaluronate and corticosteroid in front limb coffin joint derived lameness. *The Australian Equine Veterinarian* 2010;2010:75-75.

422. Osterdahl J. Evaluation of autologous conditioned serum. *Swedish Uni Agricultural Sci* 2008;67:1-16.

423. Weinberger T. Klinische Erfahrungen mit der Anwendung von ACS/ORTHOKIN/IRAP beim Pferd. *Pferdespiegel* 2008;3:111-114.

424. Erickson AE, Sun J, Levengood SKL, et al. Chitosan-based composite bilayer scaffold as an in vitro osteochondral defect regeneration model. *Biomedical microdevices* 2019;21:34.

425. Zhang J, Ming D, Ji Q, et al. Repair of osteochondral defect using icariin-conditioned serum combined with chitosan in rabbit knees. *BMC Complementary Medicine and Therapies* 2020;20:193.

426. Blázquez R, Sánchez-Margallo FM, Reinecke J, et al. Conditioned Serum Enhances the Chondrogenic and Immunomodulatory Behavior of Mesenchymal Stem Cells. *Frontiers in pharmacology* 2019;10:699-699.

427. Motaal FKA-E, Elganzoury AM, Fathalla MM, et al. Low-dose intra-articular autologous conditioned serum in treatment of primary knee osteoarthritis. *Egyptian Rheumatology and Rehabilitation* 2014;41:98.

428. Schneider U. First Results on the Outcome of Gold-induced, Autologous-conditioned Serum (GOLDIC) in the Treatment of Different Lameness-associated Equine Diseases. *Journal of Cell Science & Therapy* 2014;05.

429. Bertone AL, Ishihara A, Zekas LJ, et al. Evaluation of a single intra-articular injection of autologous protein solution for treatment of osteoarthritis in horses. *American journal of veterinary research* 2014;75:141-151.

430. O'Shaughnessey K, Matuska A, Hoeppner J, et al. Autologous protein solution prepared from the blood of osteoarthritic patients contains an enhanced profile of anti-inflammatory cytokines and anabolic growth factors. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2014;32:1349-1355.

431. King W, van der Weegen W, Van Drumpt R, et al. White blood cell concentration correlates with increased concentrations of IL-1ra and improvement in WOMAC pain scores in an open-label safety study of autologous protein solution. *J Exp Orthop* 2016;3:9.

432. Muir SM, Reisbig N, Baria M, et al. The Concentration of Plasma Provides Additional Bioactive Proteins in Platelet and Autologous Protein Solutions. *The American journal of sports medicine* 2019;47:1955-1963.

433. Matuska A, O'Shaughnessey K, King W, et al. Autologous solution protects bovine cartilage explants from IL-1alpha- and TNFalpha-induced cartilage degradation. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2013;31:1929-1935.

434. Steckbeck KE. Autologous Protein Solution Exhibits Potential to Mitigate Osteoarthritis (OA) Progression in a Human Explant Co-Culture Model. 2016.

435. O'Shaughnessey KM, Panitch A, Woodell-May JE. Blood-derived anti-inflammatory protein solution blocks the effect of IL-1β on human macrophages in vitro. *Inflammation research : official journal of the European Histamine Research Society [et al]* 2011;60:929-936.

436. Hix J, Klaassen M, Foreman R, et al. An Autologous Anti-Inflammatory Protein Solution Yielded a Favorable Safety Profile and Significant Pain Relief in an Open-Label Pilot Study of Patients with Osteoarthritis. *BioResearch open access* 2017;6:151-158.

437. Vedi V, Woodell-May J. Prospective post market data collection on patients with knee osteoarthrtis treated with autologous protein solution (APS)(progress III study). *Osteoarthritis and Cartilage* 2020;28:S156.

438. Kon E, Engebretsen L, Verdonk P, et al. Clinical Outcomes of Knee Osteoarthritis Treated With an Autologous Protein Solution Injection: A 1-Year Pilot Double-Blinded Randomized Controlled Trial. *The American journal of sports medicine* 2018;46:171-180.

439. Van Genechten W, Vuylsteke K, Swinnen L, et al. Autologous protein solution as selective treatment for advanced patellofemoral osteoarthritis in the middle-aged female patient: 54% response rate at 1 year follow-up. *Knee Surgery, Sports Traumatology, Arthroscopy* 2020:1-10.

440. Chahla J, Dean CS, Moatshe G, et al. Concentrated Bone Marrow Aspirate for the Treatment of Chondral Injuries and Osteoarthritis of the Knee: A Systematic Review of Outcomes. *Orthopaedic journal of sports medicine* 2016;4:2325967115625481.

441. Schnabel LV, Mohammed HO, Miller BJ, et al. Platelet rich plasma (PRP) enhances anabolic gene expression patterns in flexor digitorum superficialis tendons. *Journal of orthopaedic research* 2007;25:230-240.

442. McCarrel T, Fortier L. Temporal growth factor release from platelet-rich plasma, trehalose lyophilized platelets, and bone marrow aspirate and their effect on tendon and ligament gene expression. *Journal of Orthopaedic Research* 2009;27:1033-1042.

