Bone Marrow Mononuclear Cell Therapy for Equine Joint Disease

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ABSTRACT

Osteoarthritis (OA) can be debilitating and career-ending for horses. Current treatments offer temporary and symptomatic relief, but potentially deleterious side effects. Bone marrow mononuclear cells (BMNC) are a rich source of macrophage progenitors that are anti-inflammatory and promote inflammation resolution. The objective of this study was to evaluate the ability of intra-articular BMNC therapy to improve clinical signs of naturally occurring equine OA. Horses presenting with clinical and radiographic evidence of moderate OA in a single joint were randomly assigned to 1 of 3 treatments: saline (negative control), triamcinolone (positive control), or BMNC (treatment group). Horses were subjectively and objectively evaluated for lameness and synovial fluid collected (cytology and cytokine/growth factor quantification) at 0, 7, and 21 days post-injection. Data were analyzed using General Estimating Equations with significance set at $P<0.05$. There were no adverse effects noted in any treatment group. No significant differences in synovial fluid cytology parameters, objective/subjective lameness scores, nor joint circumference were found between treatment groups at any time point. Within treatment groups, joint circumference did not change over time for saline- and triamcinolone-treated horses. However, joint circumference and objective lameness decreased significantly within BMNC-treated horses between Days 0 and 21 and Days 7 and 21. Lameness improved in saline-treated horses from 0 to 21 days, but did not improve in triamcinolone-treated horses. The decreased lameness and lack of adverse effects in the BMNC-treated horses in our study support a larger clinical trial using BMNC.

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GENERAL AUDIENCE ABSTRACT

Osteoarthritis (OA) is a common source of joint pain in people and horses. Current treatments provide only partial and/or temporary relief. As a result, there is an urgent need for more effective and long-lasting treatment options. Arthritis is characterized by uncontrolled joint inflammation and progressive cartilage and bone destruction. Macrophages are cells within normal joints that function to resolve mild inflammation, maintaining joint health. However, when physiologic functions are overwhelmed, macrophages perpetuate inflammation through the recruitment of additional cell types to cope with the increased demands for repair. If this process is appropriately accomplished, macrophages resolve the inflammation, thereby enabling recovery and repair within the joint. Bone marrow aspirate is an excellent source of bone marrow-derived macrophage precursors (bone marrow mononuclear cells or BMNC) that have been shown to reduce joint inflammation and lameness in people and horses. The objective of our clinical trial was to evaluate the ability of intra-articular BMNC to improve clinical signs of naturally occurring OA in horses. BMNC treatment was compared to a placebo injection of saline and a standard-of-care in horses, corticosteroids. There were no adverse effects of BMNC treatment and BMNC-treated horses had significantly reduced joint circumference and lameness after 21 days. Synovial fluid cytology parameters did not differ significantly between treatment groups at any time point. In summary, BMNC are exciting because a horse can be treated with its own cells without the need for specialized equipment, and have the potential to naturally benefit thousands of people and horses suffering from arthritis.

DEDICATION

This project is dedicated to the most loving and compassionate confidante a person could ask for, Newton. He represents more than a dog as he was a source of companionship and beloved member of our family for the past 10.5 years. Against all odds, he was able to enjoy a full-life and bring pure joy to those around him. There is not a day goes by that a tear does not creep into my eyes or a lump does not fill the back of my throat at the thought our sweet baby love. While no longer with us, Newton will continue to touch our lives every day and be the driving force for the pursuit of knowledge and research!

ACKNOWLEDGEMENTS

To begin, this project was quite an undertaking and several thought it might be impossible to complete. Through resilience, hard-work, determination, and the help of many wonderful people along the way, we were able to execute the project and have results that are both encouraging and novel. #11,200 miles

Firstly, I would like to say thank you to my wife and best friend, Constance! Your unwavering support both personally and professionally have meant the world to me. From Knoxville to Lexington to Blacksburg and everywhere in between, you have helped guide us along on this journey. Whether it was the use of your own paid leave, 6 am jogging sessions, or late-night travels to Pennsylvania, you were there motivating and encouraging me along!

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trial. I am grateful for their support and buy-in to this project and willingness to lend a helping hand for the betterment of our equine patients!

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INTRODUCTION

Thesis Organization

This thesis is presented in a manuscript format. The introduction provides a brief overview of the research topic, which is expanded further in Chapter 1, the Literature Review, which provides an in-depth summary of literature about osteoarthritis and current therapies. The manuscript chapter, Chapter 2, is entitled "Bone Marrow Mononuclear Cell Therapy for Equine Joint Disease" and contains findings of my original clinical research project. In Chapter 3, major conclusions and future directions for investigation of mononuclear cell therapy for horses are discussed.

Introduction

Osteoarthritis (OA) is a chronic, debilitating, and often career ending condition for human and equine athletes alike. The burden of this condition is costly from both a financial and welfare standpoint. The cost of treatment can be exorbitant, which in turn affects the ability of the caretaker to adequately care for the animal. Irreversible joint damage, coupled with loss of function, result in reduced quality of life.

OA is a cyclical disease of inflammation and tissue destruction, of which the synovial membrane is a central component for continued inflammation. Synovitis is associated with both early and late symptoms of OA and has been correlated to disease progression and joint space narrowing. In fact, synovitis is often the single driver of the catabolic processes in OA. Therefore, the ability to control or alter the degree of synovitis would have implications for treatment of OA and potentially allow for the preservation of joint function and reduction in pain.

The normal synovial membrane is populated by fibroblasts, macrophages, and a small number of lymphocytes. Histologically, synovitis is characterized by thickened synovial membrane and cellular infiltration, primarily macrophages. In health, macrophages maintain an anti-inflammatory phenotype and work in conjunction with synovial fibroblasts to promote synovial health by performing phagocytosis of cartilage breakdown products, cellular debris, and foreign material, as well as secretion of regulatory cytokines and growth factors. When these regulatory and homeostatic functions are overwhelmed, macrophages become activated to an inflammatory phenotype in an effort to control and ultimately resolve inflammation and repair the injured tissue. The inflammatory phenotype produces signaling molecules that recruit additional cells (e.g., additional macrophages, neutrophils, and lymphocytes) to upregulate the inflammatory response in an effort to counteract the increased demands for repair. However, the uncontrolled production of inflammatory cytokines and the upregulated inflammatory response ultimately results in joint degeneration. Synovial macrophages are therefore the main drivers of synovial inflammation. Controlling the numbers of macrophages that display an anti-inflammatory phenotype has the potential to harness the beneficial effects of macrophages and restore joint homeostasis and resolve inflammation.

Macrophages perform the seemingly opposite functions of perpetuating synovitis and joint destruction in OA and modulating synovial homeostasis in health. It is this key observation that is the basis of this thesis. We sought to harness the effects of bone marrow mononuclear cells, which are predominantly macrophage progenitors that display an anti-inflammatory phenotype, in OA-affected joints. Our goal is to developing a novel treatment for OA that not only improves the clinical signs of lameness and joint effusion, but also causes a cellular shift

resulting in inflammation resolution, thereby creating a sustainable and reparative effect within the joint.

CHAPTER 1: LITERATURE REVIEW

Prevalence of Osteoarthritis

Osteoarthritis (OA) is a common condition that affects synovial joints and is characterized by progressive degeneration of articular tissues. In the United States, OA affects approximately 34% of people over the age of 65 and more than 50 million adults.baum[1,2] OA is the leading cause of disability and the second most costly health condition in the United States, totaling $300 billion per year.[3-6]

In horses, as in people, OA is also the most common cause of lameness across disciplines, accounting for approximately 60% of lameness problems.[7,8] OA affects an estimated 54% of Thoroughbred racehorses[9] and 10-19% of Western performance horses, primarily Quarter Horses.[10,11] There is a positive correlation between increasing age and prevalence of OA, with approximately 83.5% of horses ≥15 years old being affected.[12] Equine lameness negatively impacts the US equine industry at the rate of $678 million to 1 billion annually,[13] with the direct financial impact of OA alone estimated at $39 billion.[14,15]

Overall, OA and associated lamenesses create a substantial financial impact due to treatment-associated costs, lost earnings, and early retirement.[7,16] Current treatment options provide temporary symptomatic relief of clinical signs and fail to prevent progression of osteoarthritis. Because of the high impact of quality of life implications and the financial costs related to OA, there is a critical need to develop therapies aimed at the prevention and treatment of OA as opposed to simply blocking inflammation. Elucidating the mechanisms of the onset and progression of OA are closely tied to the ability to advance the OA treatments.

Synovial Joints: Anatomic Review

Synovial joint structure

Skeletal movement is substantially enabled by synovial joints, which allow for a large range of motion and low friction movement, and dissipation of weight-bearing forces from one long bone to another during locomotion. Synovial joints (or diarthroses) are the most common type of joint in the body and the ones most apt to sustain injury or be afflicted with disease. The general structure of a synovial joint consists of a fluid-filled (synovial) cavity surrounding the ends of long bones (Figure 1-1).[17] The synovial cavity is defined by the joint capsule, which has two main components: the fibrous joint capsule provides the outer, structural component and the synovial membrane provides the inner, metabolic component. Articular, hyaline cartilage covering the ends of bones is supported by subchondral bone. The articular cartilage is in intimate contact with, and immersed in, synovial fluid, which acts as both a lubricant for frictionless motion and a source of nourishment for the cartilage. The ligamentous and musculotendinous structures that cross joints, along with the joint capsule, stabilize synovial joints and are often intimately associated or integrated with the fibrous joint capsule.[18] A healthy synovial joint is composed of many components, each with specific structural properties that vary according to function.[19] The individual components combined act as a single functional articular unit. Dysfunction or injury to one or more components alters joint homeostasis and function, resulting in joint pathology.[17,18]

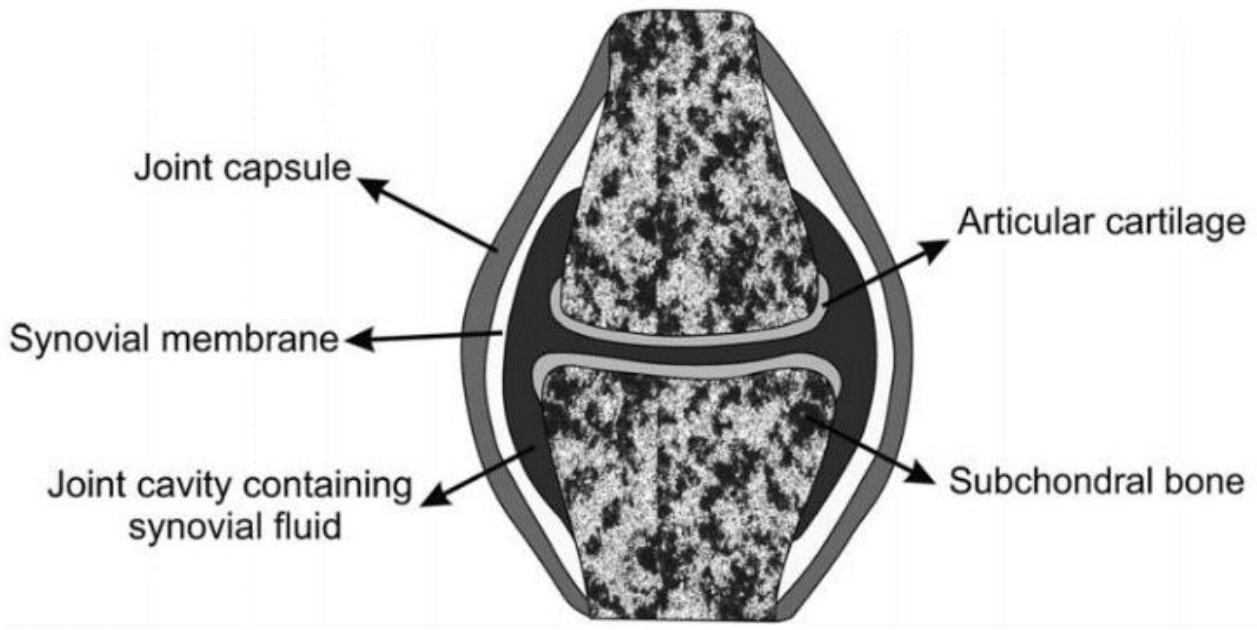

Figure 1-1. General overview of the structure of a synovial joint. From: De Grauw J.C., 2011. Molecular monitoring of equine joint homeostasis.[20] Reprinted with permission from Taylor and Francis.

Subchondral bone

The subchondral bone confers structural integrity for the overlying articular cartilage and is integrated with the articular cartilage through a layer of calcified cartilage (Figure 1-2).[21,22] Anatomically, subchondral bone is comprised of two distinct entities, the subchondral bone plate and trabecular bone.[21] The primary function of subchondral bone is to distribute the forces across a large surface area generated through weight bearing and locomotion.[22] The ossified, compact subchondral bone plate provides support, while the more elastic trabecular bone acts as an important shock-absorber during joint loading.[22] Subchondral bone has the unique ability to

disperse axial loads across a joint and physically adapts in response to the stresses placed on the joint according to Wolff's Law.[23,24] This adaptive response is prominently observed in the trabecular bone and is mediated by osteoblasts and osteoclasts, the bone cells that function to form and resorb bone, respectively.[22] In contrast to other tissues in the synovial joint, the subchondral bone responds to loading and is able to adapt by remodeling during times of injury or training through alterations in matrix turnover and mineral density. When these adaptive capabilities are exceeded, damage begins to accumulate in the subchondral bone, leading to clinical signs of joint disease and ultimately lameness.[21,24] Radiographically, subchondral bone damage can manifest as sclerosis. Functionally, sclerosis results in decreased deformability and elasticity and increased thickness within the bone plate and trabecular bone.[24] Together, these factors have a direct effect on the mechanical loading capacity of the subchondral bone/articular cartilage unit. Moreover, sclerosis confers stiffness to the subchondral bone, which alters its shock absorbing capabilities and increases shear forces on the overlying cartilage.[22] The end result of these subchondral bone changes can be cartilage damage and progression of OA.

Structure and composition of articular cartilage

Articular cartilage is a unique and multifaceted tissue that constitutes the opposing surfaces of the joint and allows for near frictionless movement during locomotion.[18,25] In addition to enabling low friction, efficient movement, articular cartilage is adapted to withstand substantial compressive loads and significant shear forces throughout the range of motion of synovial joints.[26] Grossly, articular cartilage is smooth, opaque and white (Figure 1-3). Articular cartilage is 1-4 mm thick in horses and people, depending on the joint, location within the joint (weight bearing surface or not), age of the individual, and the presence of disease.[18]

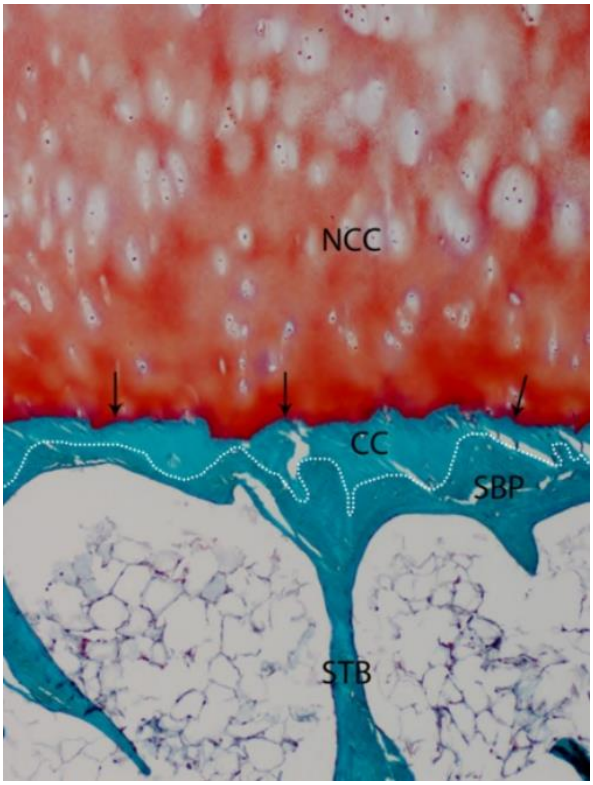

Figure 1-2. Structure of subchondral bone and articular cartilage in a normal synovial joint demonstrating incorporation of these two layers at the tidemark. CC, calcified cartilage; NCC, non-calcified cartilage; SBP, subchondral bone plate; STB, subchondral trabecular bone. Arrows denote tidemark; dotted lines indicate cement line. From: Li et al. Arthritis Research & Therapy 2013, 15:223[21] with permission from Springer Nature.

Mature articular cartilage is avascular and lacks nerves and lymphatics.[16,18,27] Most importantly, and as a result in part to its avascular nature, articular cartilage has a limited ability for healing and regeneration. Consequently, preservation of healthy articular cartilage is essential to maintaining joint health.

The lack of intrinsic vasculature, neural and lymphatic input in articular cartilage makes it dependent on synovial fluid for nutrients and waste removal.[16,18,27] Minute openings in the

superficial layer of cartilage, approximately 6 nm in diameter, allow exchange of small solutes (ions and molecules such as glucose and amino acids) between the cartilage and the synovial fluid.[28] Nutrients are produced in the layers of the synovium, diffuse into the synovial fluid and eventually reach chondrocytes embedded in the cartilage.[18,27,28]

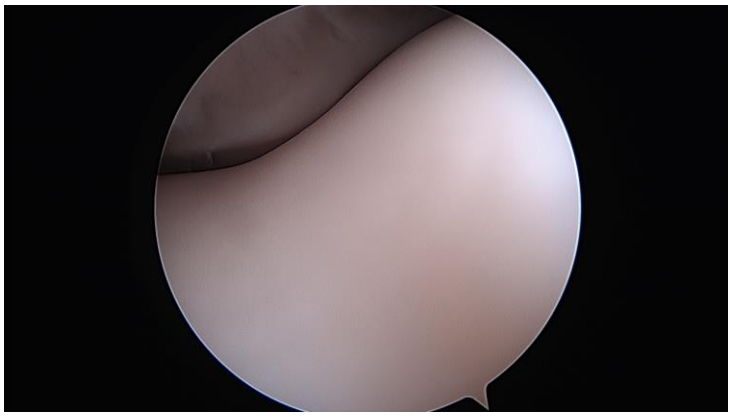

Figure 1-3. Arthroscopic photograph showing the smooth, opaque white surface of healthy articular cartilage in an adult equine metacarpophalangeal joint.

Articular cartilage is comprised of abundant extracellular matrix (ECM) containing sparsely distributed chondrocytes.[27] Water, collagen, and proteoglycan are the principal constituents of ECM with lesser amounts of noncollagenous proteins and glycoproteins present.[27] Collectively, these components help retain water within the ECM, enabling the articular cartilage to withstand stresses and loads.[17,27]

While articular cartilage appears to be a homogenous tissue macroscopically, variations in configuration exist as it transitions from the articular surface to subchondral bone.[29] The composition of articular cartilage varies between zones, with chondrocytes expressing different cellular morphology in each zone.[26,30] The mechanical properties, and thus the function of

cartilage, is dictated by the organization and interaction of its matrix components as they vary from the articular surface.[26] (Figure 1-4).

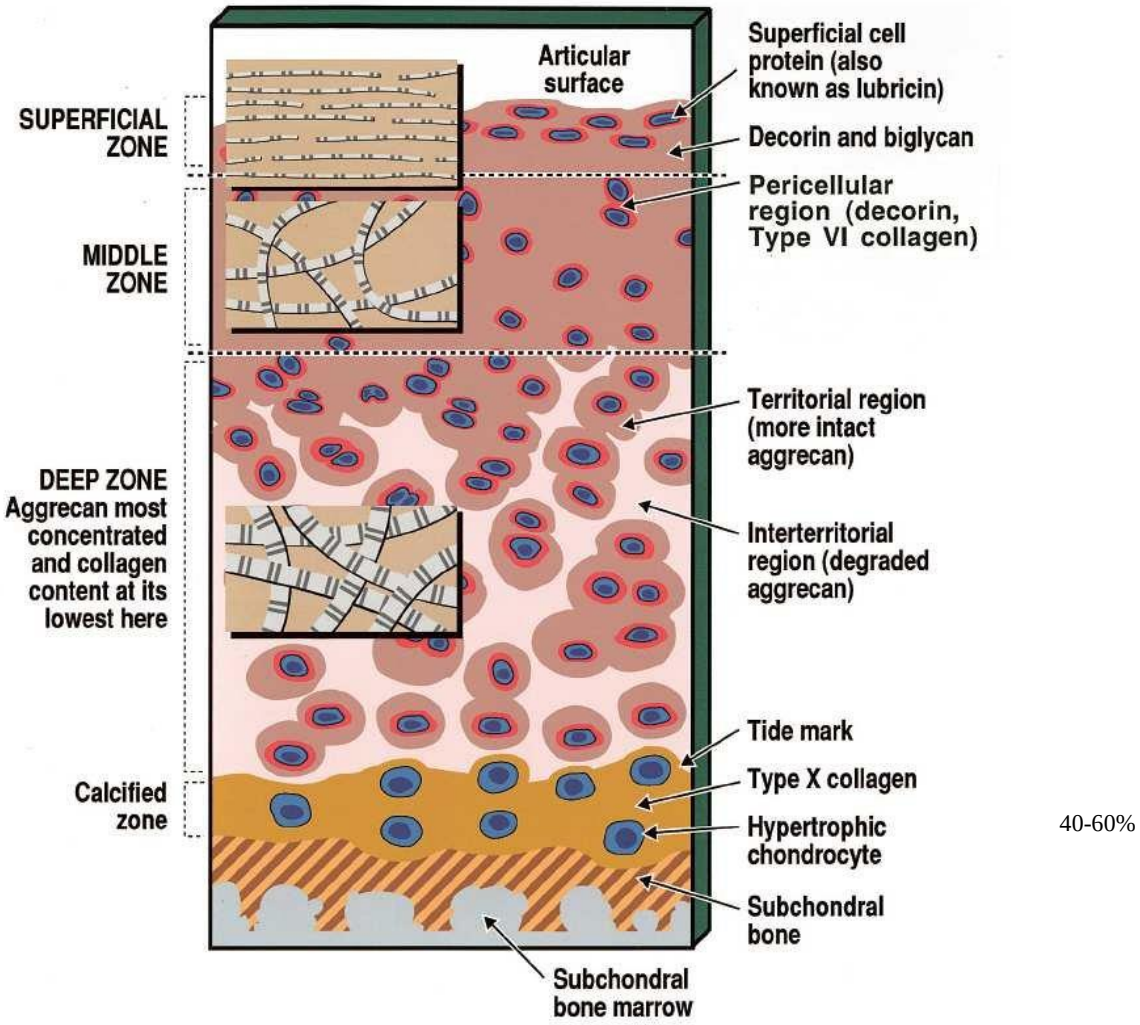

Figure 1-4. General structure of articular cartilage in regards to zones, region, and orientation of collagen fibers and chondrocytes. Modified from Poole AR: Cartilage in Health and Disease 2001.[29] Reprinted with permission from Wolters Klewer Health.

Zonal structure of mature articular cartilage

The superficial zone of cartilage, closest to the joint surface, contains the highest density of chondrocytes and comprises 10- 20% of the total cartilage thickness.[27,30] Chondrocytes in this zone are flat, elongated and orientated parallel to the articular surface. Similarly, collagen fibers (primarily type II) throughout the superficial zone are densely packed and aligned parallel to the joint surface.[27] The superficial zone contains the greatest amount of collagen, but lowest proteoglycan content, in comparison to other deeper zones. Additionally, the superficial zone contributes tensile strength to articular enabling it to withstand shear forces produced during movement while protecting the deeper layers from compressive stresses.[26,27,30,31]

Just deep to the superficial zone is the middle zone that occupies 40-60% of cartilage thickness.[26,28] In contrast to the superficial zone, the middle zone has larger, ovoid to round chondrocytes that are at a low density.[26,31] In this zone, there are larger diameter collagen fibrils that have an arched configuration.[17,27]

The deep (radiate) zone contributes approximately 30- 40% of the cartilage depth and contains chondrocytes arranged in columns.[23,28] In the deep zone, the collagen fibrils have the largest diameter, are positioned perpendicular to the surface of the joint, and cross the calcified zone and attach in the subchondral bone.[23,28] Because of its high proteoglycan content, the deep zone affords the most protection against compressive forces of all the zones.[27]

The deepest layer of cartilage is the calcified zone, which consists of hypertrophic collagen and matrix. The transition from non-calcified articular cartilage to calcified cartilage is denoted by a region referred to as the ‘tidemark’. Between the calcified cartilage and subchondral bone is the cement line.[28] The tidemark acts as an anchor point for collagen fibers

from the deep zone, securing the articular cartilage to subchondral bone. There are very few cells in the calcified zone and chondrocytes that are present are large and immature.