443. Fortier LA, Potter HG, Rickey EJ, et al. Concentrated bone marrow aspirate improves full-thickness cartilage repair compared with microfracture in the equine model. *JBJS* 2010;92:1927-1937.

444. Chu CR, Fortier LA, Williams A, et al. Minimally Manipulated Bone Marrow Concentrate Compared with Microfracture Treatment of Full-Thickness Chondral Defects: A One-Year Study in an Equine Model. *The Journal of bone and joint surgery American volume* 2018;100:138-146.

445. Han S, Sun HM, Hwang K-C, et al. Adipose-Derived Stromal Vascular Fraction Cells: Update on Clinical Utility and Efficacy. 2015;25:145-152.

446. Vidal MA, Kilroy GE, Lopez MJ, et al. Characterization of equine adipose tissue-derived stromal cells: adipogenic and osteogenic capacity and comparison with bone marrow-derived mesenchymal stromal cells. *Veterinary surgery : VS* 2007;36:613-622.

447. Frisbie DD, Kisiday JD, Kawcak CE, et al. Evaluation of adipose-derived stromal vascular fraction or bone marrow-derived mesenchymal stem cells for treatment of osteoarthritis. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2009;27:1675-1680.

448. Pittenger MF, Discher DE, Péault BM, et al. Mesenchymal stem cell perspective: cell biology to clinical progress. *npj Regenerative Medicine* 2019;4:22.

449. Jiang Y, Jahagirdar BN, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 2002;418:41-49.

450. Agung M, Ochi M, Yanada S, et al. Mobilization of bone marrow-derived mesenchymal stem cells into the injured tissues after intraarticular injection and their contribution to tissue regeneration. *Knee Surgery, Sports Traumatology, Arthroscopy* 2006;14:1307-1314.

451. Li Q, Tang J, Wang R, et al. Comparing the chondrogenic potential in vivo of autogeneic mesenchymal stem cells derived from different tissues. *Artif Cells Blood Substit Immobil Biotechnol* 2011;39:31-38.

452. Mazzotti E, Teti G, Falconi M, et al. Age-Related Alterations Affecting the Chondrogenic Differentiation of Synovial Fluid Mesenchymal Stromal Cells in an Equine Model. *Cells* 2019;8.

453. Desancé M, Contentin R, Bertoni L, et al. Chondrogenic Differentiation of Defined Equine Mesenchymal Stem Cells Derived from Umbilical Cord Blood for Use in Cartilage Repair Therapy. *Int J Mol Sci* 2018;19.

454. Gale AL, Mammone RM, Dodson ME, et al. The effect of hypoxia on chondrogenesis of equine synovial membrane-derived and bone marrow-derived mesenchymal stem cells. *BMC Vet Res* 2019;15:201.

455. De Schauwer C, Goossens K, Piepers S, et al. Characterization and profiling of immunomodulatory genes of equine mesenchymal stromal cells from non-invasive sources. *Stem cell research & therapy* 2014;5:6.

456. Youngstrom DW, LaDow JE, Barrett JG. Tenogenesis of bone marrow-, adipose-, and tendon-derived stem cells in a dynamic bioreactor. *Connect Tissue Res* 2016;57:454-465.

457. Prado AAF, Favaron PO, da Silva LCLC, et al. Characterization of mesenchymal stem cells derived from the equine synovial fluid and membrane. *BMC Veterinary Research* 2015;11:281.

458. Spaas JH, Chiers K, Bussche L, et al. Stem/progenitor cells in non-lactating versus lactating equine mammary gland. *Stem cells and development* 2012;21:3055-3067.

459. Frisbie DD, Smith RK. Clinical update on the use of mesenchymal stem cells in equine orthopaedics. *Equine veterinary journal* 2010;42:86-89.

460. Peroni JF, Borjesson DL. Anti-Inflammatory and Immunomodulatory Activities of Stem Cells. *Veterinary Clinics of North America: Equine Practice* 2011;27:351-362.

461. Wang Y, Chen X, Cao W, et al. Plasticity of mesenchymal stem cells in immunomodulation: pathological and therapeutic implications. *Nat Immunol* 2014;15:1009-1016.

462. Mancuso P, Raman S, Glynn A, et al. Mesenchymal Stem Cell Therapy for Osteoarthritis: The Critical Role of the Cell Secretome. *Frontiers in bioengineering and biotechnology* 2019;7:9-9.

463. Vidal MA, Robinson SO, Lopez MJ, et al. Comparison of chondrogenic potential in equine mesenchymal stromal cells derived from adipose tissue and bone marrow. *Veterinary surgery : VS* 2008;37:713-724.

464. Kisiday JD, Kopesky PW, Evans CH, et al. Evaluation of adult equine bone marrow- and adipose-derived progenitor cell chondrogenesis in hydrogel cultures. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2008;26:322-331.

465. Maumus M, Roussignol G, Toupet K, et al. Utility of a Mouse Model of Osteoarthritis to Demonstrate Cartilage Protection by IFNγ-Primed Equine Mesenchymal Stem Cells. *Frontiers in Immunology* 2016;7.

466. Carter-Arnold JL, Neilsen NL, Amelse LL, et al. In vitro analysis of equine, bone marrow-derived mesenchymal stem cells demonstrates differences within age- and gender-matched horses. *Equine veterinary journal* 2014;46:589-595.

467. Ardanaz N, Vázquez FJ, Romero A, et al. Inflammatory response to the administration of mesenchymal stem cells in an equine experimental model: effect of autologous, and single and repeat doses of pooled allogeneic cells in healthy joints. *BMC veterinary research* 2016;12:65-65.

468. Pigott JH, Ishihara A, Wellman ML, et al. Inflammatory effects of autologous, genetically modified autologous, allogeneic, and xenogeneic mesenchymal stem cells after intra-articular injection in horses. *Vet Comp Orthop Traumatol* 2013;26:453-460.

469. Williams LB, Koenig JB, Black B, et al. Equine allogeneic umbilical cord blood derived mesenchymal stromal cells reduce synovial fluid nucleated cell count and induce mild self-limiting inflammation when evaluated in an lipopolysaccharide induced synovitis model. *Equine veterinary journal* 2016;48:619-625.

470. Colbath A, Dow S, Hopkins L, et al. Allogeneic vs. autologous intra-articular mesenchymal stem cell injection within normal horses: Clinical and cytological comparisons suggest safety. *Equine veterinary journal* 2020;52:144-151.

471. Colbath AC, Dow SW, Hopkins LS, et al. Single and repeated intra-articular injections in the tarsocrural joint with allogeneic and autologous equine bone marrow-derived mesenchymal stem cells are safe, but did not reduce acute inflammation in an experimental interleukin-1β model of synovitis. *Equine veterinary journal* 2020;52:601-612.

472. Joswig A-J, Mitchell A, Cummings KJ, et al. Repeated intra-articular injection of allogeneic mesenchymal stem cells causes an adverse response compared to autologous cells in the equine model. *Stem cell research & therapy* 2017;8:42.

473. Schnabel LV, Pezzanite LM, Antczak DF, et al. Equine bone marrow-derived mesenchymal stromal cells are heterogeneous in MHC class II expression and capable of inciting an immune response in vitro. *Stem cell research & therapy* 2014;5:13.

474. Barrachina L, Cequier A, Romero A, et al. Allo-antibody production after intraarticular administration of mesenchymal stem cells (MSCs) in an equine osteoarthritis model: effect of repeated administration, MSC inflammatory stimulation, and equine leukocyte antigen (ELA) compatibility. *Stem cell research & therapy* 2020;11:52.

475. Yamada ALM, de Mattos Carvalho A, Moroz A, et al. Mesenchymal stem cell enhances chondral defects healing in horses. *Stem Cell Discovery* 2013;2013.

476. Grady ST, Britton L, Hinrichs K, et al. Persistence of fluorescent nanoparticle-labelled bone marrow mesenchymal stem cells in vitro and after intra-articular injection. *Journal of tissue engineering and regenerative medicine* 2019;13:191-202.

477. Broeckx S, Zimmerman M, Crocetti S, et al. Regenerative therapies for equine degenerative joint disease: a preliminary study. *PloS one* 2014;9:e85917.

478. McIlwraith CW, Frisbie DD, Rodkey WG, et al. Evaluation of intra-articular mesenchymal stem cells to augment healing of microfractured chondral defects. *Arthroscopy : the journal of*

arthroscopic & related surgery : official publication of the Arthroscopy Association of North America and the International Arthroscopy Association 2011;27:1552-1561.

479. Zayed M, Newby S, Misk N, et al. Xenogenic implantation of equine synovial fluid-derived mesenchymal stem cells leads to articular cartilage regeneration. *Stem cells international* 2018;2018.

480. Ferris DJ, Frisbie DD, Kisiday JD, et al. Clinical outcome after intra-articular administration of bone marrow derived mesenchymal stem cells in 33 horses with stifle injury. *Veterinary surgery : VS* 2014;43:255-265.

481. Goodrich LR, Chen AC, Werpy NM, et al. Addition of Mesenchymal Stem Cells to Autologous Platelet-Enhanced Fibrin Scaffolds in Chondral Defects: Does It Enhance Repair? *The Journal of bone and joint surgery American volume* 2016;98:23-34.

482. Jevotovsky DS, Alfonso AR, Einhorn TA, et al. Osteoarthritis and stem cell therapy in humans: a systematic review. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2018;26:711-729.

483. Zarini E, Supino R, Pratesi G, et al. Biocompatibility and tissue interactions of a new filler material for medical use. *Plastic and reconstructive surgery* 2004;114:934-942.

484. Fernandez-Cossio S, Castano-Oreja MT. Biocompatibility of two novel dermal fillers: histological evaluation of implants of a hyaluronic acid filler and a polyacrylamide filler. *Plastic and reconstructive surgery* 2006;117:1789-1796.

485. Christensen LH, Nielsen JB, Mouritsen L, et al. Tissue integration of polyacrylamide hydrogel: an experimental study of periurethral, perivesical, and mammary gland tissue in the pig. *Dermatologic surgery : official publication for American Society for Dermatologic Surgery [et al]* 2008;34 Suppl 1:S68-77; discussion S77.