Extracellular matrix components

Chondrocytes are metabolically active, synthesize the components of the ECM, and produce degradative enzymes responsible for the maintenance and repair of the ECM.[27,29] Further, chondrocytes function in a low oxygen environment, exhibit low metabolic turnover, and rely mostly on diffusion-derived nutrition from the synovial fluid.[32] The size, shape, and number of chondrocytes vary depending on the zone in which they are embedded, as described above.[27] Zonal variation combined with mechanical queues from the surrounding matrix dictate the production and remodeling of ECM components.[29]

Chondrocytes use cytoplasmic processes that extend into the cartilage ECM to sense mechanical loading and the pericellular microenvironment.[17,27] Articular cartilage loading causes alterations in intracellular water content, pH, and ions (Na+, K+ and H+), which in turn creates a series of signals that regulate production of matrix components.[32] Physiological loading results in increased proteoglycan and collagen content of articular cartilage matrix; however, excessive loading can also cause reductions in proteoglycans and cause catabolism of the collagen matrix.[27,32] The inability to maintain the osmotic balance of ion concentrations such as Na+ and K+ dramatically affects cell metabolism, membrane transport and macromolecule synthesis, ultimately effecting the ECM turnover. Therefore, the ECM and chondrocytes intimately rely on each other to maintain cartilage homeostasis.

In addition to sensing changes in the nearby cartilage matrix, chondrocytes are sensitive to alterations in other joint tissues through the production and release of inflammatory cytokines

into the matrix or synovial fluid by neighboring chondrocytes and synoviocytes.[32] During physiologic remodeling, low levels of interleukin (IL)-1β induce chondrocytes to produce matrix metalloproteinases (MMP) that cause collagen cleavage and remodeling.[32] For example, type IX collagen molecules are cleaved from the surface of type II fibrils by stromelysins (MMP-3) and replaced with type II collagen molecules, increasing the diameter of the fibril without removal of the entire construct.[33] Anabolic effects mediated by growth factors such as transforming growth factor-β (TGF)- β, stimulate chondrocytes to lay down matrix. Catabolism inhibitors serve to counteract matrix degradation and include tissue inhibitor of metalloproteinase and plasminogen activator inhibitor.[34] In OA, trauma or aging disrupt chondrocyte metabolism to favor catabolic processes, causing net degradation of the cartilage matrix.

Collagen is the cartilage component that provides a framework for the other matrix molecules. Several types of collagens are described in the articular cartilage and are classified as fibril-forming (types I, II, III, V, and XI) and non-fibril-forming collagens.[18] Small amounts of the non-fibrillar collagens (types V, VI, IX, X, and XI) help to form and stabilize the type II collagen network in cartilage.[31,35,36]

An individual type II collagen molecule is formed by a trimer of three identical α_1 polypeptide chains that form a triple helix.[17,37] Trimers are assembled intracellularly as propeptides and are cleaved to the mature peptide once transported extracellularly.[17] Collagen fibrils are assembled in a quarter stagger along fashion along their length, with each molecule offset from the next molecule by 25%.[17,18] Types IX and XI collagen are intimately associated with type II collagen and are crucial to the crosslinking and mechanical stability of the fibrillar network.[18]

Proteoglycans have a hyaluronic acid (HA) filamentous core that is attached to multiple glycosaminoglycans (GAG) via link proteins (Figure 1-5).[18,28] The two major classes of proteoglycans found in articular cartilage are large aggregating proteoglycan monomers, such as aggrecan, and small proteoglycans, such as decorin.[38] Aggrecan resides in the fibrillar collagen network and increases in amount with the distance from the cartilage surface.[17]

Glycosaminoglycans are long, unbranched, and highly negatively charged disaccharide molecules.[36] The most clinically relevant GAGs are HA, keratan sulfate, chondroitin-4-sulfate, and chondroitin-6-sulfate.[28,38] HA is the largest GAG produced by chondrocytes and in contrast to the other GAG, does not contain negatively-charged sulfate ester groups.[36] Furthermore, HA plays a vital role in linking chondrocyte metabolism and turnover of ECM through CD44 cell surface receptors.[29] Due to their high density of negative charges and the massive numbers of adjacent GAG molecules attached to each core protein, GAG repel each other, creating positive pressure between them that attracts and retains water molecules.[23,25,28] This retention of water is absolutely critical to the ability of cartilage to withstand repetitive compressive forces.

Joint capsule, synovial membrane, and synovial fluid

As described above, the two main layers of the joint capsule, the fibrous capsule and the synovial membrane, each perform unique roles critical to the function of healthy joints.[18] The fibrous joint capsule is contiguous with the periosteum of the bone to which it is attached, and provides structural integrity to the joint capsule, uniting the articulating bones of each synovial joint.[18,39] The mechanical stability of the fibrous joint capsule is in part created through its intimate relationships with extra-articular structures, such as collateral ligaments.[17,18] Proprioceptive

nerve endings within the fibrous joint capsule contribute to active stabilization by providing real time information regarding joint position to the brain.[17]

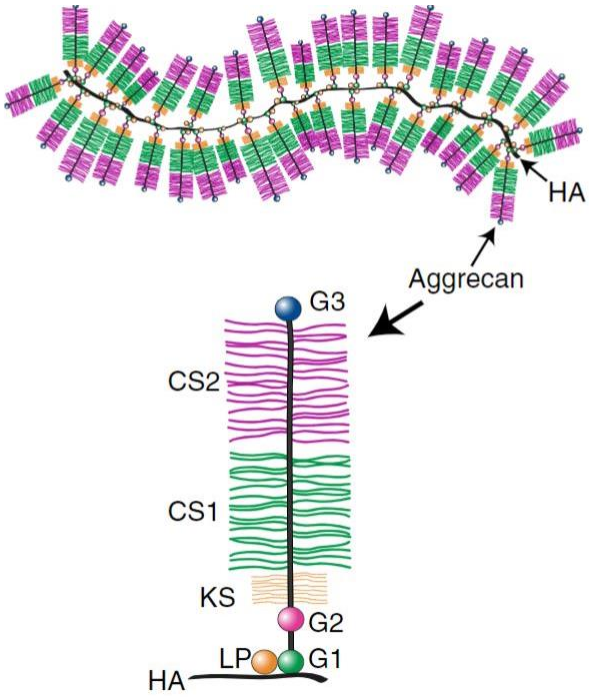

Figure 1-5. Schematic of proteoglycan aggregates with central HA with aggrecan and link proteins attached. From Roughley et al 2014 Journal of Experimental Orthopedics.[40] Reprinted with permission from Springer Nature.

The synovial membrane is metabolically active tissue that performs important functions such as providing nutrition for chondrocytes embedded in the articular cartilage and lubrication of the joint surfaces.[16,41] The composition and volume of synovial fluid, which is essential for preserving the normal physiologic state of articular cartilage, is maintained by cells within the synovial membrane.[28]Additionally, the synovial membrane provides a surface area for immune and homeostatic functions of the joint. The synovial membrane is composed of the superficial

intimal layer, a capillary plexus, a deeper network of lymph and vessels, and a subintima composed of adipose, areolar, and/or fibrous tissue.[42] The subintima has a rich blood supply and innervation that originates from peripheral and muscular nerve branches.[18] The intima represents the interface between the synovial cavity itself and the subintimal layer. The intimal layer is 1-4 cell layers thick, contains no basement membrane, and is largely responsible for regulating the volume and composition of the synovial fluid.[18] The intima contains intercellular gaps and fenestrated capillaries that favor exchange between capillaries and the joint cavity. The structure of the synovial intima varies depending on mechanical stress. In areas of high biomechanical stress, such as areas underlying tendons, the intima is flat.[42] In synovial recesses, where mechanical stresses are low, the intima has a villous architecture.[18]

The intimal layer is composed of synovial lining cells, or synoviocytes arranged on the luminal side of the intima and extends 20-40 μm beneath the luminal surface.[41,43] The two main kinds of synovial lining cells are type A (macrophage-like) and type B (fibroblast-like) synoviocytes.[44] Synovial macrophages (type A synoviocytes) are engaged in phagocytosis or pinocytosis, clearing unwanted particles from the joint.[18] Synovial fibroblasts (type B synoviocytes) are the major cell type in normal synovium, accounting for 55-70% of the cell population.[41,45] Type B synoviocytes are responsible for the secretion of proteins such as HA affect the anabolism and catabolism of the synovial joint.[18] Dialogue between synovial macrophages and fibroblasts is required in health and disease to ensure proper function. One of these functions is the coordinated secretion of signaling and structural molecules (e.g., cytokines, chemokines, growth factors, hyaluronan, lubricin, and prostaglandin E_2 (PGE_2) that balance the anabolic and the catabolic metabolism of the joint.[17,18] Importantly, sustained joint inflammation

dysregulates the anabolic and catabolic pathways, resulting in altered turnover of cartilage components and development of disease.[17,44]

Regulation of the composition and rate of production of synovial fluid is an important function of the synovium.[18] A permeable barrier, created by intercellular gaps between ECM components, allows plasma molecules ≤10 kDa to cross the synovial vessels into the joint cavity, but excludes large molecules.[18] Examples of plasma molecules that readily cross the synovial vessels and contribute to the composition of synovial fluid include glucose, oxygen, carbon dioxide, and other small proteins.[17] The regulation of solute exchanges depends on the degree of synovial inflammation, which affects vascular permeability, lymphatic drainage, and intra-articular pressure.[17] Combined, these alterations affect the size and concentration of molecules in the synovial fluid.[18] Pressure within the joint is primarily subatmospheric (-2 to -6 cm H_2O), which favors synovial ultrafiltration. Inflammation leading to joint effusion and increased intra-articular pressure negatively affect this pressure gradient.[17,18,46] Synovial membrane homeostasis is dependent on lubricant secretion of molecules such as HA and lubricin by synovial cells and drainage by the synovium lymphatics. Additionally, this physiologic balance is required for adequate steric exclusion of larger molecules from the synovial cavity.[18]

Lubricin and HA are produced by type B synoviocytes and are critical in reducing friction at the articular surface through boundary lubrication. Under normal conditions, high molecular weight (MW) molecules like lubricin and HA are not readily permeable, while small molecules like growth factors and cytokines readily diffuse through the synovial membrane. This property allows for the retention of high MW lubricating molecules within the joint and prevents entrance and deposition of high MW plasma proteins on the articular surface, altering the

viscosity and composition of the synovial fluid [41,44]. When a joint is inflamed, alterations in membrane permeability result in changes in HA and lubricin concentrations in synovial fluid.[41,47]

In health, all of the components of a synovial joint must communicate effectively with each other to ensure proper function. Alterations in one tissue of the joint can indirectly or directly interfere with the normal homeostatic mechanisms of the joint as a whole. Moreover, the balance between anabolism and catabolism is essential for optimal joint function. In OA, alterations in the molecular machinery essential for homeostasis are perturbed, metabolic demands exceed the capacity for repair, and a catabolic state characterized by joint swelling and loss of joint function develops.

Etiology of Equine OA

Osteoarthritis is a degenerative condition of synovial joints that causes pain and loss of joint function. The manner of causation is varied, but commonly entails impairment of normal tissue homeostasis, trauma, and joint instability. Alterations in homeostatic function perpetuate a cycle of low-grade inflammation and tissue degradation, which, in turn, causes changes in the cellular and molecular composition of all joint tissues, including articular cartilage, subchondral bone, and synovium.[48] Characteristic changes associated with joint tissues include cartilage degeneration, osteophyte production, subchondral bone sclerosis, synovitis, and periarticular fibrosis.[8,49] OA was once considered a non-inflammatory disease of normal wear and tear, but current evidence shows that inflammation of all of the joint tissues, especially the synovium, is integral for initiation and propagation of OA.[50]

In horses, cyclic and repetitive trauma is a well-defined risk factor for OA.[51] Inflammation is elicited in joint tissues by micro-trauma that prohibits homeostasis or a single

traumatic event that causes gross structural alterations.[50]For example, normal healthy cartilage has specialized biomechanical properties that allow it to distribute forces between joint surfaces while providing nearly friction-free movement.[17] Excessive mechanical load or a major traumatic event can shear off a cartilage fragment that is subsequently released into the synovial cavity. The presence of the damaged hyaline cartilage fragments free in the joint and along the margins of the original site of injury initiates synovial inflammation. In response to synovial inflammation, synoviocytes produce pro-inflammatory mediators that serve to recruit immune cells, increase angiogenesis in the synovial membrane, and alter the physical properties of chondrocytes.[23,50] The culmination of these responses perpetuates a viscous cycle as chondrocytes produce additional proteolytic enzymes and cytokines.[52] Cartilage degradation ensues and induces more synovial inflammation.

Importantly, inflammation of the synovium (synovitis) is strongly associated with the pathophysiology equine OA.[53] The production of inflammatory mediators such as IL-1β and tumor necrosis factor (TNF)-α by the inflamed synovium stimulates chondrocytes to produce ECM-degrading proteolytic enzymes such as MMP and A Disintegrin and Metalloproteinase with Thrombospondin Motifs (ADAMTS).[8,18,38,54] In addition to producing proteolytic enzymes, chondrocytes produce and secrete several inflammatory mediators such as IL- β, TNF-α, IL-6, IL-7, and IL-18.[41,55] These inflammatory cytokines disrupt the normal metabolic homoeostasis of cartilage by favoring catabolic processes, with MMP activation leading to alterations in chondrocyte metabolism and cartilage destruction.[55]

Pathophysiology of OA

Synovitis

Synovitis plays a central role in the initiation and propagation of OA and persists throughout all stages.[19,30,56,57] During the early stages of OA, synovitis does not manifest by overt signs of inflammation such as soft tissue swelling or joint effusion. Pain, however, may be present. During the early stages of synovitis, arthroscopic changes reflect only localized synovial inflammation and proliferation.[41,58] In the more advanced stages of OA, synovitis is associated with loss of function and pain, with correlation to the Kellgren-Lawrence scoring system, as osteophytes, sclerosis and joint space narrowing increase.[54,59] In fact, people with higher grades of synovitis are nine times more likely to experience knee pain.[54] Moreover, this provides evidence to demonstrate that synovitis is a significant factor in the pathophysiology of OA.

Histologically the synovium in OA patients is characterized by synovial lining hyperplasia, sublining fibrosis, and stromal vascularization.[41] The overall cellular infiltration in the synovium of OA-affected joints tends to be lower compared to rheumatoid arthritis (RA)-affected joints in people.[52] However, macrophage numbers in the synovium are comparable to RA-affected joints and, in some cases, higher in the synovium of OA-affected joints.[57,60] A predominance of macrophage infiltration in the synovium is especially common during the early stages of OA.[57] In end-stage OA, fibrous tissue replaces synovial cells, which compromises the basic functions of the synovium.[57]

OA-inflamed synovium synthesizes a wide range of cytokines and chemokines (e.g., IL-1β, IL-6, IL-8, TNF-α), nitric oxide (NO), PGE_2, and proteases.[41,52] A number of these inflammatory mediators have catabolic effects, accelerating destruction of cartilage, and are increased in the tissues and synovial fluid of osteoarthritic joints.[41,53] In addition, these cytokines

cause upregulation of transcriptional pathways, such as nuclear factor kappa B (NF-kB) and mitogen-activated protein (MAP) kinase, which induce synovial cells to increase production of collagenase, proteolytic enzymes (e.g., MMP) and other lipid mediators, causing vascular hyperplasia in the synovium.[41,52]

In addition to affecting the joint at the biochemical level, synovitis affects the function of the entire joint. During OA, synoviocytes produce lower quantities of HA, and that produced has a decreased MW.[61,62] As inflammation and hyperplasia change during synovitis, so does the permeability of the synovial membrane. Hyperplasia of inflamed synovium causes increased permeability and loss of HA from the joint space into peripheral circulation.[63,64] In fact, measurement of increased blood serum concentration of HA[63] has been used as a marker of OA in people.[64]

In summary, synovitis is present and contributes to all stages of OA. The hallmark of OA-affected synovium is the macrophage, which plays a critical role in the pathogenesis of OA. Through the induction of inflammatory mediators, growth factors and proteinases, macrophages within the inflamed synovium alter anabolic mechanisms in favor of catabolism and cartilage destruction. Therefore, therapies targeting macrophages and macrophage-associated inflammatory pathways may have the capacity to provide symptomatic relief and prevent the progression of OA.

Synovial macrophages

Synovial macrophages are the main drivers of inflammation within the joint and play key roles in the innate and adaptive immune systems, which play major roles in the development of OA.[65] The importance of macrophages and their role in mediating synovitis was first noted when

systemic depletion of macrophages in a mouse model of OA dramatically reduced the development of OA.[66,67] Additionally, articular macrophage depletion in human patients with RA aided in controlling joint inflammation.[68] The anti-inflammatory effects of macrophage depletion are associated with decreased expression of MMP-1, -3, -9, and -13, and IL-6 and IL-8.[60,68-72] Furthermore, a recent *in vivo* study by Kraus et al. revealed that the quantity of activated macrophages in OA-affected tissue is directly related to disease activity, severity, and pain in people.[73] The association between pain, activated macrophage quantity, and radiographic signs of OA suggests that drugs targeting synovial macrophages or synovial macrophage-driven inflammation are potential therapeutic targets.[65,73]

Macrophages are the most abundant immune cells within the osteoarthritic synovium and they communicate with neighboring synovial fibroblasts and T-cells through a group of cytokines that participate in modulation of different immune cells.[74] Synovial fibroblasts, the main stromal cell of the synovium, play an important role in the perpetuation of the inflammatory response during the development of joint disease.[75] *In vitro* stimulation of fibroblasts in osteoarthritic synovium with TNF-α, IL-1β, and interferon results in production of various inflammatory mediators, including IL-6, IL-8, and high concentrations of macrophage chemoattractant protein (MCP)-1.[76] Importantly, communication between synovial macrophages and chondrocytes can markedly affect the inflammatory reaction from these cytokines and chemokines. For example, synovial fibroblasts express Toll-like receptors (TLR)-2 and -4, which then induce production of additional cytokines, chemokines, and MMP.[75] One ligand of TLR-2 and TLR-4 is the CD14 molecule,[74,75] which is highly expressed by activated macrophages and exists in two forms during active inflammation, cell-bound or soluble .[75,76] The interaction of soluble CD14 from arthritic synovial fluid with TLR receptors on synovial fibroblasts triggers

the production of many cytokines involved in the initiation and progression of OA.[77] Additionally, synovial fibroblasts are stimulated to produce MMP-1 and MMP-3, which are associated with inflammation amplification and cartilage degeneration.[78]

The combination of sustained inflammation and concurrent production of new inflammatory cytokines and proteolytic enzymes leads to joint degeneration.[60,79] In addition to interaction with other joint tissues, molecular signaling pathways (e.g., NF-κB, STAT and Pi3K) are persistently activated in macrophages within arthritic synovium.[57,60] Benito, 2005 #407} Importantly, these signaling pathways serve as major sources of inducible NO and cytokines (e.g., IL-1β and TNF-α).[57] Furthermore, macrophages also produce MMP-2, MMP-3, and MMP-9, which are associated with articular cartilage degradation.[80] MMP-3 causes direct destruction of cartilage through degradation of ECM components and activates activation of aggrecanase II (ADAMTS-5), while MMP-2 affects articular cartilage indirectly by inducing chondrocytes to express other MMP.[55,74]

In contrast to their perceived deleterious role in synovitis, macrophages can also be key mediators of joint homeostasis.[74] Under physiologic conditions, macrophages and synovial fibroblasts work together to maintain the joint health.[81,82] One of the ways that macrophages control joint homeostasis is by performing phagocytic functions and by secreting anabolic cytokines, chemokines, and growth factors.[81-83] When phagocytic and regulatory functions become overwhelmed, synovial macrophages recruit and prime other immune cells, such as lymphocytes and neutrophils, to upregulate inflammation, in response to the increased demands for repair.[65,81,82] Synovial macrophages are the main source of IL-10, an anti-inflammatory cytokine essential for maintaining cartilage homeostasis and for tissue repair.[84,85] Absence of IL-10 inhibits recovery of damaged articular cartilage.[86] Importantly, complete depletion of

macrophages negatively affects chondrocyte metabolism and chondrocytic differentiation, as synovial macrophages modulate the transcriptional machinery of chondrocytes and synovial progenitor cells in a paracrine fashion.[83] In fact, macrophage modulation of transcriptional machinery is absolutely required for the expression of *SOX9* and *COL2*,central genetic markers of chondrogenesis and synthesis of collagen type II.[83]

Macrophages are somehow able to perform opposite functions in the joint, as they are responsible for both perpetuating synovitis and joint destruction in OA as well as modulating synovial homeostasis in health.[87] It has been proposed that these opposing functions are related to phenotype polarization of macrophages.[60,88-90] *In vitro* and *ex vivo* studies demonstrate that macrophages undergo phenotypic changes in response to environmental stimuli, polarizing into two distinctly different types of cells – inflammatory (M1) cells and suppressive/regulatory (M2) cells.[91] On the other hand, current *in vivo* findings suggest that macrophages in a non-inflammatory environment are by default homeostatic cells (M2).[74,92] Upon injury, an M1, inflammatory response is induced through activation of pattern recognition receptors by pathogen-associated molecular patterns (PAMP) which causes M1 macrophages to produce oxygen and nitrogen radicals and pro-inflammatory cytokines which leads to local tissue damage.[74,88-90] Activation of M2 macrophages is induced by IL-4 and IL-13.[88-90] During the resolution of inflammation, there is a shift in macrophage activation toward an M2 phenotype in order to promote clearance of debris, inhibit the production of inflammatory mediators, and restore tissue homeostasis[74,88-90] M2 macrophages produce anti-inflammatory cytokines such as IL-10, and promote cellular clearance, regeneration, and wound healing.[88-90,92]

Macrophages play such a pivotal role in resolving inflammation and promoting tissue repair that their depletion results in compromised wound healing and limb regeneration, or leads

to chronic inflammation.[93-95] In a salamander amputation model, systemic depletion of macrophages resulted in failure of limb regeneration, with extensive fibrosis and dysregulation of ECM component gene expression.[95] Moreover, full regenerative capacity was re-established by re-amputation once macrophage populations were restored.[95] These findings clearly demonstrate that macrophage function is essential for effective tissue repair and resolution of inflammation. In the face of chronic inflammation, macrophages become overwhelmed, thereby perpetuating inflammation. Therefore, it is reasonable to infer that increasing the numbers of macrophages in an inflamed joint could optimize and harness the desired M2 macrophage phenotype to counteract damage, favoring resolution of synovitis.

Cytokines

Cytokines are small regulatory proteins that control the anabolic and catabolic metabolism of tissues such as articular cartilage, and possess both pro- and anti-inflammatory functions.[55,96] Inflammatory cytokines are the most important compounds participating in the pathogenesis of OA. Their involvement disrupts normal cartilage homoeostasis by favoring catabolic processes that ultimately result in destruction of cartilage.[55]

The key inflammatory cytokines involved in OA, IL-1β and TNF-α, are produced by chondrocytes, synoviocytes, and infiltrating mononuclear cells.[96] Binding of IL-1β and TNF-α to membrane receptors alters chondrocyte metabolism, increasing production of proteolytic enzymes such as MMP-3, and driving degradation of important ECM components such as type II collagen and aggrecan.[55,96] In general, IL-1β and TNF-α concentrations in synovial fluid of normal and OA samples is low.[97,98] However, minimal changes in concentration (3-4 fold) is enough to dramatically increase the activity of their downstream effectors.[99]

Increases in IL-1β are associated with cartilage matrix degradation and inadequately repaired tissues that are unable to withstand normal mechanical stress. Further, increased IL-1β alters synthesis of type II collagen and proteoglycans[100-102] and stimulates production of enzymes that cleave cartilage components (e.g., MMP-1, -2, and -3).[101,102] Active IL-1β signaling also leads to nuclear translocation of NF-κB and results in expression of genes leading to the production of MMP and ADAMTS, prostaglandin, and nitric oxide.[103] The IL-1β induced translocation of NF-κB also stimulates the production of additional cytokines such as IL-6 as well as acts as autocrine loop by upregulating the production of additional IL-1β.[104] Of note, IL-1β concentrations required to induce expression of matrix degrading enzymes is substantially higher than that required for inhibiting matrix synthesis.[105] Combined, the effects of IL-1β on chondrocytes include induction of catabolic gene expression, downregulation of anabolic gene expression, and induction of chondrocyte apoptosis.[55,106]

TNF-α is considered an additional key inflammatory cytokine involved in the progression of OA.[55] Furthermore, TNF-α is synthesized as a transmembrane protein that requires cleavage by ADAM-17 (also called tumor necrosis factor converting enzyme) to be activated.[107] The secretion and presence of TNF-α in human OA is very similar to that of IL-1β, as they are secreted by the same cells within the joint and are elevated in human OA cartilage, subchondral bone, synovial membrane, and synovial fluid.[55] Similar to IL-1β, TNF-α incites the production of cartilage degradation through activating matrix metalloproteinases and aggrecanases (ADAMTS) and impairs synthesis of proteoglycans and collagen by chondrocytes.[102] Importantly, the effects of TNF-α alone appear to be less deleterious than when combined with IL-1β.[55]

IL-1β and TNF-α also cause downstream production of other inflammatory cytokines and chemokines, including IL-8, IL-6, MCP-1, NO, and PGE_2.[50,55] PGE_2 has a complex role as an

immunomodulator, which may be concentration and context dependent.[108] Classically, PGE_2 is associated with inflammation, as it mimics central signs of inflammation when injected into normal tissues and is present in inflamed tissue.[108] However, very low concentrations of PGE2 were anti-inflammatory in *in vitro* models of OA.[108,109] PGE_2 regulates the inflammatory response of synoviocytes by regulating expression of NF-kB, inhibiting MMP expression and apoptosis of chondrocytes, and suppressing cartilage collagen cleavage.[110-113] Of note, PGE_2 produces anti-inflammatory effects via transition of macrophages to an anti-inflammatory phenotype that then produces IL-10.[110,113-115]

As mentioned above when discussing macrophage phenotypes, the cytokine IL-10 exerts anti-inflammatory and chondroprotective effects, favoring chondrocyte recovery from damage.[86,116] Importantly, IL-10 has the capacity to inhibit expression of inflammatory cytokines while concurrently increasing expression of additional anti-inflammatory cytokines, such as IL-1 receptor antagonist protein (IL-1ra), a natural antagonist of IL-1. While, IL-10 exerts anti-inflammatory effects, it also increases proportionate to inflammation following injury to counteract the production of pro-inflammatory mediators. This explains why IL-10 concentrations can be increased in OA, but are especially in highly inflamed joints with RA.[117,118] During chronic low-grade inflammation, IL-10 concentrations in synovial fluid regress close to baseline.[117] Interestingly, in an *in vitro* study evaluating LPS-challenged macrophages in OA and healthy human patients, people whose cells showed no significant IL-10 increase following challenge with LPS were three times more likely to develop OA compared to those responding with a significant increase, reinforcing the role of IL-10 in driving resolution of inflammation and promoting joint homeostasis.[119]

Summary of the pathogenesis of OA

Osteoarthritis is the leading cause of lameness in horses and is a major source of medical expenditures.[8] Chronic synovitis, a major component of OA, is driven mainly by synovial macrophages.[65,69] Synovial macrophages have opposing functions in osteoarthritic joints, making them key drivers of both synovial inflammation and of synovial homeostasis.[41,50] Synovial macrophages function to maintain the health of a joint by engaging in phagocytic clearance and by secreting anti-inflammatory cytokines, chemokines, and growth factors.[17,83]

In the non-inflammatory state, most macrophages display an anti-inflammatory phenotype to maintain tissue homeostasis.[74] Once stimulated, macrophages activate and shift to an M1 phenotype, secreting inflammatory cytokines that incite inflammation and breakdown of articular cartilage.[74,120] In addition to paracrine control, activated macrophages alter intercellular signaling pathways in chondrocytes, promoting the degradation of ECM components by proteolytic enzymes.[74,120] Degraded ECM acts as damage-associated molecular patterns and perpetuates a continuous cycle of inflammation and degradation of articular cartilage.[43,52,74] This is the classic explanation of the pathogenesis of OA.

However, with the advent of discovery comes the development of newer, more modern models of the pathogenesis of OA such as the CSU osteochondral fragment model and LPS-induced synovitis that are instrumental in evaluation of pathobiological events contributing to OA. At the onset of joint injury, regulatory functions are often able to effectively modulate inflammation. Only if these homeostatic functions become overwhelmed do synovial macrophages signal other immune cells, such as neutrophils and lymphocytes, to counteract increased demands for repair, increasing inflammation.[43] If joint damage is sustained, resolution of inflammation is impeded, and catabolic processes predominate, resulting in degeneration of

the joint.[41,65] If inflammation can be effectively modulated to reverse the progression OA, and to create an environment conducive to the repair of the synovium, then perhaps OA can be prevented or reversed.[121] Perhaps the tendency for macrophages to predominantly default to the M2 phenotype can be harnessed to promote tissue repair and restore tissue homeostasis.[74,94] By capitalizing on the potential beneficial effects of M2 macrophages, there is the potential to revolutionize patient care by providing long-lasting pain relief, improved joint function, and inflammation reduction and/or resolution in OA-affected tissues.

Treatment of OA

OA is a common medical problem in horses that presents a significant treatment challenge, due in part to the complexity of the inflammatory response governing the progression of OA. Irreversible joint damage results in progressive loss of function and increased morbidity, considerable financial losses, and reduced quality of life. Treatment of OA can be symptomatic and disease modifying. Symptomatic treatment, as the name implies, reduces clinical signs associated with OA, such as heat, pain, swelling, and lameness, but has no effect on disease progression. Disease modifying osteoarthritis drugs (DMOAD) effect a positive outcome by altering both disease progression symptomatic relief of pain. Currently, there are no pharmacological therapies that effectively prevent the progression of OA. The majority of OA treatments focus on reducing clinical signs such as joint pain and swelling, improving function of the affected joint and increasing the patient's quality of life and enabling continued athletic activity. Although drugs that seek to modify the progression of OA exist, such as DMOAD, they currently do not effectively alter the course of tissue destruction and continued inflammation.

A multimodal approach to the treatment of equine joint disease is commonly undertaken. Non-steroidal anti-inflammatory drugs (NSAIDs), intra-articular corticosteroids, orthobiological therapies, and viscosupplementation are used to enhance patient comfort and reduce clinical signs. While these therapies are instrumental in providing symptomatic relief from OA, they fail to produce lasting effects and, in some cases, can have detrimental effects on cartilage metabolism and reparative mechanisms that are innately required for efficient tissue healing.[8,122-125] Some produce unwanted systemic side effects such as gastric ulceration, kidney injury, and laminitis.[8,122-125] Therefore, a critical need exists for new therapies capable of promoting inflammation resolution, while simultaneously promoting tissue healing.

Non-steroidal anti-inflammatory drugs (NSAID)

NSAIDs provide symptomatic relief from OA symptoms by exerting analgesic and anti-inflammatory effects through the inhibition of cyclooxygenase (COX) enzymes in the arachidonic acid cascade.[126] COX inhibition results in the downstream inhibition of prostaglandins and thromboxanes, thereby decreasing pain and inflammation.[127] Although highly efficacious, NSAIDs are not without risks, which vary based on the COX selectivity of each individual NSAID. Inhibition of COX-1, which is constitutively expressed and essential for regulatory functions, is known to cause mucosal damage and ulceration within the gastrointestinal tract.[127] COX-2 is an inducible enzyme associated with inflammatory events that plays a minor role in normal physiology.[127] To help reduce the unwanted side effects of NSAIDs, newer NSAIDs that more selectively block the COX-2 pathway have been developed.

While COX-2-selective NSAIDs do have fewer negative effects related to the gastrointestinal tract, they do also inhibit prostaglandins such as PGE_2, which are responsible for

chondrocyte proliferation and synthesis of ECM components.[125,128] Long-term inhibition of PGE_2 interferes with cartilage metabolism and inhibits normal tissue repair, resulting in accelerated articular cartilage destruction.[128] *In vitro*, phenylbutazone negatively impacts cartilage metabolism and results in decreased proteoglycan[129] and type II collagen synthesis.[130] In the face of decreased proteoglycan synthesis and altered cartilage reparative abilities, continued exercise could lead to ongoing cartilage breakdown and increasing severity of disease. In the short-term, NSAID relieve symptoms of OA by blocking inflammation and mitigating discomfort. However, long-term inhibition of prostaglandins through chronic NSAIDs therapy has the potential for insurmountable systemic and joint-specific side effects.[131,132]

Intra-articular corticosteroids

Corticosteroids are potent inhibitors of inflammation and exert their action via inhibition of phospholipase A_2. More specifically, corticosteroids bind to nuclear glucocorticoid receptors and cause inhibition of NF-kB , thereby inhibiting downstream production of prostaglandins and leukotrienes.[124,133] Importantly, the amelioration of pain is attributed to the inhibition of prostaglandin synthesis by corticosteroids.[134] By affecting the metabolism of arachidonic acid at the level of phospholipase A_2, corticosteroids significantly reduce pain and synovial effusion which, in turn, decreases the severity of lameness.[135] Corticosteroids inhibit multiple pathways within the arachidonic acid pathway, are more effective at reducing inflammation, and have an increased risk of side effects, compared to NSAID.

The potent anti-inflammatory effects of corticosteroids due to inhibition of the NF-kB signaling pathway is essential for the anti-inflammatory effects seen with corticosteroids; however, there may be undesirable consequences may be exacerbated with increased dose and

frequency of administration. NF-kB modulates gene expression of inflammatory regulators (IL-6, IL-8, IL-10, MCP-1) and ECM synthesis (type II collagen).[136,137] Moreover, inhibition of gene expression via the NF-kB pathway can ultimately affect homeostatic repair mechanisms within the joint, and cartilage turnover, by antagonizing common cellular pathways that are innately required for efficient tissue homeostasis and repair (e.g., IL-10, prostaglandin, and NF-kB).[84,122] While intra-articular corticosteroids mitigate pain and decrease inflammation, improving quality of life and potentially slowing joint destruction, they can also exert detrimental changes to articular cartilage through alterations in homeostatic pathways.[138]

Ultimately, the clinical effects of corticosteroids are based on a host of factors such as drug, dosage, frequency of administration, and stage of OA. As such, judicious use of corticosteroids is needed when deciding in dosage and frequency of administration due to the potential for corticosteroid arthropathy. This phenomenon is believed to occur as a result of corticosteroid suppressed synthesis of chondrocyte matrix and is related to frequency of injection, drug administered, underlying inflammation within the joint and exercise following injection.[134] For example, when IA methylprednisolone acetate was injected at increased frequency in both intercarpal joints in a horse with a 3rd carpal bone slab fracture, there was depletion in cartilage matrix, decreased viscosity of joint fluid, and decreased HA in the fractured joint.[139] While intra-articular injection of corticosteroids can be effective in rapidly reducing pain and enhancing joint function, the effects can be relatively short-lived, lasting on average 1-6 weeks.[140,141] Recent data suggests that there is a decreased clinical response to intra-articular corticosteroids in more chronically-affected joints with more structural damage.[142] Moreover, the clinical response and degree of damage can be affected by the frequency of injection. In fact, a recent study in humans suggests that serial injection of triamcinolone

performed every three months for two years increases cartilage volume loss (MRI-quantitated) and does not provide a significant reduction of knee pain compared to intra-articular saline.[143]

Intra-articular injection of corticosteroids is without question an effective treatment for horses with joint pain. However, there is long-standing controversy regarding the effects of corticosteroids on articular cartilage, as both beneficial and deleterious effects are cited.[144,145] Using the osteochondral fragment model of OA, intra-articular injection of triamcinolone in exercised horses resulted in significantly improved lameness and histomorphologic measurements compared to saline controls. Specifically, triamcinolone-treated horses (12mg triamcinolone/joint) had increased HA and GAG concentrations in synovial fluid, less inflammatory infiltration and fibrosis of the synovial membrane, and no gross evidence of articular cartilage deterioration.[144]

Using a similar model of OA, intra-articular methylprednisolone acetate (100 mg MPA/joint) had minimal effects on lameness, resulted in decreased synovial fluid PGE_2 concentrations, and caused articular cartilage erosion.[145] Overall, this study demonstrates that methylprednisolone acetate if administered at doses exceeding the biological activity of corticosteroids can have detrimental effects on cartilage healing and turnover, with limited clinical benefit in high-motion joints. Moreover, studies have shown that repeated injections of methylprednisolone acetate can significantly reduce cartilage thickness and increase the shear modulus of cartilage.[146] In turn, methylprednisolone acetate alters the biomechanical properties of cartilage, resulting in premature cartilage degradation. Similar to *in vivo* studies, *in vitro* studies have shown that intra-articular methylprednisolone acetate administration can have negative effects on cartilage metabolism. Specifically, methylprednisolone acetate resulted in

decreased proteoglycan production by equine chondrocytes and decreased matrix protein markers (type II procollagen and fibronectin) of chondrocyte differentiation.[147,148]

The positive and negative effects of IA corticosteroids on equine articular cartilage have been evaluated in a number of *in vitro* and *in vivo* studies at varying doses.[144,146-153] At high doses, MPA has a catabolic effect on articular cartilage morphology and chondrocyte viability.[153,154] This is in contrast to triamcinolone, which appears to have a chondroprotective effect such as enhanced GAG synthesis.[144]

While increased doses of corticosteroids can be deleterious to articular cartilage, recent research has demonstrated that therapeutic effects may be obtained at levels that do not adversely affect articular cartilage matrix protein synthesis.[151,155] For example, a lower, more physiologic dose of MPA (between 10 and 40 mg/joint) appears to decrease inflammation while preserving articular cartilage integrity.[152] Similarly, Trahan et al 2018 demonstrated that equine synovial and osteochondral explants treated with IL-1β were better at mitigating production of PGE_2 and MMP-13 at lower concentrations (10^{-7} and 10^{-10} M) of triamcinolone, MPA, and isoflupredone compared to higher concentrations (10^{-4} M).[151] Although low-dose corticosteroid administration decreases deleterious side effects, it does not totally eliminate them, especially if administered at frequent intervals. In fact, there is some research to suggest that low-dose corticosteroid therapy is not less detrimental than higher doses.[149] There is also evidence to show that increased loading within 6 days of exposure to corticosteroids can make articular cartilage more susceptible to cartilage matrix damage.[156] Corticosteroids should be used in the clinical scenario with knowledge of each individual medication and understanding of its side effects. Case selection is equally important. Like so much in veterinary medicine, there is no drug or dose that is one size fits all. As potent anti-inflammatories, corticosteroids can be a useful clinical tool if used

judiciously, especially for joint pain. While their benefit appears benign, corticosteroids affect cartilage metabolism with increasing effects correlating with increased dose and frequency of administration. The clinical response from corticosteroid administration is generally rapid, and as seen in people is not sustained.[140] Further the response can be dependent on the stage of OA, with chronic cases of OA tending to have a decreased clinical response. Deceptively, lameness improves drastically following intra-articular injection as decreased prostaglandin synthesis causes decreased pain and improved lameness. Over-use in an already inflamed joint coupled with corticosteroid-induced inhibition of transcriptional pathways further exacerbates the progression of OA through inhibition of normal regulatory functions and altered cartilage metabolism ultimately inciting a state of catabolism.

Disease modifying OA drugs

1. Hyaluronic acid

In health, HA, or hyaluronan, is a high MW, non-sulfated GAG that is an important component of both articular cartilage and synovial fluid. Additionally, HA is a principal lubricant of synovial soft tissues, shock absorber, and regulator of water balance in the synovial joint.[157,158] OA-induced alterations in the biochemical composition of the synovial joint cause changes in the viscoelastic properties of the synovial fluid.[158] In fact, HA concentration, functionality, and MW are decreased in OA joints compared to healthy joints. Changes in HA content and MW, in turn, contribute to pain and instability.[159,160] By attempting to harness the desired properties of HA and restore endogenous concentrations within the OA-affected joint, the concept of viscosupplementation was developed. Through injection of exogenous HA, the

goal is to enhance viscoelasticity, decrease friction within the joint, and counteract degradation of HA by hyaluronidase and reduced synthesis in OA.[161-163]

The primary effects of viscosupplementation with HA are not sustained. The intra-articular half-life of injected HA is short, with most products lasting <48 hours.[164,165] Although, physical retention of HA in the joint is often short, benefits such as decreased pain and increased mobility have more lasting effects.[165] In fact, clinical studies in people show that improvements in clinical signs can last up to one year following a single HA injection.[165] Furthermore, improvements in HA viscoelasticity and HA concentration have been shown to occur for up to six months following an intra-articular injection of HA.[161,165,166] Given the discrepancy between drug retention and therapeutic effect, additional biologic effects other than primary augmentation of synovial fluid viscoelasticity govern the improvement in clinical signs.[162] It is now recognized that HA exerts it beneficial effects by several mechanisms.[162,165,167-169]

In addition to mechanical and structural functions in the joint, HA is essential for molecular signaling, as it binds to ECM molecules and cell surface receptors which, in turn, allow it to modulate both the macro- and micro-environment of the joint.[158,169] Specifically, HA binds to CD44, which causes inhibition of IL-1β expression and reduced expression of MMP-1 and -3.[170] By reducing the production and activity of various inflammatory mediators, ECM synthesis is favored over degradation. The biological effects of HA on the ECM include enhanced production of endogenous HA and increased synthesis of proteoglycans and GAG (chondroitin and keratan sulfate).[167]

The role of MW in HA preparations is controversial and thought to play a role in effects on exogenous HA. *In vitro,* the degree of proteoglycan synthesis and inflammation reduction are linked to the MW of HA.[171,172] In addition to *in vitro* models, clinical trials in people have found

that high MW HA (average $6x10^6$ Daltons) reduces symptoms and has greater pain-relieving effects compared to low MW HA.[173,174] Conversely, intra-articular injections of crosslinked HA at very high MW ($23x10^6$ Daltons) can increase the risk of side effects because patients are twice as likely to experience adverse events such as joint effusions or flares.[175]

HA preparations for veterinary use include intra-articular and intravenous formulations. In horses, HA has been shown to promote anti-inflammatory effects with decreases in free radicals and prostaglandin synthesis.[176,177] The intravenous administration of HA decreased lameness, PGE_2 concentrations, and synovial inflammation compared to placebo-treated controls in an experimental equine model.[178] Moreover, when utilized prophylactically, racing Quarter Horses treated with HA had improvements in speed index, number of starts, and tended to race longer before requiring joint injections.[179] In contrast, an IV preparation of HA that also contained chondroitin sulfate and N-acetyl-D-glucosamine (Polyglycan) caused worsening of radiographic scores, greater degree of bone edema, and no improvement in lameness.[180]

Intra-articular HA has also been evaluated in horses and has been shown to exert some positive benefits. Using the osteochondral fragment model, intra-articular HA resulted in less cartilage fibrillation in HA-treated joints compared to polysulfated glycosaminoglycan.[181] Similarly, another study demonstrated a greater reduction in lameness for horses treated with intra-articular high MW HA ($3x10^6$ Daltons) compared to low MW HA ($0.3x10^6$ Daltons) administered IV and intra-articular.[182] Overall, HA has anti-inflammatory and analgesic effects in equine OA. Additionally, it has been shown to have benefit whether administered intra-articular or IV. Further, whether it is administered IV or intra-articular, HA is quickly eliminated from both the plasma and the synovial fluid.[183] As such consideration should be given to which preparation is administered because some formulations may have undesirable side effects.

2. Polysulfated glycosaminoglycan (PSGAG)

PSGAG is derived from bovine tracheal cartilage, comprised of repeating disaccharides, principally composed of chondroitin sulfate, and similar to GAG already present in cartilage.[134] There is *in vitro* evidence to show PSGAG inhibits production of MMP-3 from stimulated equine synovial lining cells.[184] In fact, PSGAG was compared to a number of drugs, including phenylbutazone, flunixin meglumine, betamethasone, and HA, and was the only one noted to decrease MMP-3 production.[184] Additionally, there is some evidence to suggest PSGAG may have a positive effect on collagen and GAG synthesis. In an *in vitro* study evaluating normal and arthritic tissue, PSGAG resulted in the increased synthesis of collagen and GAG from both equine chondrocytes and articular cartilage explants.[185] Likewise, several studies have provided evidence for the use of intra-articular PSGAG.

Studies evaluating intra-articular PSGAG were primarily performed using induced models of OA. Using the sodium monoiodoacetate synovitis model, intra-articular PSGAG demonstrated a significant decrease in chondrocyte death, articular cartilage fibrillation and erosion, and improved GAG staining.[186] Nevertheless, there was no observable benefit in the healing of pre-existing articular cartilage lesions following intra-articular PSGAG administration.[186] Similarly, intra-articular PSGAG administration using the osteochondral chip fragment model resulted in decreased subintimal fibrosis, vascularity and joint effusion, with a trend of decreased cartilage fibrillation.[181] Interestingly, intra-articular PSGAG did not improve lameness and response to flexion in this model.[181] In contrast to the potential benefits, an increase in septic arthritis after intra-articular administration was been reported in one study using 250 mg of intra-articular PSGAG.[187] The slight increase in septic arthritis in treated horses was attributed to likely potentiation of subclinical *Staphylococcus* contamination upon

injection.[187,188] Moreover, the risks appear to have been mitigated by concurrent injection of 125 mg amikacin.[188]

Due to the potential for increased synovial sepsis, intramuscular PSGAG has become more common in equine practice. As common as its use, there is limited evidence to support is efficacy. When 500 mg intramuscular PSGAG was administered every 4 days for 7 treatments using the sodium monoiodoacetate-induced synovitis model, there was relatively insignificant effects seen with only slightly improvement noted in articular cartilage GAG staining.[189] It should be noted that this is an extreme model of inflammation and does adequately represent the clinical progression of naturally-occurring OA. Similar findings were also noted with the osteochondral chip fragment model, with decreased serum concentration of GAG levels 14 days post treatment .[190] Another study using the osteochondral chip fragment model demonstrated increased levels of HA in synovial fluid following IM PSGAG administration.[191]

Based on the literature, intra-articular PSGAG shows more beneficial effects in equine OA. Concurrent use of intra-articular amikacin may be useful in mitigating the risks of synovial sepsis.[188] On the other hand, intra muscular administration is popular and appears safe, and has been shown to increase synovial fluid HA following administration.[191]

Orthobiological therapies

Cell-based therapies are commonly used for the treatment of equine OA and can be derived from a variety of sources, including peripheral blood and bone marrow. They typically contain some combination of growth factors, cytokines, and cells capable of eliciting anti-inflammatory, inflammatory, and/or anabolic responses.[192] The goal of biological therapies in the treatment of

OA is to both alter the inflammatory component of OA and aid in regeneration of damaged tissues.[192,193]

1. Mesenchymal stem cells (MSC)

Mesenchymal stem cells are adult stem cells that display regenerative, anti-inflammatory, and immunomodulatory functions.[194] MSC wield their positive effects on cartilage and synovium through the release of soluble mediators such as interferon gamma (IFNγ), macrophage-derived TNF-α, and PGE_2.[195] In a study evaluating MSC-conditioned media exposed to TNF-α and IFNγ, there was reduced gene expression for IL-1β, MMP-1 and MMP-13 from synovium, and increased expression of IL-1 receptor antagonist protein (IL-1ra).[196] Moreover, MSC modulates the activity of other cells within the joint through paracrine mechanisms, exerting an anti-inflammatory effect through intermediate cells, such as macrophages.[2,197] A study evaluating the influence of MSC on macrophage function showed that LPS-stimulated macrophages co-cultured with MSC shifted from a TNF-α-producing inflammatory state to an anti-inflammatory state with increased production of IL-10 and -12.[114,115,198] Furthermore, MSC secrete the key mediator PGE_2, which modulates the inflammatory activity and proliferation of T-cells and antigen presentation of dendritic cells, resulting in reduced TNF-α and increased IL-10 production.[199] In fact, PGE_2 secreted by MSC in an inflammatory environment promoted a switch from pro-inflammatory to a reparative macrophage phenotype.[198]

Similarly, MSC provided protection from sepsis in a murine cecal-puncture ligation model.[200] Protection from sepsis was demonstrated via MSC-derived PGE_2 stimulation of IL-10-producing macrophages.[200] Importantly, the beneficial effect of MSC were ameliorated with depletion of macrophages. Macrophages from septic lung tissue produced more IL-10 compared

to controls and LPS-induced macrophages produced more IL-10 when co-cultured with MSC.[200] In short, this demonstrates the ability of MSC to modulate macrophages through the release of PGE_2, and indicates the importance of macrophage function in repair and inflammation.[200] Furthermore, PGE_2 modulation of inflammation in MSC is counter to the effects of corticosteroids and NSAID on PGE_2. MSC studies have demonstrated that PGE_2 can actually be beneficial in modulating inflammation, and therefore repair.