486. Brahm J, Lessel R, Ditlev S, et al. Flux of selected body fluid constituents and benzylpenicillin in polyacrylamide hydrogel (PAAG). *Journal of tissue engineering and regenerative medicine* 2012;6:793-802.

487. Bello G, Jackson IT, Keskin M, et al. The Use of Polyacrylamide Gel in Soft-Tissue Augmentation: An Experimental Assessment. *Plastic and reconstructive surgery* 2007;119:1326-1336.

488. Borzacchiello A, Mayol L, Schiavinato A, et al. Effect of hyaluronic acid amide derivative on equine synovial fluid viscoelasticity. *Journal of Biomedical Materials Research Part A* 2010;92A:1162-1170.

489. Petersen J. Polyacrylamide hydrogel for arthritis: Google Patents, 2007.

490. Zar V, Troitsky V, Stepanov A, et al. Future of viscoelastic behavior of 3-D structure of artificial and natural samples of articular liquids at the pressure 0.1–100 MPa. *Трудьı* 2007;16.

491. Christensen LH, Breiting VB, Aasted A, et al. Long-term effects of polyacrylamide hydrogel on human breast tissue. *Plastic and reconstructive surgery* 2003;111:1883-1890.

492. Barrachina L, Remacha AR, Romero A, et al. Assessment of effectiveness and safety of repeat administration of proinflammatory primed allogeneic mesenchymal stem cells in an equine model of chemically induced osteoarthritis. *BMC Veterinary Research* 2018;14:241.

493. Nicpoń J, Marycz K, Grzesiak J. Therapeutic effect of adipose-derived mesenchymal stem cell injection in horses suffering from bone spavin. *Pol J Vet Sci* 2013;16:753-754.

494. Wilke MM, Nydam DV, Nixon AJ. Enhanced early chondrogenesis in articular defects following arthroscopic mesenchymal stem cell implantation in an equine model. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2007;25:913-925.

495. McClure SR, Yaeger M, Wang C. Clinical and Histologic Evaluation of Polyacrylamide Gel in Normal Equine Metacarpal /Metatarsal-Phalangeal Joints. *Journal of Equine Veterinary Science* 2017;54:70-77.

496. Christensen L, Camitz L, Illigen KE, et al. Synovial incorporation of polyacrylamide hydrogel after injection into normal and osteoarthritic animal joints. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2016;24:1999-2002.

497. Tnibar A, Persson AB, Jensen HE. Mechanisms of Action of an Intraarticular 2.5% Polyacrylamide Hydrogel (Arthramid Vet) in a Goat Model of Osteoarthritis: Preliminary Observations. *SM J Biomed Eng* 2017;3:1022.

498. de Souza AF, Paretsis NF, De Zoppa ALdV. What is the Evidence of Hyaluronic Acid and Polyacrylamide Hydrogel in Intra-articular Therapy in Equines? Systematic Literature Review. *Journal of Equine Veterinary Science* 2020;86:102909.

499. Henriksen M, Overgaard A, Hartkopp A, et al. Intra-articular 2.5% polyacrylamide hydrogel for the treatment of knee osteoarthritis: an observational proof-of-concept cohort study. *Clin Exp Rheumatol* 2018;36:1082-1085.

500. Tnibar A, Schougaard H, Camitz L, et al. An international multi-centre prospective study on the efficacy of an intraarticular polyacrylamide hydrogel in horses with osteoarthritis: a 24 months follow-up. *Acta veterinaria Scandinavica* 2015;57:20.

501. Janssen I, Koene M, Lischer C. Intraarticular applikation of polyacrylamide hydrogel as a treatment of osteoarthritis in the distal interphalangeal joint: Case series with 12 horses. *Pferdeheilkunde* 2012;28:650-656.

502. Zar V, Zagorodniy N, Martinov D. Effectiveness and safety of injectable endoprosthetics of synovial fluid by cross-linked polymer NOLTREX for treatment OA knee. *European Journal of Musculoskeletal Diseases* 2012;1:23-32.

503. de Clifford LT, Lowe JN, McKellar CD, et al. Use of a 2.5% Cross-Linked Polyacrylamide Hydrogel in the Management of Joint Lameness in a Population of Flat Racing Thoroughbreds: A Pilot Study. *Journal of Equine Veterinary Science* 2019;77:57-62.

504. McClure SR, Wang C. A preliminary field trial evaluating the efficacy of 4% polyacrylamide hydrogel in horses with osteoarthritis. *Journal of Equine Veterinary Science* 2017;54:98-102.

505. David F. Is it worth considering 2.5% polyacrylamide gel as a first intention joint therapy? Northeast Association of Equine Practitioners, 2017.

506. Tonbul M, Adas M, Bekmezci T, et al. Intra-articular polyacrylamide hydrogel injections are not innocent. *Case reports in orthopedics* 2014;2014:150709.