In addition to modulating the inflammatory functions of macrophages, MSC regulate macrophage chemotaxis and recruitment, which are important for regenerative processes.[201] Once injected into target tissue, MSC home to injured tissue via the release of trophic factors such as stromal cell-derived factor 1 (SDF-1), which is secreted by activated macrophages.[202,203] Once to the site of injury, exogenous MSC stimulate recruitment of endogenous MSC. Endogenous MSC activity is dependent on the presence of inflammation. Proliferation of endogenous synovial MSC occurred in the presence of articular cartilage injury, but did not occur in the absence of injury in a murine model.[204]

In horses, MSC can be harvested from several sources, with bone marrow being most common and therefore the focus of this review. Bone marrow is commonly harvested from the sternum or tuber coxae in horses with age of the horse dictating the density of MSC at the respective site.[205,206] The collected sample is predominantly composed of mononuclear cells (myeloid and hematopoietic progenitors) with a minute fraction consisting of MSC (0.001–0.01%) and bone spicules.[207] Once collected, MSC are culture expanded for 2-4 weeks to achieve large numbers of a relatively homogeneous cell population.[192,208]

The utility of MSC in equine OA has been evaluated with mixed results and varied outcomes. The degree of inflammation present in the joint appears to dictate the MSC response

and the response varied between experimental and naturally-occurring OA.[208] Induction of an anti-inflammatory niche is called 'cytokine licensing' and can be regulated by IFN-γ, TNF-α, IL-1β, and IL-17.[2,192] Priming of MSC with these molecules has been shown to improve chondroprotection and production of immunomodulators such as IL-6 and PGE_2 in inflamed environments.[192,209] It is this licensing mechanism that may explain discrepancies between studies evaluating the use of IA MSC in different OA models.[192] The osteochondral fragment model, created to mimic traumatic arthritis, incites a low level of inflammation, while LPS-induced synovitis is a potent inflammatory model.[210] Intra-articular injection of MSC following induction of the osteochondral fragment chip model had no effect on lameness and only showed appreciable decreases in levels of PGE_2.[211] In contrast, IA MSC injections in an induced model of LPS-synovitis demonstrated an ability to modulate inflammation by causing a significant reduction in total nucleated and mononuclear cell counts compared to control limbs.[210,211] Further, injection of MSC in a non-inflamed joint can cause transient synovitis that appears to be self-limiting.[210] As such, models with varied spectrums of inflammation make efficacy difficult to ascertain as stem cell niche induce stem cells into anti-inflammatory phenotypes.

2. Platelet Rich Plasma (PRP)

PRP is the plasma portion of the patient's own blood that is centrifuged or filtered to create a product that contains an increased concentration of platelets compared to normal blood. PRP has been shown to decrease cartilage catabolism through the inhibition of MMP-13 and enhanced production of endogenous HA.[212] The therapeutic effects are thought to arise from growth factor and cytokine release following platelet degranulation.[213] Examples of growth factors that have demonstrated beneficial effects on tissue repair include, platelet derived growth

factor (PDGF), TGF-β1, vascular endothelial growth factor, and insulin growth factor-1 (IGF-1).[214,215]

PRP is autologous, readily available, and can be harvested patient-side. There is some clinical evidence regarding the effects of PRP in OA-affected horses. IA injection of PRP has been shown to increase synovial growth factor concentrations such as IGF-1 in both normal and OA-affected joints, yet OA-affected joints had a less dramatic increase in growth factor concentrations.[216] Furthermore, IA PRP incited a transient inflammatory reaction in both normal and OA-affected with increased synovial effusion noted in OA-affected joints compared to normal joints.[216] In a pilot study of 4 horses treated with intra-articular PRP, lameness was improved, with maximal effects noted two months post injection and persistent improvement for eight months.[217] Similarly, treatment with intra-articular PRP lysate improved lameness scores compared to saline controls in 10 horses with OA of the distal interphalangeal joint.[218]

Although these results are promising, several factors, such as small sample size, incomplete analysis of growth factor content, and variation in PRP-product used should be taken into consideration when interpreting the efficacy of PRP for the intra-articular treatment of lameness in horses. One of the key areas of debate is the white blood cell (WBC) content of PRP, especially for intra-articular use. Commercially available devices for making PRP products for horses vary in their ability to concentrate platelets, the extent to which they may also concentrate WBC, and perhaps more importantly, the platelet:WBC ratio.[192] The importance of platelet numbers and whether an optimal number of platelets is required for various treatments is an often debated issue. Growth factor levels do appear to be directly correlated to the number of platelets.[219] However, increasing platelet numbers can have a detrimental effect on type II and III collagen gene expression.[220] The importance of leukocyte numbers in PRP preparations for intra-

articular use is a debated topic, with some studies suggesting the use of leukocyte reduced PRP over concerns for release of inflammatory factors from WBC and the negative impact this may have within the joint.[214,221,222] Inflammatory cytokines such as IL-1β, IL-6 and IL-8 are related to leukocyte count and can be reduced by depletion of leukocytes.[212]

3. Autologous conditioned serum (ACS)

ACS is an autologous biologic produced by collecting peripheral blood and incubating it with medical grade glass beads at 37°C for 24 hours. The conditioned serum is then harvested following centrifugation, filtered and stored at -20°C for serial intra-articular injections. Incubation induces monocytes to release increased concentrations of anti-inflammatory cytokines such as IL-1ra, IL-4, and IL-10 and growth factors such as basic fibroblast growth factor and TGF-β1.[223,224] Clinical results for the treatment of equine OA with ACS have been encouraging.[225] Horses with experimentally-induced OA treated with 4 weekly injections of ACS demonstrated improved lameness and decreased cartilage fibrillation and synovial thickness compared to saline-treated controls.[225] The clinical effects observed with ACS may be related to frequency of injection and content of IGF-1 and IL-1ra.[226] Similarly, horses with naturally occurring OA treated with ACS every two days for a total of 3 injections had significantly decreased synovial fluid IL-1β concentrations and significantly increased IL-1ra concentrations 42 days post treatment compared to horses injected at 7 day intervals.[227]

Although clinical results with intra-articular ACS are promising, *in vitro* studies have shown mixed results in regards to chondroprotection. Despite increased concentrations of IL-1ra and IGF-1 in equine cartilage explants treated with ACS in an *in vitro* inflammatory model, there was no significant difference in MMP-3 production or synthesis and proteoglycan loss between ACS

and serum-treated samples.[228] While the mechanisms of the benefits observed following ACS treatment are not completely understood, ACS administration is widely used in clinical practice. The anti-inflammatory effects of ACS are generally viewed to be much less potent than corticosteroids, hence it is primarily recommended for treatment of mild to moderate OA and synovitis, especially in young horses where the detrimental effects of corticosteroids are viewed to be most devastating.

4. Bone marrow mononuclear cell therapy (BMNC)

BMNC are a heterogeneous population of nucleated cells derived from bone marrow aspirate by density gradient centrifugation using Ficoll, a high-mass polysaccharide to isolate the mononuclear cell fraction from bone spicules, erythrocytes, and granulocyte precursors.[229] The result is a population of cells that includes >50% monocyte and macrophage progenitors along with mixed hematopoietic progenitor cells (~25%) lymphoid progenitors (lymphocytes, plasma cells- ~10%), fibroblastic reticular cells (~10%), and a very small proportion of MSC (0.01-0.0001%).[230,231]

BMNC is an exciting new targeted OA therapy that is autologous and point-of-care, with the ability to modulate the homeostatic effects of regulatory macrophages.[232,233]

BMNC provide potent anti-inflammatory and trophic effects in chronically inflamed and injured tissues, including the central nervous system and lungs.[230,234] BMNC decreased the inflammatory phase of healing while concurrently improving tissue quality in both tendons and ligaments in horses, with treated horses demonstrating increased inflammatory cell infiltration and extracellular matrix synthesis.[235,236] Specifically, there were increased levels of cartilage oligomeric matrix protein and type I collagen in treated horses versus placebo-treated controls.[236]

BMNC have also provided improvement in murine models of acute liver failure and related survival.[237,238] BMNC therapy was demonstrated more pronounced benefits such as improved lung function, alveolar collapse and increased levels of TGF- and VEGF than MSC in a murine asthmatic model.[239] Moreover, macrophage progenitors from BMNC exhibited potent anti-inflammatory effects comparable to corticosteroids in equine and murine chronic inflammatory airway disease.[84,230] IL-10 concentrations in bronchoalveolar lavage fluid from horses treated with BMNC were 30-fold higher compared to horses treated with dexamethasone.[84] Additionally, BMNC-treated horses demonstrated significantly decreased levels of neutrophils and increased number of macrophages on days 7 and 21 compared to dexamethasone-treated horse.[84] IL-10 is a potent anti-inflammatory cytokine important in tissue homeostasis and repair, and plays an essential role in the protection of chondrocytes and other tissue from inflammatory insults.[84,116,240,241]

Reports of the use of BMNC for the treatment of OA are limited; however, existing reports are promising. BMNC reduced the clinical signs in people with tennis elbow and moderate knee OA.[233,242,243] Intra-articular injection of BMNC was not associated with any adverse effects and provided significant clinical improvement and better pain relief when compared to hyaluronic acid treatment in human knee OA.[233,243] By taking advantage of the natural bias of macrophage progenitors toward the anti-inflammatory, M2 phenotype when exposed to an inflammatory environment, it may be possible to modulate synovial inflammation and establish an environment conducive to tissue healing.[84,95,230,244] By promoting synovial inflammation resolution, BMNC therapy has the potential to benefit innumerable human and veterinary patients suffering from OA.

Synovial inflammation is a major component of joint disease and is significantly mediated by macrophages.[41] Under physiologic conditions, macrophages work together with synovial fibroblasts to maintain joint health.[81,82] Once these homeostatic mechanisms are overwhelmed, macrophages incite joint inflammation in order to signal the physiologic need for the body to respond and return the synovial environment back to normal. If inflammation is not resolved, then chronic inflammation ultimately leads to pathologic cartilage and bone changes characteristic of OA.[245] Although macrophages are commonly thought of as causing negative effects, such as inflammation, under physiological conditions, macrophage-mediated inflammation is actually self-limiting and performs a critical role in inflammation resolution.[80,86] This physiologic response is regulated, and involves the recruitment of other types of immune cells, such as neutrophils and lymphocytes, to help eliminate foreign materials, aid in tissue repair, and eventually restore tissue homeostasis.[81,91] These apparently contradictory macrophage functions are incompletely understood, and are the topic of a number of recent and ongoing lines of investigation.[88-90] Chronic joint injury hinders inflammation resolution and associated cytokines allowing catabolic enzymes to perpetuate joint degradation.[43,246] Therefore, inflammation resolution is an essential step in mitigating the catabolic processes of OA and shifting cellular balances in the joint toward an anabolic response and homeostatic result.[50,121]

Macrophage progenitors are M2-biased and are the primary cells shown to counteract the deleterious effects of inflammation by promoting an anti-inflammatory response *in vitro* and *in vivo*.[115,244,247] *In vitro*, macrophages undergo phenotypic changes in response to environmental stimuli, polarizing into distinctly different cell types – inflammatory (M1) and suppressive/regulatory (M2).[248] These variations in activation states gave rise to the concept of macrophage polarization.[249] Importantly, the M2 phenotype can be induced by exposure to an

inflammatory environment, such as a diseased joint.[84,244] *In vitro*, chondrogenic differentiation of progenitor cells is impeded by M1 macrophages from the osteoarthritic synovium[79]; however, repolarization to the M2 phenotype improves clinical and histological signs of joint disease in a mouse model.[250]

Recent studies have suggested that macrophage polarization is not as straightforward as once postulated and that they rather exhibit a continuum of marker expression and exist in a hybrid state – producing both pro-and anti-inflammatory cytokines that are essential for resolution of inflammation.[251] *In vitro* culture of equine BMNC in normal and inflamed synovial fluid resulted in macrophage-rich cultures with phenotypes similar those seen in normal synovial macrophages and increasing levels of IL-10+ macrophages and IL-10 and IGF-1 concentrations, and decreasing IL-1β concentrations.[251] Interestingly, changes were more marked in inflamed synovial fluid compared to normal.[251] These findings strengthen the argument for increasing IL-10-producing macrophages in inflamed joints as a means of tipping the response in favor of resolving inflammation.

In a recent study *in* vivo using an equine model of LPS-induced synovial inflammation, autologous intra-articular BMNC injection resulted in marked reduction of acute inflammation without adverse effects.[251] BMNC-treated joints showed gross and laboratorial improvements in synovial fluid and synovial membrane parameters, with increased numbers of regulatory macrophages and synovial fluid IL-10 concentrations compared with saline-treated controls.[251] In fact, inflamed joints with BMNC had 24% higher macrophage counts and 10% more IL-10^{+} cells than saline-treated controls.[251] BMNC-treated joints were comparable to healthy joints grossly and histologically, whereas saline-treated control joints remained abnormal 6 days after injection.[251] In summary, these studies support the idea that capitalizing on the macrophage-

derived effects of BMNC has the potential to reestablish joint homeostasis and are the seminal studies serving as the groundwork for this thesis project.

References

1. Lawrence RC, Felson DT, Helmick CG, et al: Estimates of the prevalence of arthritis and other rheumatic conditions in the United States: Part II. Arthritis Rheum 58:26-35, 2008.
2. Wang Y, Chen X, Cao W, et al: Plasticity of mesenchymal stem cells in immunomodulation: pathological and therapeutic implications. Nat Immunol 15:1009-1016, 2014.
3. Boring MA, Hootman JM, Liu Y, et al: Prevalence of arthritis and arthritis-attributable activity limitation by urban-rural county classification - United States, 2015. MMWR Morb Mortal Wkly Rep 66:527-532, 2017.
4. Cisternas MG, Murphy L, Sacks JJ, et al: Alternative methods for defining osteoarthritis and the impact on estimating prevalence in a US population-based survey. Arthritis Care Res (Hoboken) 68:574-580, 2016.
5. Torio CM, Moore BJ: National inpatient hospital costs: The most expensive conditions by payer, 2013: Statistical Brief #204, in Healthcare Cost and Utilization Project (HCUP) Statistical Briefs, Rockville, Agency for Healthcare Research and Quality (US), 2006.
6. De Filippis L, Gulli S, Caliri A, et al: Epidemiology and risk factors in osteoarthritis: literature review data from "OASIS" study. Reumatismo 56:169-184, 2004.
7. Oke S MC, Moyer W: Review of the economic impact of osteoarthritis and oral joint-health supplements in horses. Proc Am Assoc Equine Pract 56:12-16, 2010.
8. McIlwraith CW, Frisbie DD, Kawcak CE: The horse as a model of naturally occurring osteoarthritis. Bone Joint Res 1:297-309, 2012.
9. Fubini SL, Erb HN, Freeman KP, et al: Prognostic factors affecting survival of 507 horses with joint disease: (1983 to 1990). Can J Vet Res 63:253-260, 1999.

10. Dabareiner RM, Cohen ND, Carter GK, et al: Lameness and poor performance in horses used for team roping: 118 cases (2000-2003). J Am Vet Med Assoc 226:1694-1699, 2005.
11. Dabareiner RM, Cohen ND, Carter GK, et al: Musculoskeletal problems associated with lameness and poor performance among horses used for barrel racing: 118 cases (2000-2003). J Am Vet Med Assoc 227:1646-1650, 2005.
12. Ireland JL, Clegg PD, McGowan CM, et al: Disease prevalence in geriatric horses in the United Kingdom: veterinary clinical assessment of 200 cases. Equine Vet J 44:101-106, 2012.
13. Seitzinger AH, Kane AJ, Kopral CA, et al: A comparison of the economic costs of equine lameness, colic, and equine protozoal myeloencephalitis (EPM). 9th International Symposium on Veterinary Epidemiology and Economics:1-3, 2000.
14. Deloitte Consulting, LLC: The economic impact of the horse industry in the United States: American Horse Council, 2005.
15. Schlueter A, Orth M: Equine osteoarthritis: a brief review of the disease and its causes. Equine Comp Exerc Physiol 1:221-231, 2004.
16. Frisbie DD, Al-Sobayil F, Billinghurst RC, et al: Changes in synovial fluid and serum biomarkers with exercise and early osteoarthritis in horses. Osteoarthritis Cartilage 16:1196-1204, 2008.
17. van Weeren PR: General anatomy and physiology of joints, in McIlwraith CW, Frisbie DD, Kawcak CE, et al (eds): Joint Disease in the Horse (Second Edition), Vol. Edinburgh, W.B. Saunders, 2016, pp 1-24.
18. Frisbie DD: Synovial joint biology and pathobiology, in Auer JA, Stick JA (eds): Equine Surgery (Fourth Edition), Saint Louis, W.B. Saunders, 2012, pp 1096-1114.

19. Bijlsma JW, Berenbaum F, Lafeber FP: Osteoarthritis: an update with relevance for clinical practice. Lancet 377:2115-2126, 2011.
20. de Grauw JC: Molecular monitoring of equine joint homeostasis. Vet Q 31:77-86, 2011.
21. Li G, Yin J, Gao J, et al: Subchondral bone in osteoarthritis: insight into risk factors and microstructural changes. Arthritis Res Ther 15:223, 2013.
22. Stewart HL, Kawcak CE: The importance of subchondral bone in the pathophysiology of osteoarthritis. Front Vet Sci 5:178, 2018.
23. Goldring MB, Goldring SR: Articular cartilage and subchondral bone in the pathogenesis of osteoarthritis. Ann NY Acad Sci 1192:230-237, 2010.
24. Kawcak CE, McIlwraith CW, Norrdin RW, et al: The role of subchondral bone in joint disease: a review. Equine Vet J 33:120-126, 2001.
25. Luria A, Chu CR: Articular cartilage changes in maturing athletes: New targets for joint rejuvenation. Sports Health 6:18-30, 2014.
26. Decker RS, Koyama E, Pacifici M: Articular cartilage: Structural and developmental intricacies and questions. Curr Osteoporos Rep 13:407-414, 2015.
27. Sophia Fox AJ, Bedi A, Rodeo SA: The basic science of articular cartilage: Structure, composition, and function. Sports Health 1:461-468, 2009.
28. Athanasiou KA, Darling EM, DuRaine GD, et al: Structure and function of cartilage, in Articular Cartilage. Baton Rouge, CRC Press LLC, 2013.
29. Poole AR, Kojima T, Yasuda T, et al: Composition and structure of articular cartilage. Clinical Orthop Rel Res 391:S26-S33, 2001.
30. Carballo CB, Nakagawa Y, Sekiya I, et al: Basic Science of Articular Cartilage. Clin Sports Med 36:413-425, 2017.

31. Baumann CA, Hinckel BB, Bozynski CC, et al: Articular cartilage: Structure and restoration, in Yanke, Adam B, Cole, Brian J (eds.) Joint Preservation of the Knee Vol Springer International Publishing, pp 3-24, 2019.
32. Milner P, Wilkins R, Gibson J: Cellular physiology of articular cartilage in health and disease, in Rothschild BM (ed), Principles of Osteoarthritis - Its Definition, Character, Derivation and Modality-Related Recognition, Rijeka, Croatia, IntechOpen, pp 567-590, 2012.
33. Eyre DR, Wu JJ, Woods PE: The cartilage collagens: structural and metabolic studies. J Rheumatol Suppl 27:49-51, 1991.
34. Caterson B, Flannery CR, Hughes CE, et al: Mechanisms involved in cartilage proteoglycan catabolism. Matrix Biol 19:333-344, 2000.
35. Bhosale AM, Richardson JB: Articular cartilage: structure, injuries and review of management. Br Med Bull 87:77-95, 2008.
36. Camarero-Espinosa S, Rothen-Rutishauser B, Foster EJ, et al: Articular cartilage: from formation to tissue engineering. Biomater Sci 4:734-767, 2016.
37. Poole AR, Kobayashi M, Yasuda T, et al: Type II collagen degradation and its regulation in articular cartilage in osteoarthritis. Ann Rheum Dis 61:ii78-ii81, 2002.
38. Buckwalter JA, Mankin HJ: Articular cartilage: tissue design and chondrocyte-matrix interactions. Instr Course Lect 47:477-486, 1998.
39. Ralphs JR, Benjamin M: The joint capsule: structure, composition, ageing and disease. J Anat 184:503-509, 1994.
40. Roughley PJ, Mort JS: The role of aggrecan in normal and osteoarthritic cartilage. J Exp Orthop 1:8, 2014.

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

42. Bertone AL: Joint physiology: responses to exercise and training, in Hinchcliff KW, Geor RJ, Kaneps AJ (eds): Equine Exercise Physiology, Edinburgh, W.B. Saunders, 2008, pp 132-142.

43. 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 Rheum 62:647-657, 2010.

44. Veale DJ, Firestein GS: Synovium, in Firestein GS, Budd RC, Gabriel SE, et al (eds): Kelley and Firestein's Textbook of Rheumatology (Tenth Edition), Elsevier, 2017, pp 20-33.

45. Zvaifler NJ: Macrophages and the synovial lining. Scand J Rheumatol Suppl 101:67-75, 1995.

46. Tamer TM: Hyaluronan and synovial joint: function, distribution and healing. Interdiscip Toxicol 6:111-125, 2013.

47. Stafford CT, Niedermeier W, Holley HL, et al: Studies on the concentration and intrinsic viscosity of hyaluronic acid in synovial fluids of patients with rheumatic diseases. Ann Rheum Dis 23:152-157, 1964.

48. Miller RE, Miller RJ, Malfait AM: Osteoarthritis joint pain: The cytokine connection. Cytokine 70:185-193, 2014.

49. Kidd JA, Fuller C, Barr ARS: Osteoarthritis in the horse. Equine Vet Educ 13:160-168, 2001.

50. Goldring MB, Otero M: Inflammation in osteoarthritis. Curr Opin Rheumatol 23:471-478, 2011.

51. McIlwraith C: Traumatic arthritis and posttraumatic osteoarthritis in the horse, in McIlwraith C (ed), Joint Disease in the Horse (2nd ed) 2016, pp 33-48.
52. Mathiessen A, Conaghan PG: Synovitis in osteoarthritis: current understanding with therapeutic implications. Arthritis Res Ther 19:1-18, 2017.
53. Kamm JL, Nixon AJ, Witte TH: Cytokine and catabolic enzyme expression in synovium, synovial fluid and articular cartilage of naturally osteoarthritic equine carpi. Equine Vet J 42:693-699, 2010.
54. Wang X, Hunter DJ, Jin X, et al: The importance of synovial inflammation in osteoarthritis: current evidence from imaging assessments and clinical trials. Osteoarthritis Cartilage 26:165-174, 2018.
55. Wojdasiewicz P, Poniatowski LA, Szukiewicz D: The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of osteoarthritis. Mediators Inflamm 2014:561459, 2014.
56. Lane NE, Brandt K, Hawker G, et al: OARSI-FDA initiative: defining the disease state of osteoarthritis. Osteoarthritis Cartilage 19:478-482, 2011.
57. Benito MJ: Synovial tissue inflammation in early and late osteoarthritis. Ann Rheum Dis 64:1263-1267, 2005.
58. Sellam J, Berenbaum F: The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis. Nat Rev Rheumatol 6:625-635, 2010.
59. Baker K, Grainger A, Niu J, et al: Relation of synovitis to knee pain using contrast-enhanced MRIs. Ann Rheum Dis 69:1779-1783, 2010.

60. 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 8:R187, 2006.

61. Barker SA, Bayyuk SH, Brimacombe JS, et al: Fingerprinting the hyaluronic acid component of normal and pathological synovial fluids. Clin Chim Acta 8:902-909, 1963.

62. Dahl LB, Dahl IM, Engstrom-Laurent A, et al: Concentration and molecular weight of sodium hyaluronate in synovial fluid from patients with rheumatoid arthritis and other arthropathies. Ann Rheum Dis 44:817-822, 1985.

63. Mazieres B, Garnero P, Gueguen A, et al: Molecular markers of cartilage breakdown and synovitis at baseline as predictors of structural progression of hip osteoarthritis. The ECHODIAH Cohort. Ann Rheum Dis 65:354-359, 2006.

64. Goldberg RL, Huff JP, Lenz ME, et al: Elevated plasma levels of hyaluronate in patients with osteoarthritis and rheumatoid arthritis. Arthritis Rheum 34:799-807, 1991.

65. Bondeson J: Activated synovial macrophages as targets for osteoarthritis drug therapy. Curr Drug Targets 11:576-585, 2010.

66. van Lent PL, Blom AB, van der Kraan P, et al: Crucial role of synovial lining macrophages in the promotion of transforming growth factor beta-mediated osteophyte formation. Arthritis Rheum 50:103-111, 2004.

67. van Lent PL, Krijger GC, Hofkens W, et al: Selectively induced death of macrophages in the synovial lining of murine knee joints using 10B-liposomes and boron neutron capture synovectomy. Int J Radiat Biol 85:860-871, 2009.

68. Barrera P, Blom A, van Lent PL, et al: Synovial macrophage depletion with clodronate-containing liposomes in rheumatoid arthritis. Arthritis Rheum 43:1951-1959, 2000.

69. 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 18:83, 2016.

70. Kandahari AM, Yang X, Dighe AS, et al: recognition of Immune Response for the Early Diagnosis and Treatment of Osteoarthritis. J Immunol Res 2015:192415, 2015.

71. Van Lent PLEM, Blom AB, Van Der Kraan P, et al: Crucial role of synovial lining macrophages in the promotion of transforming growth factor ?-mediated osteophyte formation. Arthritis Rheum 50:103-111, 2004.

72. Blom AB, van Lent PL, Holthuysen AE, et al: Synovial lining macrophages mediate osteophyte formation during experimental osteoarthritis. Osteoarthritis Cartilage 12:627-635, 2004.

73. Kraus VB, McDaniel G, Huebner JL, et al: Direct in vivo evidence of activated macrophages in human osteoarthritis. Osteoarthritis Cartilage 24:1613-1621, 2016.

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

75. Ospelt C: Synovial fibroblasts in 2017. RMD Open 3:e000471, 2017.

76. Lin J, Ke Y, Li Z, et al: The secretion of proinflammatory cytokines and chemokines in stimulated fibroblast-like synoviocytes of osteoarthritis. Osteoarthritis Cartilage 20:S240, 2012.

77. Nair A, Kanda V, Bush-Joseph C, et al: Synovial fluid from patients with early osteoarthritis modulates fibroblast-like synoviocyte responses to toll-like receptor 4 and toll-like receptor 2 ligands via soluble CD14. Arthritis Rheum 64:2268-2277, 2012.

78. Machado CRL, Resende GG, Macedo RBV, et al: Fibroblast-like synoviocytes from fluid and synovial membrane from primary osteoarthritis demonstrate similar production of interleukin 6, and metalloproteinases 1 and 3. Clin Exp Rheumatol 37:306-309, 2019.

79. Fahy N, de Vries-van Melle ML, Lehmann J, et al: Human osteoarthritic synovium impacts chondrogenic differentiation of mesenchymal stem cells via macrophage polarisation state. Osteoarthritis Cartilage 22:1167-1175, 2014.

80. Smeets TJ, Barg EC, Kraan MC, et al: Analysis of the cell infiltrate and expression of proinflammatory cytokines and matrix metalloproteinases in arthroscopic synovial biopsies: comparison with synovial samples from patients with end stage, destructive rheumatoid arthritis. Ann Rheum Dis 62:635-638, 2003.

81. Kennedy A, Fearon U, Veale DJ, et al: Macrophages in synovial inflammation. Front Immunol 2:52, 2011.

82. Dimitrova P, Ivanovska N: How Important are Innate Immunity Cells in Osteoarthritis Pathology, in Rothschild BM (ed), Principles of Osteoarthritis - Its Definition, Character, Derivation and Modality-Related Recognition, Rijeka, Croatia, IntechOpen, pp 519-544, 2012.

83. Fichadiya A, Bertram KL, Ren G, et al: Characterizing heterogeneity in the response of synovial mesenchymal progenitor cells to synovial macrophages in normal individuals and patients with osteoarthritis. J Inflamm (Lond) 13:12, 2016.

84. Barussi FC, Bastos FZ, Leite LM, et al: Intratracheal therapy with autologous bone marrow-derived mononuclear cells reduces airway inflammation in horses with recurrent airway obstruction. Respir Physiol Neurobiol 232:35-42, 2016.
85. Sanin DE, Prendergast CT, Mountford AP: IL-10 Production in macrophages is regulated by a TLR-driven CREB-mediated mechanism that is linked to genes involved in cell metabolism. J Immunol 195:1218-1232, 2015.
86. Behrendt P, Feldheim M, Preusse-Prange A, et al: Chondrogenic potential of IL-10 in mechanically injured cartilage and cellularized collagen ACI grafts. Osteoarthritis Cartilage 26:264-275, 2018.
87. Kurowska-Stolarska M, Alivernini S: Synovial tissue macrophages: friend or foe? RMD Open 3:e000527, 2017.
88. Mills CD, Ley K: M1 and M2 macrophages: the chicken and the egg of immunity. J Innate Immun 6:716-726, 2014.
89. Mills CD: M1 and M2 macrophages: oracles of health and disease. Crit Rev Immunol 32:463-488, 2012.
90. Mills CD: Anatomy of a discovery: M1 and M2 macrophages. Front Immunol 6:212, 2015.
91. Murray PJ: Macrophage polarization. Annu Rev Physiol 79:541-566, 2017.
92. Dey A, Allen J, Hankey-Giblin PA: Ontogeny and polarization of macrophages in inflammation: blood monocytes versus tissue macrophages. Front Immunol 5:683, 2015.
93. Stables MJ, Shah S, Camon EB, et al: Transcriptomic analyses of murine resolution-phase macrophages. Blood 118:e192-e208, 2011.

94. Wu C-L, McNeill J, Goon K, et al: Conditional macrophage depletion increases inflammation and does not inhibit the development of osteoarthritis in obese macrophage fas-induced apoptosis-transgenic mice. Arthritis Rheumatol 69:1772-1783, 2017.

95. Godwin JW, Pinto AR, Rosenthal NA: Macrophages are required for adult salamander limb regeneration. Proc Natl Acad Sci U S A 110:9415-9420, 2013.

96. Goldring MB: Osteoarthritis and cartilage: the role of cytokines. Curr Rheumatol Rep 2:459-465, 2000.

97. McNulty AL, Rothfusz NE, Leddy HA, et al: Synovial fluid concentrations and relative potency of interleukin-1 alpha and beta in cartilage and meniscus degradation. J Orthop Res 31:1039-1045, 2013.

98. Moreira JJ, Moraes AP, Brossi PM, et al: Autologous processed plasma: cytokine profile and effects upon injection into healthy equine joints. J Vet Sci 16:47-55, 2015.

99. Martel-Pelletier J, McCollum R, DiBattista J, et al: The interleukin-1 receptor in normal and osteoarthritic human articular chondrocytes. Identification as the type I receptor and analysis of binding kinetics and biologic function. Arthritis Rheum 35:530-540, 1992.

100. Platt D, Bayliss MT: An investigation of the proteoglycan metabolism of mature equine articular cartilage and its regulation by interleukin-1. Equine Vet J 26:297-303, 1994.

101. Morris EA, Treadwell BV: Effect of interleukin 1 on articular cartilage from young and aged horses and comparison with metabolism of osteoarthritic cartilage. Am J Vet Res 55:138-146, 1994.

102. Richardson DW, Dodge GR: Effects of interleukin-1beta and tumor necrosis factor-alpha on expression of matrix-related genes by cultured equine articular chondrocytes. Am J Vet Res 61:624-630, 2000.

103. Tetlow LC, Adlam DJ, Woolley DE: Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: associations with degenerative changes. Arthritis Rheum 44:585-594, 2001.

104. Fan Z, Söder S, Oehler S, et al: Activation of interleukin-1 signaling cascades in normal and osteoarthritic articular cartilage. Am J Pathol 171:938-946, 2007.

105. Arner EC, Pratta MA: Independent effects of interleukin-1 on proteoglycan breakdown, proteoglycan synthesis, and prostaglandin E2 release from cartilage in organ culture. Arthritis Rheumatol 32:288-297, 1989.

106. Daheshia M, Yao JQ: The interleukin 1β pathway in the pathogenesis of osteoarthritis. J Rheumatol 35:2306, 2008.

107. Black RA, Rauch CT, Kozlosky CJ, et al: A metalloproteinase disintegrin that releases tumour-necrosis factor-α from cells. Nature 385:729-733, 1997.

108. Funk CD: Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 294:1871-1875, 2001.

109. Manferdini C, Maumus M, Gabusi E, et al: Adipose-derived mesenchymal stem cells exert antiinflammatory effects on chondrocytes and synoviocytes from osteoarthritis patients through prostaglandin E2. Arthritis Rheum 65:1271-1281, 2013.

110. Tchetina EV, Di Battista JA, Zukor DJ, et al: Prostaglandin PGE2 at very low concentrations suppresses collagen cleavage in cultured human osteoarthritic articular cartilage: this involves a decrease in expression of proinflammatory genes, collagenases and COL10A1, a gene linked to chondrocyte hypertrophy. Arthritis Res Ther 9:R75, 2007.

111. Scher JU, Pillinger MH: The anti-inflammatory effects of prostaglandins. J Investig Med 57:703-708, 2009.

112. Nishitani K, Ito H, Hiramitsu T, et al: PGE2 inhibits MMP expression by suppressing MKK4-JNK MAP kinase-c-JUN pathway via EP4 in human articular chondrocytes. J Cell Biochem 109:425-433, 2010.
113. Gomez PF, Pillinger MH, Attur M, et al: Resolution of inflammation: prostaglandin E2 dissociates nuclear trafficking of individual NF-kappaB subunits (p65, p50) in stimulated rheumatoid synovial fibroblasts. J Immunol 175:6924-6930, 2005.
114. Kim J, Hematti P: Mesenchymal stem cell-educated macrophages: a novel type of alternatively activated macrophages. Exp Hematol 37:1445-1453, 2009.
115. Maggini J, Mirkin G, Bognanni I, et al: Mouse bone marrow-derived mesenchymal stromal cells turn activated macrophages into a regulatory-like profile. PLoS One 5:e9252, 2010.
116. Iannone F, De Bari C, Dell'Accio F, et al: Interleukin-10 and interleukin-10 receptor in human osteoarthritic and healthy chondrocytes. Clin Exp Rheumatol 19:139-145, 2001.
117. Cush JJ, Splawski JB, Thomas R, et al: Elevated interleukin-10 levels in patients with rheumatoid arthritis. Arthritis Rheum 38:96-104, 1995.
118. Lettesjö H, Nordström E, Ström H, et al: Synovial fluid cytokines in patients with rheumatoid arthritis or other arthritic lesions. Scand J Immunol 48:286-292, 1998.
119. Riyazi N, Slagboom E, de Craen AJ, et al: Association of the risk of osteoarthritis with high innate production of interleukin-1beta and low innate production of interleukin-10 ex vivo, upon lipopolysaccharide stimulation. Arthritis Rheum 52:1443-1450, 2005.
120. Culemann S, Gruneboom A, Nicolas-Avila JA, et al: Locally renewing resident synovial macrophages provide a protective barrier for the joint. Nature, 2019.

121. Caron JP, Gandy JC, Brown JL, et al: Omega-3 fatty acids and docosahexaenoic acid oxymetabolites modulate the inflammatory response of equine recombinant interleukin1beta-stimulated equine synoviocytes. Prostaglandins Other Lipid Mediat 142:1-8, 2019.
122. de Souza MV: Osteoarthritis in horses - Part 2: a review of the intra-articular use of corticosteroids as a method of treatment. Broeckx
123. de Grauw JC, van de Lest CH, Brama PA, et al: In vivo effects of meloxicam on inflammatory mediators, MMP activity and cartilage biomarkers in equine joints with acute synovitis. Equine Vet J 41:693-699, 2009.
124. Ostergaard M, Halberg P: Intra-articular corticosteroids in arthritic disease: a guide to treatment. BioDrugs 9:95-103, 1998.
125. Kunkle B JS, D Attebery, N Targa, D Larsen and P Hanson: Effects of firocoxib and phenylbutazone dosed for 42 days in horses. Proc Am Assoc Equine Pract :54, 2010.
126. 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. Sem Arthritis Rheum 43:701-712, 2014.
127. Osafo N, Agyare C, Obiri DD, et al: Mechanism of Action of Nonsteroidal Anti-Inflammatory Drugs, in, Nonsteroidal Anti-Inflammatory Drugs, Al-kaf AG (ed). InTech Open, 2017.
128. Ross A. Hauser M: The acceleration of articular cartilage degeneration in osteoarthritis by nonsteroidal anti-inflammatory drugs. J Prolother:305-322, 2010.

129. Beluche LA, Bertone AL, Anderson DE, et al: Effects of oral administration of phenylbutazone to horses on in vitro articular cartilage metabolism. Am J Vet Res 62:1916-1921, 2001.

130. 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. Vet J 201:51-56, 2014.

131. Frean SP, Abraham LA, Lees P: In vitro stimulation of equine articular cartilage proteoglycan synthesis by hyaluronan and carprofen. Res Vet Sci 67:183-190, 1999.

132. Jolly WT, Whittem T, Jolly AC, et al: The dose-related effects of phenylbutazone and a methylprednisolone acetate formulation (Depo-Medrol) on cultured explants of equine carpal articular cartilage. J Vet Pharmacol Ther 18:429-437, 1995.

133. Creamer P: Intra-articular corticosteroid treatment in osteoarthritis. Curr Opin Rheumatol 11:417-421, 1999.

134. Caron JP: Intra-articular injections for joint disease in horses. Vet Clin North Am Equine Pract 21:559-573, 2005.

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

136. Marcu KB, Otero M, Olivotto E, et al: NF-kappaB signaling: multiple angles to target OA. Curr Drug Targets 11:599-613, 2010.

137. Kydd AS, Reno CR, Tsao HW, et al: Early inflammatory arthritis in the rabbit: the influence of intraarticular and systemic corticosteroids on mRNA levels in connective tissues of the knee. J Rheumatol 34:130-139, 2007.

138. Fubini SL, Boatwright CE, Todhunter RJ, et al: Effect of intramuscularly administered polysulfated glycosaminoglycan on articular cartilage from equine joints injected with methylprednisolone acetate. Am J Vet Res 54:1359-1365, 1993.
139. Owen RA, Marsh JA, Hallett FR, et al: Intra-articular corticosteroid- and exercise-induced arthropathy in a horse. J Am Vet Med Assoc 184:302-308, 1984.
140. Bellamy N, Campbell J, Robinson V, et al: Intraarticular corticosteroid for treatment of osteoarthritis of the knee. Cochrane Database Syst Rev:CD005328, 2006.
141. Jüni P, Hari R, Rutjes AWS, et al: Intra-articular corticosteroid for knee osteoarthritis. Cochrane Database Syst Rev 10:CD005328, 2015.
142. Maricar N, Parkes MJ, Callaghan MJ, et al: Structural predictors of response to intra-articular steroid injection in symptomatic knee osteoarthritis. Arthritis Res Ther 19:88, 2017.
143. McAlindon TE, LaValley MP, Harvey WF, et al: Effect of intra-articular triamcinolone vs saline on knee cartilage volume and pain in patients with knee osteoarthritis: a randomized clinical trial. J Am Med Assoc 317:1967-1975, 2017.
144. Frisbie DD, Kawcak CE, Trotter GW, et al: Effects of triamcinolone acetonide on an in vivo equine osteochondral fragment exercise model. Equine Vet J 29:349-359, 1997.
145. Frisbie DD, Kawcak CE, Baxter GM, et al: Effects of 6alpha-methylprednisolone acetate on an equine osteochondral fragment exercise model. Am J Vet Res 59:1619-1628, 1998.
146. Murray RC, DeBowes RM, Gaughan EM, et al: The effects of intra-articular methylprednisolone and exercise on the mechanical properties of articular cartilage in the horse. Osteoarthritis Cartilage 6:106-114, 1998.

147. Byron CR, Benson BM, Stewart AA, et al: Effects of methylprednisolone acetate and glucosamine on proteoglycan production by equine chondrocytes in vitro. Am J Vet Res 69:1123-1128, 2008.

148. Fubini SL, Todhunter RJ, Burton-Wurster N, et al: Corticosteroids alter the differentiated phenotype of articular chondrocytes. J Orthop Res 19:688-695, 2001.

149. 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 Vet J 35:444-450, 2003.

150. Todhunter RJ, Fubini SL, Vernier-Singer M, et al: Acute synovitis and intra-articular methylprednisolone acetate in ponies. Osteoarthritis Cartilage 6:94-105, 1998.

151. Trahan RA, Byron CR, Dahlgren LA, et al: In vitro effects of three equimolar concentrations of methylprednisolone acetate, triamcinolone acetonide, and isoflupredone acetate on equine articular tissue cocultures in an inflammatory environment. Am J Vet Res 79:933-940, 2018.

152. 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. Vet Surg 29:546-557, 2000.

153. Trotter GW, McIlwraith CW, Yovich JV, et al: Effects of intra-articular administration of methylprednisolone acetate on normal equine articular cartilage. Am J Vet Res 52:83-87, 1991.

154. Shoemaker RS, Bertone AL, Martin GS, et al: Effects of intra-articular administration of methylprednisolone acetate on normal articular cartilage and on healing of experimentally induced osteochondral defects in horses. Am J Vet Res 53:1446-1453, 1992.

155. Richardson D, Dodge G: Dose-dependent effects of corticosteroids on expression of matrix-related genes in normal and cytokine-treated articular chondrocytes. Inflamm Res 52:39-49, 2003.

156. Farquhar T, Todhunter RJ, Fubini SL, et al: Effect of methylprednisolone and mechanical loading on canine articular cartilage in explant culture. Osteoarthritis Cartilage 4:55-62, 1996.

157. Papakonstantinou E, Roth M, Karakiulakis G: Hyaluronic acid: A key molecule in skin aging. Dermatoendocrinol 4:253-258, 2012.

158. Gupta RC, Lall R, Srivastava A, et al: Hyaluronic acid: molecular mechanisms and therapeutic trajectory. Front Vet Sci 6:1-24, 2019

159. Wimmer MA, Grad S, Kaup T, et al: Tribology approach to the engineering and study of articular cartilage. Tissue Eng 10:1436-1445, 2004.

160. Kurisawa M, Chung JE, Yang YY, et al: Injectable biodegradable hydrogels composed of hyaluronic acid-tyramine conjugates for drug delivery and tissue engineering. Chem Commun (Camb):4312-4314, 2005.

161. Balazs EA, Denlinger JL: Viscosupplementation: a new concept in the treatment of osteoarthritis. J Rheumatol Suppl 39:3-9, 1993.

162. Balazs EA: Viscosupplementation for treatment of osteoarthritis: from initial discovery to current status and results. Surg Technol Int 12:278-289, 2004.

163. Forsey RW, Fisher J, Thompson J, et al: The effect of hyaluronic acid and phospholipid based lubricants on friction within a human cartilage damage model. Biomaterials 27:4581-4590, 2006.

164. Brown T, Laurent U, Fraser: Turnover of hyaluronan in synovial joints: elimination of labelled hyaluronan from the knee joint of the rabbit. Exp Physiol 76:125-134, 1991.

165. Bagga H, Burkhardt D, Sambrook P, et al: Longterm effects of intraarticular hyaluronan on synovial fluid in osteoarthritis of the knee. J Rheumatol 33:946-950, 2006.

166. Pal S, Thuppal S, Reddy KJ, et al: Long-term (1-Year) safety and efficacy of a single 6-ml injection of hylan G-F 20 in indian patients with symptomatic knee osteoarthritis. Open Rheumatol J 8:54-68, 2014.

167. Altman RD, Manjoo A, Fierlinger A, et al: The mechanism of action for hyaluronic acid treatment in the osteoarthritic knee: a systematic review. BMC Musculoskelet Disord 16:321, 2015.

168. Bannuru R, Natov N, Obadan I, et al: Therapeutic trajectory of hyaluronic acid versus corticosteroids in the treatment of knee osteoarthritis: a systematic review and meta-analysis. Arthritis Rheum 61:1704-1711, 2009.

169. Webb D, Naidoo P: Viscosupplementation for knee osteoarthritis: a focus on Hylan G-F 20. OrthopRes Rev 10:73-81, 2018.

170. Sasaki A, Sasaki K, Konttinen YT, et al: Hyaluronate inhibits the interleukin-1beta-induced expression of matrix metalloproteinase (MMP)-1 and MMP-3 in human synovial cells. Tohoku J Exp Med 204:99-107, 2004.

171. Chang C-C, Hsieh M-S, Liao S-T, et al: Hyaluronan regulates PPARγ and inflammatory responses in IL-1β-stimulated human chondrosarcoma cells, a model for osteoarthritis. Carbohydr Polym 90:1168-1175, 2012.

172. Homandberg GA, Ummadi V, Kang H: The role of insulin-like growth factor-I in hyaluronan mediated repair of cultured cartilage explants. Inflamm Res 53:396-404, 2004.

173. Berenbaum F, Grifka J, Cazzaniga S, et al: A randomised, double-blind, controlled trial comparing two intra-articular hyaluronic acid preparations differing by their molecular weight in symptomatic knee osteoarthritis. Ann Rheum Dis 71:1454-1460, 2012.
174. Wobig M, Bach G, Beks P, et al: The role of elastoviscosity in the efficacy of viscosupplementation for osteoarthritis of the knee: a comparison of hylan G-F 20 and a lower-molecular-weight hyaluronan. Clin Ther 21:1549-1562, 1999.
175. Reichenbach S, Blank S, Rutjes AW, et al: Hylan versus hyaluronic acid for osteoarthritis of the knee: a systematic review and meta-analysis. Arthritis Rheum 57:1410-1418, 2007.
176. Clegg PD, Jones MD, Carter SD: The effect of drugs commonly used in the treatment of equine articular disorders on the activity of equine matrix metalloproteinase-2 and 9. J Vet Pharmacol Ther 21:406-413, 1998.
177. Lynch TM, Caron JP, Arnoczky SP, et al: Influence of exogenous hyaluronan on synthesis of hyaluronan and collagenase by equine synoviocytes. Am J Vet Res 59:888-892, 1998.
178. Kawcak CE, Frisbie DD, Trotter GW, et al: Effects of intravenous administration of sodium hyaluronate on carpal joints in exercising horses after arthroscopic surgery and osteochondral fragmentation. Am J Vet Res 58:1132-1140, 1997.
179. McIlwraith CW GN, Frisbie DD: Prospective study on the prophylactic value of intravenous hyaluronan in 2-year-old racing Quarter Horses. Proc Am Assoc Equine Pract 44:269, 1998.
180. Frisbie DD, McIlwraith CW, Kawcak CE, et al: Efficacy of intravenous administration of hyaluronan, sodium chondroitin sulfate, and N-acetyl-d-glucosamine for prevention or treatment of osteoarthritis in horses. Am J Vet Res 77:1064-1070, 2016.

181. 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. Am J Vet Res 70:203-209, 2009.

182. White GW, Stites T, Hamm J, et al: Evaluation of the efficacy of various preparations of sodium hyaluronate in an induced equine carpitis model. J Equine Vet Sci 19:331-337, 1999.

183. Popot MA, Bonnaire Y, Guéchot J, et al: Hyaluronan in horses: physiological production rate, plasma and synovial fluid concentrations in control conditions and following sodium hyaluronate administration. Equine Vet J 36:482-487, 2004.

184. May SA, Hooke RE, Lees R: The effect of drugs used in the treatment of osteoarthrosis on stromelysin (proteoglycanase) of equine synovial cell origin. Equine Vet J 20:28-32, 2010.

185. Glade MJ: Polysulfated glycosaminoglycan accelerates net synthesis of collagen and glycosaminoglycans by arthritic equine cartilage tissues and chondrocytes. Am J Vet Res 51:779-785, 1990.

186. Yovich JV, Trotter GW, McIlwraith CW, et al: Effects of polysulfated glycosaminoglycan on chemical and physical defects in equine articular cartilage. Am J Vet Res 48:1407-1414, 1987.

187. Gustafson SB, McIlwraith CW, Jones RL: 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. Am J Vet Res 50:2014-2017, 1989.

188. 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. Am J Vet Res 50:2018-2022, 1989.

189. 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 53:224-230, 1989.

190. Frisbie DD, Kawcak CE, McIlwraith CW: Evaluation of the effect of extracorporeal shock wave treatment on experimentally induced osteoarthritis in middle carpal joints of horses. Am J Vet Res 70:449-454, 2009.