507. Tnibar A, Schoogaard H, Koene M, et al. A controlled clinical trial on the efficacy of an intra-articular Polyacrylamide Hydrogel in horses with osteoarthritis [abstract]. *Veterinary Surgery* 2014;43:E138.

508. Muzzonigro TS, Ghivizzani SC, Robbins PD, et al. THE ROLE OF GENE THERAPY: Fact or Fiction? *Clinics in Sports Medicine* 1999;18:223-239.

509. Brower-Toland BD, Saxer RA, Goodrich LR, et al. Direct adenovirus-mediated insulin-like growth factor I gene transfer enhances transplant chondrocyte function. *Human gene therapy* 2001;12:117-129.

510. Evans CH, Gouze J, Gouze E, et al. Osteoarthritis gene therapy. *Gene therapy* 2004;11:379-389.

511. Nixon AJ, Goodrich LR, Scimeca MS, et al. Gene therapy in musculoskeletal repair. *Annals of the New York Academy of Sciences* 2007;1117:310-327.

512. Frisbie D, Ghivizzani S, Robbins PD, et al. Treatment of experimental equine osteoarthritis by in vivo delivery of the equine interleukin-1 receptor antagonist gene. *Gene therapy* 2002;9:12-20.

513. Goodrich LR, Brower-Toland BD, Warnick L, et al. Direct adenovirus-mediated IGF-I gene transduction of synovium induces persisting synovial fluid IGF-I ligand elevations. *Gene Ther* 2006;13:1253-1262.

514. Goodrich LR, Hidaka C, Robbins PD, et al. Genetic modification of chondrocytes with insulin-like growth factor-1 enhances cartilage healing in an equine model. *J Bone Joint Surg Br* 2007;89:672-685.

515. Ruan MZ, Erez A, Guse K, et al. Proteoglycan 4 expression protects against the development of osteoarthritis. *Science translational medicine* 2013;5:176ra134-176ra134.

516. Caron JP, Fernandes JC, Martel-Pelletier J, et al. Chondroprotective effect of intraarticular injections of interleukin-1 receptor antagonist in experimental osteoarthritis. Suppression of collagenase-1 expression. *Arthritis and rheumatism* 1996;39.

517. Wooley PH, Whalen JD, Chapman DL, et al. The effect of an interleukin-1 receptor antagonist protein on type II collagen–induced arthritis and antigen-induced arthritis in mice. *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology* 1993;36:1305-1314.

518. Watson Levings RS, Broome TA, Smith AD, et al. Gene therapy for osteoarthritis: pharmacokinetics of intra-articular self-complementary adeno-associated virus interleukin-1 receptor antagonist delivery in an equine model. *Human gene therapy Clinical development* 2018;29:90-100.

519. Moss KL, Jiang Z, Dodson ME, et al. Sustained interleukin-10 transgene expression following intra-articular AAV5-IL-10 administration to horses. *Human gene therapy* 2020;31:110-118.

520. Nixon AJ, Grol MW, Lang HM, et al. Disease-modifying osteoarthritis treatment with interleukin-1 receptor antagonist gene therapy in small and large animal models. *Arthritis & Rheumatology* 2018;70:1757-1768.

521. Goodrich L, Grieger J, Phillips J, et al. scAAVIL-1ra dosing trial in a large animal model and validation of long-term expression with repeat administration for osteoarthritis therapy. *Gene therapy* 2015;22:536-545.

522. Ortved KF, Begum L, Mohammed HO, et al. Implantation of rAAV5-IGF-I Transduced Autologous Chondrocytes Improves Cartilage Repair in Full-thickness Defects in the Equine Model. *Molecular Therapy* 2015;23:363-373.

523. Menendez MI, Clark DJ, Carlton M, et al. Direct delayed human adenoviral BMP-2 or BMP-6 gene therapy for bone and cartilage regeneration in a pony osteochondral model. *Osteoarthritis and Cartilage* 2011;19:1066-1075.

524. Bogers SH. Cell-Based Therapies for Joint Disease in Veterinary Medicine: What We Have Learned and What We Need to Know. *Frontiers in veterinary science* 2018;5:70.

525. Wilson JM, McKenzie E, Duesterdieck-Zellmer K. International Survey Regarding the Use of Rehabilitation Modalities in Horses. *Frontiers in veterinary science* 2018;5:120.

526. Walmsley JP. Diagnosis and treatment of ligamentous and meniscal injuries in the equine stifle. *The Veterinary clinics of North America Equine practice* 2005;21:651-672, vii.

527. Weiss ARR, Lee O, Eggenhofer E, et al. Differential effects of heat-inactivated, secretome-deficient MSC and metabolically active MSC in sepsis and allogenic heart transplantation. *STEM CELLS* 2020;38:797-807.

528. Hegewald AA, Ringe J, Bartel J, et al. Hyaluronic acid and autologous synovial fluid induce chondrogenic differentiation of equine mesenchymal stem cells: a preliminary study. *Tissue Cell* 2004;36:431-438.

529. Bohannon LK, Owens SD, Walker NJ, et al. The effects of therapeutic concentrations of gentamicin, amikacin and hyaluronic acid on cultured bone marrow-derived equine mesenchymal stem cells. *Equine veterinary journal* 2013;45:732-736.