191. Burba DJ, Collier MA, Default LE, et al: In vivo kinetic study on uptake and distribution of intramuscular tritium-labeled polysulfated glycosaminoglycan in equine body fluid compartments and articular cartilage in an osteochondrial defect model. J Equine Vet Sci 13:696-703, 1993.

192. Bogers SH: Cell-based therapies for joint disease in veterinary medicine: what we have learned and what we need to know. Front Vet Sci 5:70, 2018.

193. Wenham CY, McDermott M, Conaghan PG: Biological therapies in osteoarthritis. Curr Pharm Des 21:2206-2215, 2015.

194. Shi Y, Hu G, Su J, et al: Mesenchymal stem cells: a new strategy for immunosuppression and tissue repair. Cell Res 20:510-518, 2010.

195. Madrigal M, Rao KS, Riordan NH: A review of therapeutic effects of mesenchymal stem cell secretions and induction of secretory modification by different culture methods. J Transl Med 12:260, 2014.

196. Van Buul GM, Villafuertes E, Bos PK, et al: Mesenchymal stem cells secrete factors that inhibit inflammatory processes in short-term osteoarthritic synovium and cartilage explant culture. Osteoarthritis Cartilage 20:1186-1196, 2012.
197. Zhou Y, Yamamoto Y, Xiao Z, et al: The immunomodulatory functions of mesenchymal stromal/stem cells mediated via paracrine activity. J Clin Med 8:1025, 2019.
198. Ulivi V, Tasso R, Cancedda R, et al: Mesenchymal stem cell paracrine activity is modulated by platelet lysate: induction of an inflammatory response and secretion of factors maintaining macrophages in a proinflammatory phenotype. Stem Cells Dev 23:1858-1869, 2014.
199. Aggarwal S, Pittenger MF: Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 105:1815-1822, 2005.
200. Németh K, Leelahavanichkul A, Yuen PST, et al: Bone marrow stromal cells attenuate sepsis via prostaglandin E2–dependent reprogramming of host macrophages to increase their interleukin-10 production. Nature Med 15:42-49, 2009.
201. Pajarinen J, Lin T, Gibon E, et al: Mesenchymal stem cell-macrophage crosstalk and bone healing. Biomaterials 196:80-89, 2019.
202. Mouiseddine M, François S, Semont A, et al: Human mesenchymal stem cells home specifically to radiation-injured tissues in a non-obese diabetes/severe combined immunodeficiency mouse model. Br J Radiol 80:S49-55, 2007.
203. Kitaori T, Ito H, Schwarz EM, et al: Stromal cell–derived factor 1/CXCR4 signaling is critical for the recruitment of mesenchymal stem cells to the fracture site during skeletal repair in a mouse model. Arthritis Rheum 60:813-823, 2009.

204. Kurth TB, Dell'Accio F, Crouch V, et al: Functional mesenchymal stem cell niches in adult mouse knee joint synovium in vivo. Arthritis Rheum 63:1289-1300, 2011.

205. Delling U, Lindner K, Ribitsch I, et al: Comparison of bone marrow aspiration at the sternum and the tuber coxae in middle-aged horses. Can J Vet Res 76:52-56, 2012.

206. Adams MK, Goodrich LR, Rao S, et al: Equine bone marrow-derived mesenchymal stromal cells (BMDMSCs) from the ilium and sternum: are there differences? Equine Vet J 45:372-375, 2013.

207. Pittenger MF, Mackay AM, Beck SC, et al: Multilineage Potential of Adult Human Mesenchymal Stem Cells. Science 284:143-147, 1999.

208. Frisbie DD, Smith RK: Clinical update on the use of mesenchymal stem cells in equine orthopaedics. Equine Vet J 42:86-89, 2010.

209. Cassano JM, Schnabel LV, Goodale MB, et al: Inflammatory licensed equine MSCs are chondroprotective and exhibit enhanced immunomodulation in an inflammatory environment. Stem Cell Res Ther 9:82, 2018.

210. 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 Vet J 48:619-625, 2016.

211. 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. J Orthop Res 27:1675-1680, 2009.

212. Sundman EA, Cole BJ, Karas V, et al: The anti-inflammatory and matrix restorative mechanisms of platelet-rich plasma in osteoarthritis. Am J Sports Med 42:35-41, 2014.

213. Fontenot RL, Sink CA, Werre SR, et al: Simple tube centrifugation for processing platelet-rich plasma in the horse. Can Vet J 53:1266-1272, 2012.

214. Schnabel LV, Mohammed HO, Miller BJ, et al: Platelet rich plasma (PRP) enhances anabolic gene expression patterns in flexor digitorum superficialis tendons. J Orthop Res 25:230-240, 2007.

215. Waselau M, Sutter WW, Genovese RL, et al: Intralesional injection of platelet-rich plasma followed by controlled exercise for treatment of midbody suspensory ligament desmitis in Standardbred racehorses. J Am Vet Med Assoc 232:1515-1520, 2008.

216. 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. J S Afr Vet Assoc 90:e1-e9, 2019.

217. Carmona JU, Argüelles D, Climent F, et al: Autologous platelet concentrates as a treatment of horses with osteoarthritis: a preliminary pilot clinical Study. J Equine Vet Sci 27:167-170, 2007.

218. Tyrnenopoulou P, Diakakis N, Karayannopoulou M, et al: Evaluation of intra-articular injection of autologous platelet lysate (PL) in horses with osteoarthritis of the distal interphalangeal joint. Vet Q 36:56-62, 2016.

219. Boswell SG, Schnabel LV, Mohammed HO, et al: Increasing platelet concentrations in leukocyte-reduced platelet-rich plasma decrease collagen gene synthesis in tendons. Am J Sports Med 42:42-49, 2014.

220. Yoshida R, Cheng M, Murray MM: Increasing platelet concentration in platelet-rich plasma inhibits anterior cruciate ligament cell function in three-dimensional culture. J Orthop Res 32:291-295, 2014.

221. McCarrel TM, Minas T, Fortier LA: Optimization of leukocyte concentration in platelet-rich plasma for the treatment of tendinopathy. J Bone Joint Surg Am 94:e143(141-148), 2012.

222. McLellan J, Plevin S: Does it matter which platelet-rich plasma we use? Equine Vet Educ 23:101-104, 2011.

223. Wright-Carpenter T, Opolon P, Appell HJ, et al: Treatment of muscle injuries by local administration of autologous conditioned serum: animal experiments using a muscle contusion model. Int J Sports Med 25:582-587, 2004.

224. Meijer H, Reinecke J, Becker C, et al: The production of anti-inflammatory cytokines in whole blood by physico-chemical induction. Inflamm Res 52:404-407, 2003.

225. 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. Am J Vet Res 68:290-296, 2007.

226. 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 Vet Res 16:181, 2020.

227. 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. J Equine Vet Sci 60:35-42.e32, 2018.

228. Carlson ER, Stewart AA, Carlson KL, et al: Effects of serum and autologous conditioned serum on equine articular chondrocytes treated with interleukin-1β. Am J Vet Res 74:700-705, 2013.

229. Cuende N, Rico L, Herrera C: Concise Review: Bone Marrow Mononuclear Cells for the Treatment of Ischemic Syndromes: Medicinal Product or Cell Transplantation? Stem Cells 1:403-408, 2012.

230. Cruz FF, Borg ZD, Goodwin M, et al: CD11b+ and Sca-1+ cells exert the main beneficial effects of systemically administered bone marrow-derived mononuclear cells in a murine model of mixed Th2/Th17 allergic airway inflammation. Stem Cells Transl Med 5:488-499, 2016.

231. Alvarez-Viejo M, Menendez-Menendez Y, Blanco-Gelaz MA, et al: Quantifying mesenchymal stem cells in the mononuclear cell fraction of bone marrow samples obtained for cell therapy. Transplant Proc 45:434-439, 2013.

232. 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 J 33:14337-14353, 2019.

233. Goncars V, Kalnberzs K, Jakobsons E, et al: Treatment of Knee Osteoarthritis with Bone Marrow-Derived Mononuclear Cell Injection: 12-Month Follow-up. Cartilage 10:26-35, 2019.

234. Agadi S, Shetty AK: Concise Review: Prospects of Bone Marrow Mononuclear Cells and Mesenchymal Stem Cells for Treating Status Epilepticus and Chronic Epilepsy. Stem Cells 33:2093-2103, 2015.

235. Barreira AP, Alves A, Saito M, et al: Autologous implant of bone marrow mononuclear cells as treatment of induced equine tentinitis. J Appl Res Vet Med 6:46-54, 2008.

236. Crovace A, Lacitignola L, Rossi G, et al: Histological and immunohistochemical evaluation of autologous cultured bone marrow mesenchymal stem cells and bone marrow mononucleated cells in collagenase-induced tendinitis of equine superficial digital flexor tendon. Vet Med Int 2010:250978, 2010.

237. Jin SZ, Meng XW, Sun X, et al: Granulocyte colony-stimulating factor enhances bone marrow mononuclear cell homing to the liver in a mouse model of acute hepatic injury. Dig Dis Sci 55:2805-2813, 2010.

238. Jin SZ, Meng XW, Han MZ, et al: Stromal cell derived factor-1 enhances bone marrow mononuclear cell migration in mice with acute liver failure. World J Gastroenterol 15:2657-2664, 2009.

239. Abreu SC, Antunes MA, de Castro JC, et al: Bone marrow-derived mononuclear cells vs. mesenchymal stromal cells in experimental allergic asthma. Respir Physiol Neurobiol 187:190-198, 2013.

240. Wang C, Yu X, Cao Q, et al: Characterization of murine macrophages from bone marrow, spleen and peritoneum. BMC Immunology 14:6, 2013.

241. Behrendt P, Feldheim M, Preusse-Prange A, et al: Chondrogenic potential of IL-10 in mechanically injured cartilage and cellularized collagen ACI grafts. Osteoarthritis Cartilage 26:264-275, 2018.

242. Singh A, Gangwar DS, Singh S: Bone marrow injection: A novel treatment for tennis elbow. J Nat Sci Biol Med 5:389-391, 2014.

243. Goncars V, Jakobsons E, Blums K, et al: The comparison of knee osteoarthritis treatment with single-dose bone marrow-derived mononuclear cells vs. hyaluronic acid injections. Medicina (Kaunas) 53:101-108, 2017.

244. Olingy CE, San Emeterio CL, Ogle ME, et al: Non-classical monocytes are biased progenitors of wound healing macrophages during soft tissue injury. Sci Rep 7:447, 2017.
245. Griffin TM, Scanzello CR: Innate inflammation and synovial macrophages in osteoarthritis pathophysiology. Clin Exp Rheumatol 37 Suppl 120:57-63, 2019.
246. Loeser RF, Collins JA, Diekman BO: Ageing and the pathogenesis of osteoarthritis. Nature Rev Rheumatol 12:412+, 2016.
247. Shizhu J, Xiangwei M, Xun S, et al: Bone marrow mononuclear cell transplant therapy in mice with CCl4-induced acute liver failure. Turk J Gastroenterol 23:344-352, 2012.
248. Murray PJ, Allen JE, Biswas SK, et al: Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity 41:14-20, 2014.
249. Gordon S, Plüddemann A, Martinez Estrada F: Macrophage heterogeneity in tissues: phenotypic diversity and functions. Immunol Rev 262:36-55, 2014.
250. Jain S, Tran TH, Amiji M: Macrophage repolarization with targeted alginate nanoparticles containing IL-10 plasmid DNA for the treatment of experimental arthritis. Biomaterials 61:162-177, 2015.
251. Menarim BC, Gillis KH, Oliver A, et al: Inflamed synovial fluid induces a homeostatic response in bone marrow mononuclear cells in vitro: Implications for joint therapy. FASEB J 34:4430-4444, 2020.

CHAPTER 2: BONE MARROW MONONUCLEAR CELL THERAPY FOR EQUINE JOINT DISEASE

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Abstract

Osteoarthritis (OA) in horses can be debilitating and career-ending. Current treatments offer temporary relief of clinical signs, but potentially deleterious side effects. Bone marrow mononuclear cells (BMNC) are a rich source of macrophage progenitors that have the ability to reduce OA symptoms in people and inflammation in experimentally-induced synovitis in horses. The objective of this study was to evaluate the ability of intra-articular BMNC therapy to improve clinical signs of naturally occurring equine OA. Horses presenting with clinical and radiographic evidence of moderate OA in a single joint were randomly assigned to 1 of 3 treatment groups: saline (negative control), triamcinolone (positive control), or BMNC (treatment group). Horses were subjectively and objectively evaluated for lameness and synovial fluid collected for cytologic evaluation at 0, 7, and 21 days post-injection. Data were analyzed using General Estimating Equations with significance set at $P<0.05$. There were no adverse effects noted in any treatment group. No significant differences were found in synovial fluid cytology parameters, joint circumference or lameness between treatment groups at any time point. Joint circumference did not change over time within saline- and triamcinolone-treated groups. Joint circumference reduced and lameness improved significantly within the BMNC-

treated group between Days 0 and 21 and Days 7 and 21. Lameness also improved significantly within the saline-treated group from 0 to 21 days, but did not improve within the triamcinolone-treated group. The decreased lameness and lack of adverse effects in the BMNC-treated horses in our study support a larger clinical trial using BMNC.

Introduction

Osteoarthritis (OA) is ubiquitous, complex, and a clinically challenging disease affecting both companion animals and humans.[1] OA is the most common cause of lameness in horses of all disciplines, accounting for approximately 60% of lameness problems.[1,2] Lameness related to OA has been associated with poor performance, early retirement, and failure to race in Thoroughbred racehorses.[3-5]

OA affects multiple joint tissues and causes cellular and structural pathological changes in intra-articular cartilage, synovium and bone.[6,7] Clinically OA is characterized by the presence of synovial effusion, soft-tissue swelling and lameness and/or poor performance [2] Inflammation of the synovial membrane (synovitis) is a key driver of clinical signs and pathologic processes in equine osteoarthritis as it increases production of inflammatory mediators such as metalloproteinases (MMP), cytokines and chemokines that create an unfavorable synovial environment for tissue homeostasis.[2,8-10] Inflammation in the synovium drives and perpetuates OA, and is further fueled by cytokines and break down products from other tissues leading to a cycle of inflammation and tissue destruction. Uncontrolled inflammation combined with limited self-regenerative capacity of articular cartilage and synovial tissues result in joint degeneration.[11,12]

Macrophages are significant mediators of synovial inflammation and contribute to pathologic cartilage and bone responses characteristic of OA.[13] In general, macrophages play key roles in both the innate and adaptive immune systems, both of which are increasingly thought to play a role in the development of OA.[12,14] Under physiological conditions, macrophage-mediated inflammation is self-resolving, and involves the recruitment of immune cells to help eliminate foreign materials, aid in tissue repair, and eventually return the tissue to homeostasis.[12,15] When

phagocytic and regulatory functions become overwhelmed, synovial macrophages recruit and prime other immune cells, such as lymphocytes and neutrophils, to upregulate inflammation in response to the increased demands for repair.[11,12]

Macrophages undergo phenotypic changes in response to environmental stimuli, polarizing into distinctly different cell types – inflammatory (M1) and regulatory (M2).[15,16] Importantly, the M2 phenotype can be induced by exposure to an inflammatory environment, such as a diseased joint.[14,17] Macrophage phenotype plays a direct role in the continuation or amelioration of OA. *In vitro,* chondrogenic differentiation of progenitor cells is impeded by M1 macrophages from the osteoarthritic synovium[18]; however, repolarization to the M2 phenotype improves clinical and histological signs of joint disease in a mouse model.[19] Furthermore, the ratio of M1 to M2 macrophages is markedly higher and significantly positively correlated to severity level of knee osteoarthritis in humans.[20] Due to their duplicity of function, macrophages have become the focus of significant ongoing research efforts.[12,19,21,22] Although little is known about the specific roles of macrophages and the effects of their phenotype polarization on joint homeostasis and repair, extrapolation from other tissues suggests that targeting macrophage polarization could be a novel treatment for osteoarthritic joints.[22-24] By taking advantage of the natural bias of macrophage progenitors toward the M2 phenotype when exposed to an inflammatory environment, it may be possible to modulate synovial inflammation and clinical signs, thereby establishing an environment conducive to tissue healing.[17,23-26]

Current therapeutic options for OA mainly target clinical signs by temporary anti-inflammatory effects, but fail to produce sustained or reparative effects. Furthermore, traditional treatments inhibit both pathological and homeostatic pathways with detrimental consequences. For example, corticosteroids can have detrimental effects on cartilage metabolism by

antagonizing common cellular pathways (e.g., prostaglandins and NF-kB) that are innately required for efficient tissue healing.[27,28] Orthobiological therapies such as autologous conditioned serum and mesenchymal stem cells have become popular in recent years but have shown only modest benefit in improvement of clinical signs.[29-31] A recent study demonstrated that single and repeated intra-articular injections of allogeneic and autologous bone marrow derived mesenchymal stem cells were equally ineffective in reducing the inflammatory response from acute recombinant IL-1β-induced joint inflammation in horses.[32] Consequently, there is a critical need for therapies that halt disease progression and resolve synovial inflammation, while maintaining homeostatic and reparative functions of the joint. Recent research has shown the therapeutic potential of targeting inducers of the resolution phase of inflammation as opposed to ones that inhibit the acute phase of inflammation.[33,34]

Bone marrow mononuclear cells (BMNC) are a source of macrophages that have been used to enhance tissue repair and treat chronic inflammation.[24] Macrophage progenitors (>50% naïve progenitors) are the primary cells in BMNC responsible for downregulating inflammation.[24,35] BMNC are considered M2-biased progenitors and promote an anti-inflammatory response counteracting the deleterious effects of inflammation *in vitro* and *in vivo.*[17,24] The anti-inflammatory effects of bone marrow derived macrophages are associated with increased production of interleukin (IL)-10 concentrations.[23,36] IL-10 is a potent anti-inflammatory cytokine important in tissue homeostasis and repair and plays an essential role in the protection of chondrocytes from inflammatory insults.[37,38] BMNC have been shown to provide potent anti-inflammatory and trophic effects in injured tissues, including the central nervous system, heart, and lungs.[24,39,40] Macrophage progenitors from BMNC had clinical effects similar to corticosteroids in equine and murine chronic inflammatory airway disease.[23,24] IL-10

concentrations in bronchoalveolar lavage fluid from horses treated with BMNC were 30-fold higher compared to horses treated with dexamethasone.[23] Endogenous production of IL-10 by synovial membrane macrophages is inhibited by corticosteroids.[23,28] Intra-articular injection of bone marrow-derived mononuclear cells was shown to be safe and provided significant symptomatic and pain relief for human keen OA when compared to hyaluronic acid.[41,42] By harnessing the M2-biased property of BMNC and increasing the number of cells responsible for homeostatic functions, joint health may be restored by shifting the balance in an affected joint toward a homeostatic and reparative state.

Equine BMNC are autologous and rapidly isolated from bone marrow aspirate for point-of-care administration and have the potential to substantially advance the current treatment of equine joint disease.[43] Important advantages of BMNC over cultured mesenchymal stem cells for intra-articular therapy include a cell population rich in M2-biased macrophage progenitors and the efficiency of same-day isolation and treatment with minimal equipment, manipulation, and expense.[43,44] Intra-articular BMNC therapy has the potential to provide long-lasting modulation of joint inflammation and promotion of tissue repair through regulatory macrophages In a recent study by Menarim et al, horses treated with intra-articular BMNC had no adverse reactions and treated joints showed gross improvement and demonstrated increased levels of anti-inflammatory markers within the synovial fluid, with increasing regulatory macrophages and synovial fluid IL-10 concentrations compared with saline-treated controls.[43] BMNC-treated joints were histologically comparable to healthy joints, which remained abnormal in saline-treated controls.[43]

The objective of this study was to evaluate the ability of intra-articular BMNC to improve clinical signs of naturally occurring osteoarthritis in horses. We hypothesized that intra-articular

administration of BMNC would reduce lameness and joint inflammation comparable to that achieved with intra-articular corticosteroids.

Materials and Methods

Study Design

The study was designed as a multi-center, randomized, blinded, placebo-controlled clinical trial. The study protocol was approved by the Institutional Animal Care and Use Committee and the Hospital Board. The study was performed at the Virginia Maryland College of Veterinary Medicine in collaboration with external equine practices in the mid-Atlantic region and North Carolina.

Adult horses between 3 and 16 years of age referred with lameness of at least Grade 2 on a 0-5 scale[45] and isolated to a single joint in one limb were prospectively enrolled in the study. Eligibility for inclusion was based on the following criteria: a complete medical record, including signalment, history, and presenting physical examination findings (temperature, pulse, respiratory rate); predominant limb lameness of Grade 2 or 3 on a scale of 0-5[45] isolated to a single joint yielding a positive response to intra-articular anesthesia, with radiographic evidence of moderate OA. The source of lameness was localized using a combination of subjective lameness evaluation and objective quantification using a body-mounted, wireless, inertial sensor system (Lameness Locator®), diagnostic analgesia, and radiography. Radiographic OA scores (mild, moderate, or severe) were assigned by consensus between 4 experienced clinicians (SHB, CRB, LAD, or RSP).[29,46] Moderate OA was assigned based on radiographic evidence of osteophytes, sclerosis and lysis with or without narrowing of articular margin.[29,46] Horses with moderate OA, isolated to a single joint, were eligible for enrollment in the study. Once selected

for study inclusion, a physical examination and complete blood count were performed to ensure general health.

Following completion of a written Client Consent form, horses were randomly assigned to 1 of 3 treatment groups using a random number generator (Excel, Microsoft Office 2019): saline (negative control, n=6), triamcinolone (positive control, n=6), or BMNC (treatment group, n=7; Figures 2-1 and 2-2). A 7-day washout period was observed following local anesthesia and horses then underwent repeat lameness scoring to establish baseline lameness for the study. Horses were re-evaluated at 7 and 21 days following treatment to assess lameness, collect synovial fluid for cytology, cytokine analysis, and to perform joint circumference measurements. Exclusion criteria included intra-articular injection within 3 months prior to screening, severe co-morbidity (including complete intra-articular fractures and concurrent ligamentous and tendinous injuries), and administration of other medication aimed at the musculoskeletal system (i.e., NSAID, PSGAG, and nutraceuticals) received within 4 weeks of study enrollment or during the 3 weeks of the study itself. Horses with a predominant limb lameness in the proximal and distal interphalangeal joints and distal tarsal joints were excluded due to the decreased likelihood of obtaining adequate volumes of synovial fluid.

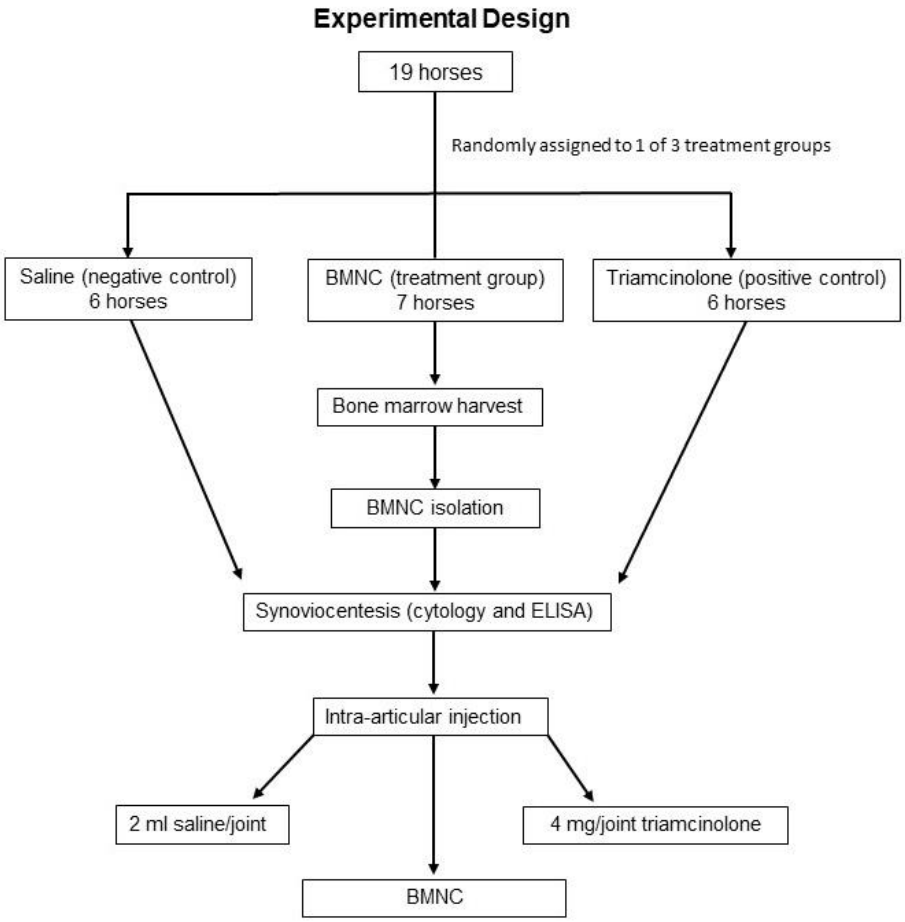

Figure 2-1. Schematic showing study design.

Objective lameness evaluation

Lameness evaluations were performed with a body-mounted inertial sensor system (Lameness Locator®) while horses were trotting in a straight line on a hard or firm surface. Sensors were attached to each horse as described.[47-51] Briefly, each horse was instrumented with a poll (head), right forelimb pastern, and pelvis sensor device secured in place for the examination.

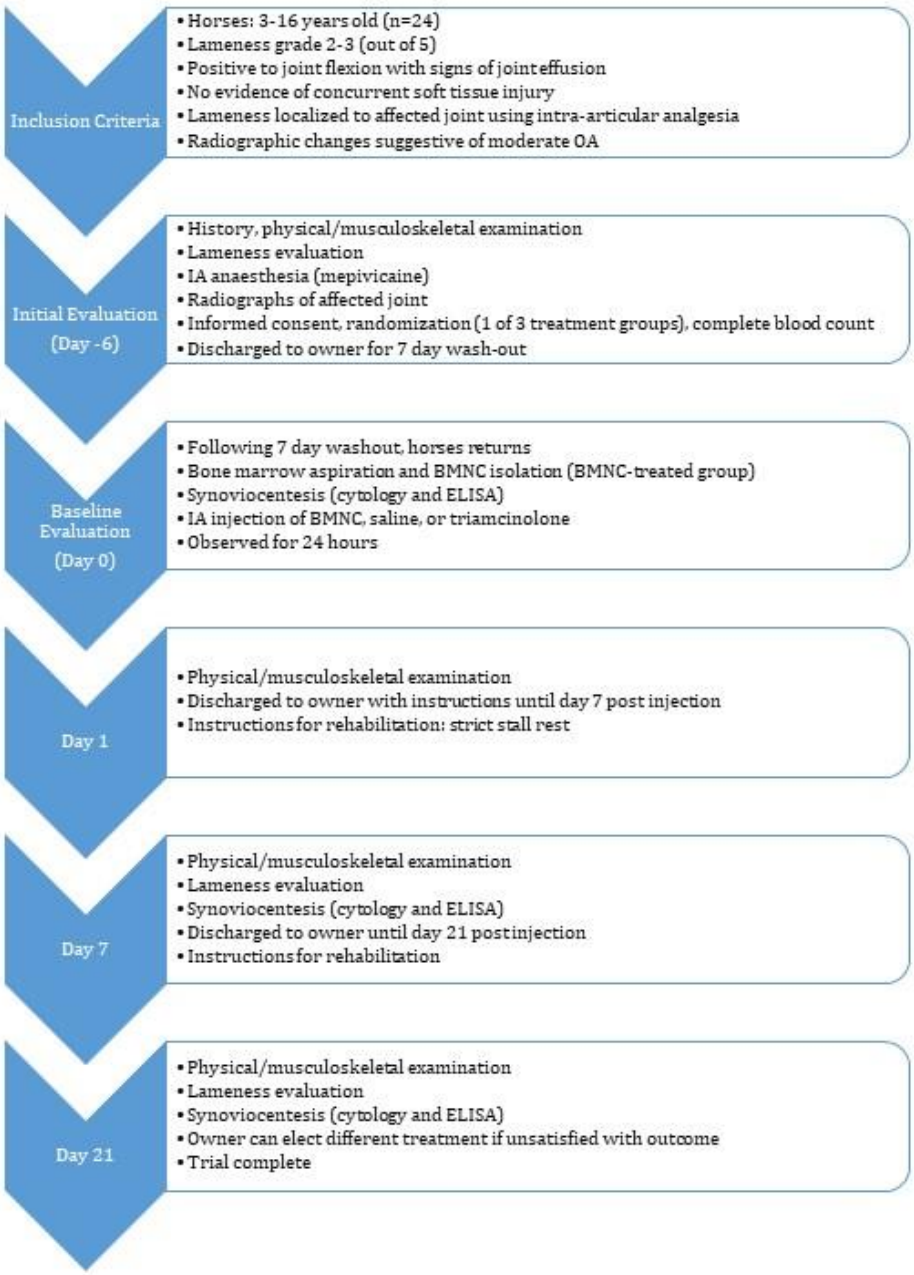

Figure 2-2. Schematic showing experimental flow for each individual horse.

Forelimb lameness was considered present when the vector sum (VS) of MaxHDiff and MinHDiff > 8.5mm.[47-51] If the VS was a positive value (MinHDiff is > 0), the lameness was

considered localized to the right forelimb. A negative VS (MinHDiff is < 0) indicated a left forelimb lameness. Data collected from vertical pelvic movement were not used in this study.

Subjective lameness evaluation

Subjective lameness grading was performed using a published grading scale (0-5) (Table 2-1).[45] Horses were trotted on asphalt (or similar hard surface) for evaluation in a straight line and limb flexion tests. Lunging of horses was performed on various types of surfaces. Videographic recordings were obtained for subjective grading; however, only the straight line was used in statistical analysis to maintain consistency with the Lameness Locator®.

Table 2-1: Table displaying criteria for Lameness Scoring (0-5).[45]

Lameness grades from 0–5 are based on observation of the horse at a trot in hand, in a straight line, on a firm or hard surface.

Score	Description
0	No lameness observed
1	Mild lameness observed while the horse is trotted in a straight line (head nod or pelvic hike inconsistent)
2	Obvious lameness is observed (head nod and pelvic hike are seen consistently)
3	Overt lameness (pronounced head nod or pelvic hike of several centimeters)
4	Severe lameness (extreme head nod or pelvic hike, but horse can still be trotted)
5	Horse is non-weight-bearing and should not be trotted.

Bone marrow harvest

Bone marrow aspirates for horses in the BMNC-treated group were performed immediately prior to intra-articular injection, as previously described.[43] Sham bone marrow aspirates were not performed on horses in the saline- or triamcinolone-treated groups as lack of aspirate was not deemed to influence the outcome and was considered an unnecessary, invasive treatment for client-owned horses. Horses were sedated with detomidine hydrochloride (0.01 mg/kg IV) and butorphanol tartrate (0.01 mg/kg IV). The sternum was clipped, aseptically prepared, and the skin, subcutaneous tissue and underling musculature anesthetized with 2% lidocaine hydrochloride (0.5-2 mg/kg) in all horses to minimize bias. Bone marrow aspirates (25 mL each) were collected from the 4th and 5th sternebrae using either an 8-gauge Komiyashiki needle or 11-gauge Jamshidi needle and a 60 mL syringe preloaded with 15,000 IU heparin and 10 ml Dulbecco's phosphate buffered saline (DPBS).[43,44]

Bone marrow mononuclear cell isolation

In a laminar flow hood, bone marrow aspirate was filtered (200µm blood administration set), gently layered over 2.5 mL of Ficoll-Paque™ Plus (GE Healthcare Life Sciences) in 15 mL conical tubes, and centrifuged (500 x g for 30 minutes at 4°C).[43,44] Supernatant plasma was aspirated and discarded up to 1.5 ml above the Ficoll ring. Mononuclear cells in Ficoll-Paque™ were aspirated, transferred to a sterile 50 ml conical tube, washed twice in DPBS, centrifuged (400 x g for 10 minutes at 4°C), and resuspended in DPBS (20 x 10^6 cells/ml DPBS). The concentration of isolated BMNC for each horse were determined using a hematocytometer. BMNC cell viability was confirmed through the application of a Dye Exclusion Test using trypan blue (Sigma-Aldrich).[52] Isolated BMNC were maintained in sterile conical centrifuge

tubes at 4°C to preserve viability and prevent clumping while transported from the laboratory to the clinic.[43,44] Cells in excess of those required for injection were cryopreserved for future studies.

Synoviocentesis, cytology, and joint treatment

Affected joints were prepared aseptically for synoviocentesis and synovial fluid (~2ml) was aspirated using a 20g x 1.5in hypodermic needle. Synovial fluid was aliquoted for cytology (EDTA tubes; Virginia Tech Animal Laboratory Services, Blacksburg, VA; Antech Diagnostics, Lake Success, NY) and prospective cytokine and growth factor quantification (Protein LoBind®; Eppendorf®, Westbury, CT). Synovial fluid samples for cytology were analyzed using an automated processor (ADVIA 2120 hematology 173 Analyzer, Siemens Healthcare Diagnostics, Inc., Tarrytown, NY). Differential cell counts were performed by a veterinary clinical pathologist (SB). EDTA-free samples were centrifuged (12,000 x g for 10 minutes at 4°C) and the supernatant stored at -80°C for cytokine and growth factor quantification. After synovial fluid was aseptically obtained for cytology and cytokine quantification and in the same synoviocentesis procedure all joints received the assigned treatment in a volume of 2 mL: BMNC-treated joints received 20 x 10^6 BMNC in DPBS, while saline and corticosteroid groups received DPBS, or 4 mg triamcinolone, respectively.

Joint circumference measurement

Joint circumference was used as a means to assess soft tissue swelling and synovial effusion following intra-articular injections. The location of joint circumference measurement was perceived as the location of maximum circumference and marked by clipping the hair. Joint

circumference (cm) was measured two times with a flexible measuring tape, consecutively, at the same location on the limb and then averaged. All measurements were performed by a single observer (JBE).

Follow-up evaluation

Following treatment of affected joints, horses were monitored for 24 hours for attitude, appetite, temperature, pulse, respiratory rate, signs of local inflammation, measurement of joint circumference (using a flexible measuring tape), and lameness in the stall. Evaluation outside the stall was performed at the walk only at 24 hours to minimize the possibility of joint trauma immediately following injection. Horses were discharged after evaluation at 24 hours with instructions to receive 7 days of stall rest, followed by 7 days of stall rest with 15 minutes of hand walking twice a day, and finally 20 minutes of trot under saddle for the final 7 days before re-evaluation. Horses were re-evaluated 7 and 21 days following treatment by performing a complete physical examination, measurement of joint circumference, and lameness evaluation with subjective and objective (Lameness Locator®) methods. Examinations were video recorded for later scoring by 3 blinded observers (SHB, CRB, RSP) and synovial fluid was collected under sedation from the treated joint for repeat cytology and cytokine quantification.

Cytokine and growth factor quantification

Aliquots of synovial fluid in Protein LoBind microfuge tubes and preserved at -80°C were used for quantification of growth factors and anti- and pro-inflammatory cytokines and chemokines [basic fibroblast growth factor-2, granulocyte monocyte colony stimulating factor, IL-1B, IL-6, macrophage chemoattractant protein 1 (MCP-1),

IL-10, TNF-A, stromal cell–derived factor 1 (SDF-1), IGF-1, IL-1 receptor antagonist (IL-1ra) and prostaglandin E_2 (PGE_2)] by equine multiplex bead-based assay (Eqcttmag-93K, Milliplex Map Equine chemokine/cytokine, Luminex 200 plate reader; MilliporeSigma). Manufacturer's modifications to include IGF-1, SDF-1, and IL-1ra and to validate inclusion of 1 additional point at the lower end of the standard curve to maximize detection of low analyte concentrations were included. PGE_2 was quantified by ELISA (KGE004B; R&D Systems, Minneapolis, MN, USA; SpectraMax M5 plate reader; Molecular Devices, Sunny- vale, CA, USA).[53]

Synovial fluid samples were thawed and digested with 10 mL hyaluronidase solution [100 IU testicular hyaluronidase in acetate buffer (LS005474; Worthington Biochemical, Lakewood, NJ, USA)] in 200 mL of synovial fluid and incubated for 30 minutes at 37°C. Samples were then centrifuged for 10 minutes at 12,000 x g and 4°C to remove any particulate matter and the supernatant was recovered (Protein LoBind microfuge tubes; Eppendorf).[53] Based on our previous experience with equine synovial fluid samples, a dilution of 1:2 was selected for PGE_2 quantification, and no dilution was deemed necessary for the Milliplex assay.[53] Following hyaluronidase digestion, PGE_2 samples were performed as previously described.[53] The remaining analytes were measured using hyaluronidase-digested samples without any additional processing.

Statistical analysis

Joint circumference data were log transformed to stabilize the model and are expressed as median and range. A Kappa statistic was used to compare intra- and inter-observer variation for subjective video analysis of lameness. Lameness Locator® data were analyzed for 17 horses with

forelimb lameness. Two horses with pelvic limb lameness were excluded because of the inability to handle the data in an identical manner as for forelimb lameness. Lameness Locator® data were collated in Microsoft Excel and inertial lameness measurements were reduced to 0 to represent a sound horse and establish baseline measurement. The Diff Max (push off lameness) or Diff Min (impact lameness) measurements were normalized to positive measurements, and entered under the limb that was lame (treated or control). Data analysis was performed using General Estimating Equations (GEE) to assess the effects of treatment and time. Chi-Square test was used for Least Squares Means for post-hoc comparisons of joint circumference data. Least square means adjustment for multiple comparisons was performed using the Tukey-Kramer test. All analyses were performed using commercial software (SAS version 9.4, SAS Institute, Inc., Cary, NC). Significance was set at $P<0.05$.

Results

Nineteen adult horses (11 castrated males and 8 females) between 3 and 16 years of age met inclusion criteria for the study. These included 18 Thoroughbreds and 1 American Quarter horse. Of the 19 horses included in the study, a predominant limb lameness was isolated to the forelimb in 17/19 (89%) and the hindlimb in 2/19 (12%; Table 2-2)

BMNC isolation

BMNC were successfully isolated from all six horses in the treatment group. There were no adverse effects noted with harvest site. BMNC concentration tended to vary by horse and age; however, the amount isolated was well in excess of what was needed for injection. Cell viability following isolation was between 80-90%.

Table 2-2: Signalment, affected joint, treatment, and outcome for horses enrolled in study.

Horse	Age (years)	Breed	Sex	Affected Joint	Treatment	Outcome
1	3	TB	Gelding	R radiocarpal	BMNC	Adopted for new career following racing
2	7	TB	Gelding	L middle carpal	BMNC	Adopted for light trail riding
3	3	TB	Mare	R radiocarpal	Triamcinolone	Adopted for new career following racing
4	3	TB	Gelding	R radiocarpal	Saline	Adopted for new career following racing
5	3	TB	Mare	L middle carpal	BMNC	Adopted for new career following racing
6	4	TB	Mare	R middle carpal	Saline	Adopted for new career following racing
7	6	TB	Gelding	RF MCP	Saline	Remained lame
8	6	TB	Gelding	RF MCP	BMNC	Adopted for new career following racing
9	3	QH	Gelding	R tibiotarsal	Triamcinolone	Returned to previous use
10	10	TB	Gelding	LF MCP	BMNC	Increased level of use; able to begin cantering and low-level jumping
11	6	TB	Mare	LF MCP	Triamcinolone	Returned to previous use
12	7	TB	Gelding	R middle carpal	Saline	Adopted for new career following racing
13	11	TB	Mare	LF MCP	Triamcinolone	Adopted for new career following racing
14	5	TB	Mare	RH MCP	BMNC	Switched to right forelimb lameness; low-level jumping
15	16	TB	Gelding	RF MCP	Triamcinolone	Adopted for new career following racing
16	3	TB	Mare	R middle carpal	Triamcinolone	Adopted for new career following racing
17	3	TB	Mare	L middle carpal	Saline	Adopted for new career following racing
18	6	TB	Gelding	RF MCP	BMNC	Adopted for new career following racing
19	3	TB	Gelding	L middle carpal	Saline	Adopted for new career following racing

TB, Thoroughbred; QH, Quarter Horse; BMNC, bone marrow mononuclear cells; R, right; L, left; RF, right front; LF, left front; MCP, metacarpophalangeal

BMNC isolation

BMNC were successfully isolated from all six horses in the treatment group. There were no adverse effects noted with harvest site. BMNC concentration tended to vary by horse and age; however, the amount isolated was well in excess of what was needed for injection. Cell viability following isolation was between 80-90%.

Intra-articular injections

Intra-articular injections and synoviocentesis were successfully performed in all horses and affected joints. Injection of BMNC caused no adverse effects in any horses. Injection with BMNC were made within 4 hours of harvesting bone marrow in all horses. The time required to harvest bone marrow and isolate BMNC decreased with time as experience with isolation protocol and cell counting improved.

Clinical exam

Body temperature, heart rate, respiratory rate, and appetite remained normal for all horses throughout the study. Horse 9 (triamcinolone) had increased synovial effusion following injection, which resolved by day 21. Horse 17 (saline) showed signs of transient post-injection synovitis with an increase in lameness between day 1 and 4 that was resolved by day 7 without intervention. Additionally, Horse 11 (triamcinolone) had increased lameness at day 21 compared to baseline. These changes were inconsistent and varied between horses.

Synovial fluid cytology

Injection of BMNC caused no adverse effects in any horses. Median TNCC remained <500 cells/uL at all time points (Fig. 2-3). The median TNCC at Day 7 in the BMNC group (440 cells/uL) was significantly higher than at Day 0 (100 cells/uL; *P*=0.003). Large mononuclear cells were the predominant cell type across treatments at all time points. Median TP was <2.25 g/dL for all treatments at all time points (Fig. 2-3). Median TP decreased significantly between 0 and 7 days in the saline-treated group (*P*=0.012). There were no other differences detected between groups or over time for TNCC, TP, or differential cell counts.

Lameness evaluation

Objective lameness data for 17/19 horses with forelimb lameness was included for statistical analysis. One horse from each of the saline and triamcinolone-treated groups, respectively were excluded in this analysis due to the presence of primary pelvic limb lameness. This was done to ensure a consistent and homogenous study population because forelimb and pelvic limb Lameness Locator® data could not be manipulated in identical manners. Overall, when all 17 horses were included in the analysis, lameness did not differ by treatment group (*P*=0.970); however, it did decrease significantly over time (*P*=0.010; Figure 2-4). In the BMNC-treated group, lameness did not decrease from Day 0 to 7 (*P*=0.947), but did decrease significantly from Day 0 to 21 (*P*=0.002) and from Day 7 to 21 (*P*=0.001). Lameness in the saline-treated group decreased significantly only from Day 0 to 21 (*P*=0.017), but not from Day 0 to 7 (*P*=0.784) or from Day 7 to 21 (*P*=0.104). In the triamcinolone-treated group, lameness did not decrease significantly between any of the time points (Day 0-7, *P*=0.193; Day 7-21, *P*=0.652; Day 0-21, *P*=0.324). There was one horse in the triamcinolone group that was an

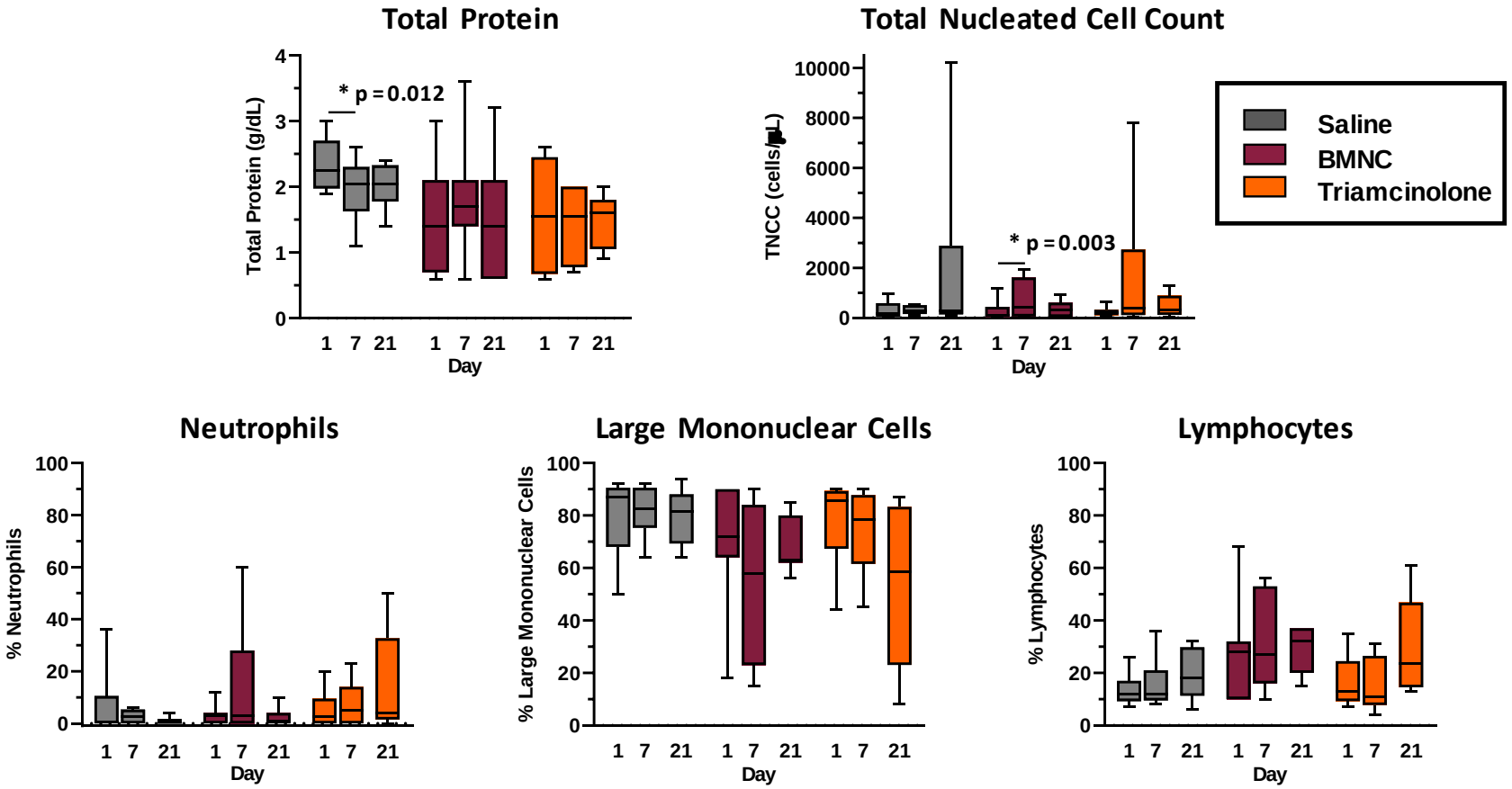

Figure 2-3. Synovial fluid cytology from saline- (n=6), BMNC- (n=7), and triamcinolone- (n=6) treated joints. * Significant difference between values indicated by the bar above. Median TNCC remained <500 cells/uL and median total protein was <2.25 g/dL for all treatments at all time points. Lines on box plots represent median value, boxes represent 25th and 75th quartile and whiskers represent 5th and 95th quartiles.

outlier and showed significantly increased lameness between 7 and 21 days, which would not be expected following corticosteroid treatment. There was no identifiable reason for this increase. Reanalysis excluding this single horse retained similar results of decreased lameness and statistical significance in the BMNC- and saline-treated groups; however, with n=4 horses in the triamcinolone group, lameness was significantly decreased between Days 7 and 21 ($P<0.001$) and Days 0 and 21 ($P<0.001$), but not for Days 0 to 7 ($P=0.248$; Figure 2-4). There were no significant differences in subjective lameness evaluation scores between treatments or within groups over time.

Joint circumference

Overall, joint circumference did not differ by treatment group ($P=0.430$) or over time ($P=0.995$; Figure 2-5). However, joint circumference did decrease significantly over time in the BMNC-treated group from Day 0 to 21 days ($P=0.004$) and Day 7 to 21 days ($P=0.001$). There were no significant differences over time for the saline ($P=0.309$) and triamcinolone groups ($P=0.994$).