530. Mishra A, Tummala P, King A, et al. Buffered platelet-rich plasma enhances mesenchymal stem cell proliferation and chondrogenic differentiation. *Tissue Eng Part C Methods* 2009;15:431-435.

531. Edmonds RE, Garvican ER, Smith RK, et al. Influence of commonly used pharmaceutical agents on equine bone marrow-derived mesenchymal stem cell viability. *Equine veterinary journal* 2017;49:352-357.

532. Parker RA, Clegg PD, Taylor SE. The in vitro effects of antibiotics on cell viability and gene expression of equine bone marrow-derived mesenchymal stromal cells. *Equine veterinary journal* 2012;44:355-360.

533. Pezzanite L, Chow L, Soontararak S, et al. Amikacin induces rapid dose-dependent apoptotic cell death in equine chondrocytes and synovial cells in vitro. *Equine veterinary journal* 2020;n/a.

534. Rinnovati R, Romagnoli N, Gentilini F, et al. The influence of environmental variables on platelet concentration in horse platelet-rich plasma. *Acta veterinaria Scandinavica* 2016;58:45.

535. Sutter WW, Kaneps AJ, Bertone AL. Comparison of hematologic values and transforming growth factor-beta and insulin-like growth factor concentrations in platelet concentrates obtained by use of buffy coat and apheresis methods from equine blood. *American journal of veterinary research* 2004;65:924-930.

536. Burkett BN, Thomason JM, Hurdle HM, et al. Effects of Firocoxib, Flunixin Meglumine, and Phenylbutazone on Platelet Function and Thromboxane Synthesis in Healthy Horses. *Veterinary surgery : VS* 2016;45:1087-1094.

537. Marohl T, Rothenbuhler R, Leach M, et al. Cox-inhibitors do not impact anti-inflammatory IL-1ra concentrations in Autologous Protein Solution. International Cartilage Repair Society 2016.

538. Marohl T, Rothenbuhler R, Leach M, et al. Steroids methylprednisolone and triamcinolone do not impact antiinflammatory IL-1ra concentrations in Autologous Protein Solution. International Cartilage Repair Society 2016.

539. Muller M, Raabe O, Addicks K, et al. Effects of non-steroidal anti-inflammatory drugs on proliferation, differentiation and migration in equine mesenchymal stem cells. *Cell biology international* 2011;35:235-248.

540. Gilbertie JM, Davis JL, Davidson GS, et al. Oral reserpine administration in horses results in low plasma concentrations that alter platelet biology. *Equine veterinary journal* 2019;51:537-543.

541. Tnibar A, Persson AB, Nielsen H, et al. Evaluation of a Polyacrylamide Hydrogel in the Treatment of Induced Osteoarthritis in a Goat Model: A Randomized Controlled Pilot Study. *Osteoarthritis and Cartilage* 2014;22:S477-S477.

542. Clarke S, Mellor D, Clements D, et al. Prevalence of radiographic signs of degenerative joint disease in a hospital population of cats. *Veterinary Record* 2005;157:793-799.

543. Clements DN, Fitzpatrick N, Carter SD, et al. Cartilage gene expression correlates with radiographic severity of canine elbow osteoarthritis. *The Veterinary Journal* 2009;179:211-218.

544. Sutton S, Clutterbuck A, Harris P, et al. The contribution of the synovium, synovial derived inflammatory cytokines and neuropeptides to the pathogenesis of osteoarthritis. *Veterinary journal (London, England : 1997)* 2009;179:10-24.

545. Arora M. Cell culture media: a review. *Mater methods* 2013;3:24.

546. Freshney RI. Defined Media and Supplements In: Freshney RI, ed. *Culture of animal cells: a manual of basic technique and specialized applications*. Hoboken, NJ: John Wiley & Sons, 2011;99-114.

547. Bettger WJ, McKeehan WL. Mechanisms of cellular nutrition. *Physiol Rev* 1986;66:1-35.

548. Russell K, Koch T. Equine platelet lysate as an alternative to fetal bovine serum in equine mesenchymal stromal cell culture–too much of a good thing? *Equine veterinary journal* 2016;48:261-264.

549. Freshney RI. Serum-free media In: Freshney RI, ed. *Culture of animal cells: a manual of basic technique and specialized applications*. Hoboken, NJ: John Wiley & Sons, 2011;115-132.

550. Azouna NB, Jenhani F, Regaya Z, et al. Phenotypical and functional characteristics of mesenchymal stem cells from bone marrow: comparison of culture using different media supplemented with human platelet lysate or fetal bovine serum. *Stem cell research & therapy* 2012;3:6.

551. Busschers E, Holt JP, Richardson DW. Effects of glucocorticoids and interleukin-1β on expression and activity of aggrecanases in equine chondrocytes. *American journal of veterinary research* 2010;71:176-185.

552. Fitzsimmons JS, Sanyal A, Gonzalez C, et al. Serum-free media for periosteal chondrogenesis in vitro. *Journal of Orthopaedic Research* 2004;22:716-725.

553. Pool R. Pathologic manifestations of joint disease in the athletic horse. *Joint disease in the horse* 1996;1:87-104.

554. Hewitt KM, Stringer MD. Correlation between the surface area of synovial membrane and the surface area of articular cartilage in synovial joints of the mouse and human. *Surg Radiol Anat* 2008;30:645-651.

555. Radonic A, Thulke S, Mackay IM, et al. Guideline to reference gene selection for quantitative real-time PCR. *Biochem Biophys Res Commun* 2004;313:856-862.

556. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta* 1986;883:173-177.

557. Oke SL, Hurtig MB, Keates RA, et al. Assessment of three variations of the 1,9-dimethylmethylene blue assay for measurement of sulfated glycosaminoglycan concentrations in equine synovial fluid. *American journal of veterinary research* 2003;64:900-906.

558. Ushiyama T, Chano T, Inoue K, et al. Cytokine production in the infrapatellar fat pad: another source of cytokines in knee synovial fluids. *Annals of the rheumatic diseases* 2003;62:108-112.

559. Mathiessen A, Conaghan PG. Synovitis in osteoarthritis: current understanding with therapeutic implications. *Arthritis research & therapy* 2017;19:18-18.

560. Schwarz C, Leicht U, Rothe C, et al. Effects of different media on proliferation and differentiation capacity of canine, equine and porcine adipose derived stem cells. *Research in veterinary science* 2012;93:457-462.

561. Franke J, Abs V, Zizzadoro C, et al. Comparative study of the effects of fetal bovine serum versus horse serum on growth and differentiation of primary equine bronchial fibroblasts. *BMC Veterinary Research* 2014;10:119.

562. Knudson CB, Knudson W. Cartilage proteoglycans. *Semin Cell Dev Biol* 2001;12:69-78.

563. Steinhagen J, Bruns J, Niggemeyer O, et al. Perfusion culture system: Synovial fibroblasts modulate articular chondrocyte matrix synthesis in vitro. *Tissue and Cell* 2010;42:151-157.

564. Van den Boom R, Van Der Harst M, Brommer H, et al. Relationship between synovial fluid levels of glycosaminoglycans, hydroxyproline and general MMP activity and the presence and severity of articular cartilage change on the proximal articular surface of P1. *Equine veterinary journal* 2005;37:19-25.

565. Sandler EA, Frisbie DD, McIlwraith CW. A dose titration of triamcinolone acetonide on insulin-like growth factor-1 and interleukin-1-conditioned equine cartilage explants. *Equine veterinary journal* 2004;36:58-63.

566. Frisbie DD, Kawcak CE, Werpy NM, et al. Clinical, biochemical, and histologic effects of intra-articular administration of autologous conditioned serum in horses with experimentally induced osteoarthritis. *American journal of veterinary research* 2007;68.

567. Clutterbuck AL, Allaway D, Harris P, et al. Curcumin reduces prostaglandin E2, matrix metalloproteinase-3 and proteoglycan release in the secretome of interleukin 1beta-treated articular cartilage. *F1000Research* 2013;2:147.

568. Zucca E, Corsini E, Galbiati V, et al. Evaluation of amniotic mesenchymal cell derivatives on cytokine production in equine alveolar macrophages: an in vitro approach to lung inflammation. *Stem cell research & therapy* 2016;7:137.

569. Darabos N, Haspl M, Moser C, et al. Intraarticular application of autologous conditioned serum (ACS) reduces bone tunnel widening after ACL reconstructive surgery in a randomized controlled trial. *Knee surgery, sports traumatology, arthroscopy : official journal of the ESSKA* 2011;19 Suppl 1:S36-46.

570. van Drumpt RA, van der Weegen W, King W, et al. Safety and Treatment Effectiveness of a Single Autologous Protein Solution Injection in Patients with Knee Osteoarthritis. *Biores Open Access* 2016;5:261-268.

571. Hix J, Klaassen M, Foreman R, et al. An Autologous Anti-Inflammatory Protein Solution Yielded a Favorable Safety Profile and Significant Pain Relief in an Open-Label Pilot Study of Patients with Osteoarthritis. *Biores Open Access* 2017;6:151-158.

572. Wanstrath AW, Hettlich BF, Su L, et al. Evaluation of a Single Intra-Articular Injection of Autologous Protein Solution for Treatment of Osteoarthritis in a Canine Population. *Veterinary surgery : VS* 2016;45:764-774.

573. Brama PA, van den Boom R, DeGroott J, et al. Collagenase-1 (MMP-1) activity in equine synovial fluid: influence of age, joint pathology, exercise and repeated arthrocentesis. *Equine veterinary journal* 2004;36:34-40.

574. Ghasemi S, Sardari K, Mirshokraei P, et al. In vitro study of matrix metalloproteinases 1, 2, 9, 13 and serum amyloid A mRNAs expression in equine fibroblast-like synoviocytes treated with doxycycline. *Can J Vet Res* 2018;82:82-88.

575. Tortorella MD, Burn TC, Pratta MA, et al. Purification and cloning of aggrecanase-1: a member of the ADAMTS family of proteins. *Science (New York, NY)* 1999;284:1664-1666.

576. Caterson B, Flannery CR, Hughes CE, et al. Mechanisms involved in cartilage proteoglycan catabolism. *Matrix Biol* 2000;19:333-344.

577. Kamm JL, Nixon AJ, Witte TH. Cytokine and catabolic enzyme expression in synovium, synovial fluid and articular cartilage of naturally osteoarthritic equine carpi. *Equine veterinary journal* 2010;42:693-699.

578. Glasson SS, Askew R, Sheppard B, et al. Characterization of and osteoarthritis susceptibility in ADAMTS-4-knockout mice. *Arthritis and rheumatism* 2004;50:2547-2558.

579. Chen S, Huang Y, Zhou Z-J, et al. Upregulation of tumor necrosis factor α and ADAMTS-5, but not ADAMTS-4, in human intervertebral cartilage endplate with modic changes. *Spine* 2014;39:E817-E825.

580. Kisiday JD, McIlwraith CW, Rodkey WG, et al. Effects of Platelet-Rich Plasma Composition on Anabolic and Catabolic Activities in Equine Cartilage and Meniscal Explants. *Cartilage* 2012;3:245-254.

581. Pinho-Ribeiro FA, Verri WA, Jr., Chiu IM. Nociceptor Sensory Neuron-Immune Interactions in Pain and Inflammation. *Trends Immunol* 2017;38:5-19.

582. Frisbie DD. Future directions in treatment of joint disease in horses. *The Veterinary clinics of North America Equine practice* 2005;21:713-724, viii.

583. Hedbom E, Hauselmann HJ. Molecular aspects of pathogenesis in osteoarthritis: the role of inflammation. *Cell Mol Life Sci* 2002;59:45-53.

584. Palmer JL, Bertone AL. Joint structure, biochemistry and biochemical disequilibrium in synovitis and equine joint disease. *Equine veterinary journal* 1994;26:263-277.

585. Seshadri V, Coyle CH, Chu CR. Lidocaine potentiates the chondrotoxicity of methylprednisolone. *Arthroscopy : the journal of arthroscopic & related surgery : official publication of the Arthroscopy Association of North America and the International Arthroscopy Association* 2009;25:337-347.

586. Murray RC, Znaor N, Tanner KE, et al. The effect of intra-articular methylprednisolone acetate and exercise on equine carpal subchondral and cancellous bone microhardness. *Equine veterinary journal* 2002;34:306-310.

587. Toth F, Schumacher J, Schramme MC, et al. Effect of anesthetizing individual compartments of the stifle joint in horses with experimentally induced stifle joint lameness. *American journal of veterinary research* 2014;75:19-25.

588. Kay AT, Bolt DM, Ishihara A, et al. Anti-inflammatory and analgesic effects of intra-articular injection of triamcinolone acetonide, mepivacaine hydrochloride, or both on lipopolysaccharide-induced lameness in horses. *American journal of veterinary research* 2008;69:1646-1654.

589. Misheff MM, Stover SM. A comparison of two techniques for arthrocentesis of the equine metacarpophalangeal joint. *Equine veterinary journal* 1991;23:273-276.

590. Menarim BC, Gillis KH, Oliver A, et al. Autologous bone marrow mononuclear cells modulate joint homeostasis in an equine in vivo model of synovitis. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2019;33:14337-14353.

591. Coleridge M, Schumacher J, DeGraves F. Comparison of lameness scores after a low 4-point nerve block to lameness scores after additional desensitisation of the dorsal metatarsal nerves in horses with experimentally induced pain in the metatarsophalangeal joint. *Equine Veterinary Education* 2020;32:199-203.

592. Kay A, Bolt D, Ishihara A, et al. Anti-inflammatory and analgesic effects of intra-articular injection of triamcinolone acetonide, mepivacaine hydrochloride, or both on lipopolysaccharide-induced lameness in horses. *American journal of veterinary research* 2009;69:1646-1654.

593. Becker C, Heidersdorf S, Drewlo S, et al. Efficacy of Epidural Perineural Injections With Autologous Conditioned Serum for Lumbar Radicular Compression: An Investigator-Initiated, Prospective, Double-Blind, Reference-Controlled Study. *Spine* 2007;32.

594. van den Boom R, van de Lest CH, Bull S, et al. Influence of repeated arthrocentesis and exercise on synovial fluid concentrations of nitric oxide, prostaglandin E2 and glycosaminoglycans in healthy equine joints. *Equine veterinary journal* 2005;37:250-256.

595. Fullerton JN, Gilroy DW. Resolution of inflammation: a new therapeutic frontier. *Nature Reviews Drug Discovery* 2016;15:551-567.

596. Buckley CD, Gilroy DW, Serhan CN. Proresolving lipid mediators and mechanisms in the resolution of acute inflammation. *Immunity* 2014;40:315-327.

597. Nelson BB, King MR, Frisbie DD. Assessment of a novel equine tarsocrural experimental joint disease model using recombinant interleukin-1β and arthroscopic articular sampling of the medial malleolus of the tibia on the standing sedated horse. *The Veterinary Journal* 2017;229:54-59.