Discussion

Intra-articular BMNC injection in horses with moderate, naturally-occurring OA was associated with no adverse effects and resulted in decreased soft tissue swelling and scores of objectively assessed lameness. In contrast to triamcinolone treatment which demonstrated no decrease in lameness, the majority of lameness improvement occurred between 7 and 21 days in the BMNC-treated horses. These findings are similar to a study performed by Barussi et al 2016 that compared tracheal infusion of BMNC in horses with recurrent airway obstruction to

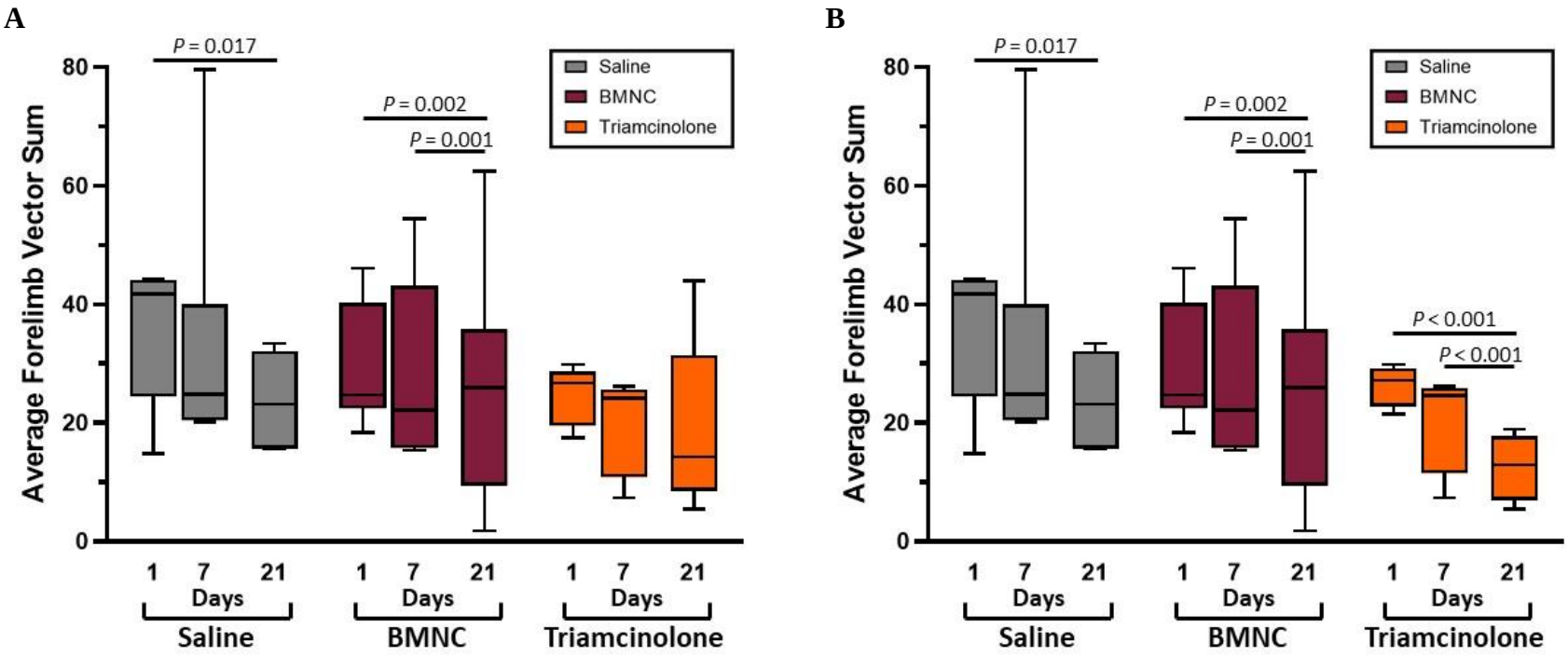

Figure 2-4. Objective lameness data collected using the Lameness Locator® for horses treated with saline, bone marrow mononuclear cells (BMNC), or triamcinolone. (A) Data from 17 horses with forelimb lameness (saline n=6, BMNC n=6, triamcinolone n=5). (B) Data from 16 horses with forelimb lameness (saline n=6, BMNC n=6, triamcinolone n=4) after omitting the one triamcinolone-treated horse that was an outlier with a marked increase in lameness between 7 and 21 days. * Significant difference between values indicated by the bar above. Lines on box plots represent median value, boxes represent 25th and 75th quartile and whiskers represent 5th and 95th quartiles.

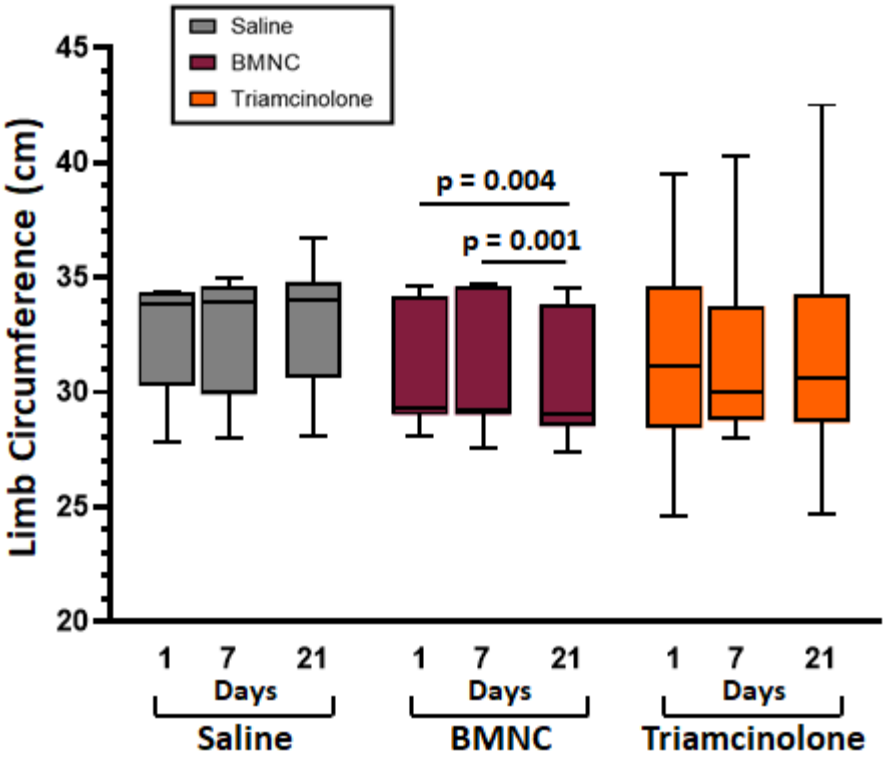

Figure 2-5. Joint circumference measurements for horses treated with saline (n=6), bone marrow mononuclear cells (BMNC; n=7), or triamcinolone (n=6). Lines on box plots represent median value, boxes represent 25th and 75th quartile and whiskers represent 5th and 95th quartiles.

dexamethasone treatment. Marked improvement of clinical signs such as coughing and respiratory effort and increases in anti-inflammatory cytokines such as IL-10 were noted at 7 and 21 days in horses treated with intratracheal BMNC.[23] Rest alone resulted in decreased lameness, regardless of treatment group. Importantly, synovial fluid parameters remained within normal limits for the 21 days following BMNC injection, with a small increase in TNCC at Day 7. This is the first controlled clinical trial investigating autologous intra-articular BMNC injection for the treatment of horses with naturally-occurring OA and provides objective data supporting a larger clinical trial. Equine BMNC are autologous, readily available, do not require extensive processing, and reduced inflammation and lameness in naturally-occurring OA in the horses studied.

Consistent with published reports in people, intra-articular BMNC did not cause adverse effects.[42,43] The transient increase in TNCC in the BMNC-treated group in our study may have been a direct response to the nucleated cells injected into the joint and/or to a recruitment of endogenous nucleated cells, possibly to initiate inflammation resolution. This finding is consistent with a study evaluating intra-articular BMNC injection into experimentally inflamed equine joints.[43] In that study, intra-articular BMNC resulted in a 20% increase in macrophages on cytology at 96 and 144 hours post injection.[43] Collectively, these findings suggest that the effect of BMNC in OA-affected joints may follow a similar pattern with regulatory macrophage progenitors aiding in resolution of inflammation.

There was a significant decrease in the joint circumference measurement at all time points for the BMNC-treated group. Joint circumference is a direct measure of synovial effusion, which is an indirect measure of inflammation within and surrounding the joint. We chose to utilize joint circumference as a direct measure of synovial effusion as opposed to a synovial

effusion grading scale which is a clinical observation and prone to more subjectivity. Consistent with published reports, the anti-inflammatory effects of BMNC, as measured by joint circumference, were readily apparent in each of the 7 horses in the BMNC group, despite variations between horses in the positive and negative control groups.[42] In a human study evaluating the use of intra-articular BMNC for knee OA, a single dose of BMNC lasted more than 12 months, was considered superior to 3 weekly injections of hyaluronan, and demonstrated clinical improvement in 96% of BMNC-treated patients.[41,42] Moreover, BMNC exhibited potent anti-inflammatory effects comparable to corticosteroids in equine and murine chronic inflammatory airway disease.[23,24] Intratracheal delivery of BMNC in horses affected with recurrent airway obstruction resulted in marked resolution of inflammation, similar to the effects provided by corticosteroids.[23] IL-10 concentrations in bronchoalveolar lavage fluid from horses treated with BMNC were 30-fold higher compared to horses treated with dexamethasone.[23] Similar, the reduction in lameness and mean joint circumference noted in our horses could represent an increased concentration of IL-10 and M2-like macrophages within the affected joint. Significant anti-inflammatory effects were also observed using BMNC to treat a murine model of airway disease.[24] In that study, BMNC depleted of macrophages eliminated the reparative and remodeling processes initiated by BMNC therapy, providing further evidence of the potent anti-inflammatory potential of BMNC-derived macrophages.[24] Together, these studies demonstrate the inflammation resolving potential of BMNC, which is mediated through macrophage-derived IL-10 production.

The lack of significance on subjective lameness evaluation is likely due to a variety of factors. In our study, we used a lameness grading scale that allowed grading of horses within the trot, thus giving more sensitivity to changes in lameness versus the American Association of

Equine Practitioners (AAEP) scale that grades across different gaits and conditions.[45] Lameness can be inconsistent between observers as demonstrated in multiple studies[48,50,51] and can vary within the same evaluation period. A study evaluating the repeatability of subjective evaluation found that when the mean AAEP lameness score was >1.5, the graders agreed 93.1% of the time but this decreased to 61.9% of the time when the mean score ≤1.[51] Moreover, clinicians only agreed 51.6% of times when asked to choose if the horse was lame and what limb was affected.[51] Graders in our study only evaluated the horses on the straight line, which could also affect interpretation without observation in a circle to the left or right. In addition, despite our efforts to consistently position the video camera, small variations in video quality may have affected the ability to accurately score lameness compared to live assessment. Together, this reinforces the poor agreement between clinicians and clinical observation. Change over time can be measured quantitatively with the Lameness Locator® and has been shown to be more sensitive detection and diagnosis of lameness compared to subjective evaluation.[48]

Objective lameness improvement was noted in all treatment groups. Yet, only the BMNC-treated horses showed a significant decrease in lameness at day 7 and 21 compared to baseline. The reduction in lameness correlates with the decrease in mean joint circumference in BMNC-treated horses suggesting that an improvement in effusion can result in improved lameness. Improved lameness in all treatment groups over the course of the study is not surprising, as rest alone is able to improve by allowing time for tissues to heal.[54,55]

Recent research has shown that synovial macrophages play an integral role in OA as synovial macrophage infiltration is associated with OA progression such as osteophyte formation and cartilage degradation, synovial inflammation, and pain and may serve as a potential therapeutic target.[6,21,56] Although macrophages are commonly thought of as causing negative

effects, such as inflammation, under physiological conditions, macrophage-mediated inflammation is actually self-limiting and performs a critical role in inflammation resolution.[57] *In vitro*, macrophages undergo phenotypic changes in response to environmental stimuli, polarizing into distinctly different cell types – inflammatory (M1) and suppressive/regulatory (M2).[16] These variations in activation states gave rise to the concept of macrophage polarization.[58] Importantly, the M2 phenotype can be induced by exposure to an inflammatory environment, such as a diseased joint.[17,23] Recent studies have suggested that macrophage polarization is not as straightforward as once postulated. Rather, macrophages exhibit a continuum of marker expression and exist in a hybrid state – producing both pro-and anti-inflammatory cytokines that are essential for resolution of inflammation.[53] *In vitro* culture of BMNC in normal and inflamed synovial resulted in macrophage-rich cultures with phenotypes, similar to those seen in normal synovial macrophages, and increasing levels of IL-10+ macrophages and IL-10 and IGF-1 concentrations, and decreasing IL-1β concentrations.[53]

The rationale for our clinical trial stems from work performed by Menarim et al.[43,53] In an *in vivo* study using an equine model of LPS-induced synovial inflammation, autologous intra-articular BMNC injection resulted in marked reduction of acute inflammation without adverse effects.[43] BMNC-treated joints showed gross, cytologic, and inflammatory biomarker improvements in synovial fluid and synovial membrane parameters, with increased numbers of regulatory macrophages and synovial fluid IL-10 concentrations compared with saline-treated controls.[43] In fact, inflamed joints with BMNC had 24% higher macrophage counts and 10% more IL-10^{+} cells than saline-treated controls.[43] BMNC-treated joints were comparable to healthy joints grossly and histologically, whereas saline-treated control joints contained swollen orange dark orange to brown synovium and increased vascularity histologically 6 days after

injection.[43] These experimental studies support the idea that capitalizing on the macrophage-derived effects of BMNC has the potential to reestablish joint homeostasis and are the seminal studies serving as the groundwork for this clinical trial.

The results of our clinical trial investigating the use of intra-articular BMNC for naturally-occurring OA demonstrated significant improvement in lameness in the BMNC-treated group similar to that seen with the triamcinolone-treated group, as hypothesized. Moreover, intra-articular BMNC treatment resulted in significant decreases in mean limb circumference. The noticeable anti-inflammatory effects of BMNC therapy in our study were likely achieved by optimizing the functions of synovial macrophages. Based on the published literature, we hypothesize that the anti-inflammatory effects and resulting improvements in hour horses are due to increased production of IL-10 by regulatory macrophage progenitors present in BMNC. Autologous BMNC from bone marrow aspirate are a promising novel point-of-care therapy and have the potential to substantially advance the current treatment of equine joint disease by producing a lasting, biological effect.[41,43,44,53] BMNC, which are rich in M2-biased macrophage progenitors, provide important advantages over available intra-articular therapies, including the detrimental effects on cartilage metabolism known to occur following corticosteroid therapy.

There are a few important limitations in our study, including the performance of saline-treated controls, sample size and the inability to readily include the two hind limb lame horses in the objective lameness analysis. Improved lameness scores in saline-treated controls highlights the difficulty of evaluating naturally-occurring disease. Compared to experimental models, disease severity and clinical signs vary widely between patients with naturally-occurring osteoarthritis.[59] Additionally, rest alone can improve lameness regardless of treatment, which is consistent with published literature and a finding in our objective analysis of lameness

scores.[54,55] In a randomized, double-blinded, placebo controlled clinical study evaluating intra-articular hyaluronan treatment in equine lameness originating from the metacarpophalangeal joint, hyaluronan injection was not better than a single saline injection for reducing lameness in horses with synovitis or mild osteoarthritis.[54] Similarly, the combination of rest alone and an intra-articular injection of 2 ml of 0.9% NaCl solution resulted in decreased lameness in horses with traumatic arthritis over a 3-week treatment period.[55] Nonetheless, it is encouraging that BMNC showed significant effects even with a lower number of horses. We look forward to the opportunity to test this hypothesis further in a larger and more diverse population of horses.

In summary, BMNC therapy has the potential to provide long-lasting resolution of joint inflammation and the promotion of tissue repair. Overall, our results agree with findings from an experimental model of synovial inflammation model in horses, as well as clinical trials of intra-articular BMNC for OA-affected human patients.[41-43] BMNC therapy was safe, caused no adverse effects, required minimal processing, and resulted in improved lameness and decreased joint circumference in all horses. Our findings support further investigation of the clinical use of BMNC in horses affected with OA.

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References

1. Oke S MC, Moyer W: Review of the Economic Impact of Osteoarthritis and Oral Joint-Health Supplements in Horses. Proc Am Assoc Equine Pract 56:12-16, 2010.
2. McIlwraith CW, Frisbie DD, Kawcak CE: The horse as a model of naturally occurring osteoarthritis. Bone Joint Res 1:297-309, 2012.
3. Jeffcott LB, Rossdale PD, Freestone J, et al: An assessment of wastage in thoroughbred racing from conception to 4 years of age. Equine Vet J 14:185-198, 1982.
4. Rossdale PD, Hopes R, Digby NJ, et al: Epidemiological study of wastage among racehorses 1982 and 1983. Vet Rec 116:66-69, 1985.
5. Fubini SL, Erb HN, Freeman KP, et al: Prognostic factors affecting survival of 507 horses with joint disease: (1983 to 1990). Can J Vet Res 63:253-260, 1999.
6. Scanzello CR, Goldring SR: The role of synovitis in osteoarthritis pathogenesis. Bone 51:249-257, 2012.
7. Smith MD, Triantafillou S, Parker A, et al: Synovial membrane inflammation and cytokine production in patients with early osteoarthritis. J Rheumatol 24:365-371, 1997.
8. Kawcak CE, McIlwraith CW, Norrdin RW, et al: The role of subchondral bone in joint disease: a review. Equine Vet J 33:120-126, 2001.
9. Clegg PD, Carter SD: Matrix metalloproteinase-2 and -9 are activated in joint diseases. Equine Vet J 31:324-330, 1999.
10. Clegg PD, Coughlan AR, Riggs CM, et al: Matrix metalloproteinases 2 and 9 in equine synovial fluids. Equine Vet J 29:343-348, 1997.

11. 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 Rheum 62:647-657, 2010.
12. Kennedy A, Fearon U, Veale DJ, et al: Macrophages in synovial inflammation. Front Immunol 2:52, 2011.
13. Griffin TM, Scanzello CR: Innate inflammation and synovial macrophages in osteoarthritis pathophysiology. Clin Exp Rheumatol 37 Suppl 120:57-63, 2019.
14. Mills CD, Ley K: M1 and M2 macrophages: the chicken and the egg of immunity. J Innate Immun 6:716-726, 2014.
15. Murray PJ: Macrophage Polarization. Annu Rev Physiol 79:541-566, 2017.
16. Murray PJ, Allen JE, Biswas SK, et al: Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity 41:14-20, 2014.
17. Olingy CE, San Emeterio CL, Ogle ME, et al: Non-classical monocytes are biased progenitors of wound healing macrophages during soft tissue injury. Sci Rep 7:447, 2017.
18. Fahy N, de Vries-van Melle ML, Lehmann J, et al: Human osteoarthritic synovium impacts chondrogenic differentiation of mesenchymal stem cells via macrophage polarisation state. Osteoarthritis Cartilage 22:1167-1175, 2014.
19. Jain S, Tran TH, Amiji M: Macrophage repolarization with targeted alginate nanoparticles containing IL-10 plasmid DNA for the treatment of experimental arthritis. Biomaterials 61:162-177, 2015.
20. Liu B, Zhang M, Zhao J, et al: Imbalance of M1/M2 macrophages is linked to severity level of knee osteoarthritis. Exp Ther Med 16:5009-5014, 2018.

21. Bondeson J: Activated synovial macrophages as targets for osteoarthritis drug therapy. Curr Drug Targets 11:576-585, 2010.
22. Fichadiya A, Bertram KL, Ren G, et al: Characterizing heterogeneity in the response of synovial mesenchymal progenitor cells to synovial macrophages in normal individuals and patients with osteoarthritis. J Inflamm (Lond) 13:12, 2016.
23. Barussi FC, Bastos FZ, Leite LM, et al: Intratracheal therapy with autologous bone marrow-derived mononuclear cells reduces airway inflammation in horses with recurrent airway obstruction. Respir Physiol Neurobiol 232:35-42, 2016.
24. Cruz FF, Borg ZD, Goodwin M, et al: CD11b+ and Sca-1+ Cells Exert the Main Beneficial Effects of Systemically Administered Bone Marrow-Derived Mononuclear Cells in a Murine Model of Mixed Th2/Th17 Allergic Airway Inflammation. Stem Cells Transl Med 5:488-499, 2016.
25. Bellner L, Marrazzo G, van Rooijen N, et al: Heme oxygenase-2 deletion impairs macrophage function: implication in wound healing. FASEB J 29:105-115, 2015.
26. Godwin JW, Pinto AR, Rosenthal NA: Macrophages are required for adult salamander limb regeneration. Proc Natl Acad Sci U S A 110:9415-9420, 2013.
27. Kydd AS, Reno CR, Tsao HW, et al: Early inflammatory arthritis in the rabbit: the influence of intraarticular and systemic corticosteroids on mRNA levels in connective tissues of the knee. J Rheumatol 34:130-139, 2007.
28. de Souza MV: Osteoarthritis in horses - Part 2: a review of the intra-articular use of corticosteroids as a method of treatment. Braz Arch Biol Technol 59:1-10, 2016.

29. 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. 68:290-296, 2007.
30. 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. J Equine Vet Sci 60:35-42.e32, 2018.
31. Broeckx SY, Martens AM, Bertone AL, et al: The use of equine chondrogenic-induced mesenchymal stem cells as a treatment for osteoarthritis: A randomised, double-blinded, placebo-controlled proof-of-concept study. Equine Vet J 51:787-794, 2019.
32. 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 Vet J 52:601-612, 2019.
33. Caron JP, Gandy JC, Brown JL, et al: Omega-3 fatty acids and docosahexaenoic acid oxymetabolites modulate the inflammatory response of equine recombinant interleukin1beta-stimulated equine synoviocytes. Prostaglandins Other Lipid Mediat 142:1-8, 2019.
34. Buckley CD, Gilroy DW, Serhan CN. Proresolving lipid mediators and mechanisms in the resolution of acute inflammation. *Immunity*. 2014;40(3):315-327.
35. Shizhu J, Xiangwei M, Xun S, et al: Bone marrow mononuclear cell transplant therapy in mice with CCl4-induced acute liver failure. Turk J Gastroenterol 23:344-352, 2012.

36. Wang C, Yu X, Cao Q, et al: Characterization of murine macrophages from bone marrow, spleen and peritoneum. BMC Immunology 14:6, 2013.

37. Behrendt P, Feldheim M, Preusse-Prange A, et al: Chondrogenic potential of IL-10 in mechanically injured cartilage and cellularized collagen ACI grafts. Osteoarthritis Cartilage 26:264-275, 2018.

38. Iannone F, De Bari C, Dell'Accio F, et al: Interleukin-10 and interleukin-10 receptor in human osteoarthritic and healthy chondrocytes. Clin Exp Rheumatol 19:139-145, 2001.

39. Liu W, Zhang X, Zhao M, et al: Activation in M1 but not M2 macrophages contributes to cardiac remodeling after myocardial infarction in rats: a critical role of the calcium sensing receptor/NRLP3 inflammasome. Cell Physiol Biochem 35:2483-2500, 2015.

40. Agadi S, Shetty AK: Concise review: prospects of bone marrow mononuclear cells and mesenchymal stem cells for treating status epilepticus and chronic epilepsy. Stem Cells 33:2093-2103, 2015.

41. Goncars V, Jakobsons E, Blums K, et al: The comparison of knee osteoarthritis treatment with single-dose bone marrow-derived mononuclear cells vs. hyaluronic acid injections. Medicina (Kaunas) 53:101-108, 2017.

42. Goncars V, Kalnberzs K, Jakobsons E, et al: Treatment of Knee Osteoarthritis with Bone Marrow-Derived Mononuclear Cell Injection: 12-Month Follow-up. Cartilage 10:26-35, 2019.

43. 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 J 33:14337-14353, 2019.

44. Corrêa F, Borlone C, Wittwer F, et al: How to obtain and isolate equine sternal bone marrow mononuclear cells with limited resources. Arch Med Vet 46:471-476, 2014.

45. Ross MW: Movement, in Ross MW, Dyson SJ (eds): Diagnosis and Management of Lameness in the Horse (Second Edition), Saint Louis, W.B. Saunders, 2011, pp 64-80.

46. Holzer N, Salvo D, Marijnissen ACA, et al: Radiographic evaluation of posttraumatic osteoarthritis of the ankle: the Kellgren–Lawrence scale is reliable and correlates with clinical symptoms. Osteoarthritis Cartilage 23:363-369, 2015.

47. Keegan KG, Kramer J, Yonezawa Y, et al: Assessment of repeatability of a wireless, inertial sensor-based lameness evaluation system for horses. Am J Vet Res 72:1156-1163, 2011.

48. McCracken MJ, Kramer J, Keegan KG, et al: Comparison of an inertial sensor system of lameness quantification with subjective lameness evaluation. Equine Vet J 44:652-656, 2012.

49. 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. Am J Vet Res 73:368-374, 2012.

50. Keegan KG, Yonezawa Y, Pai PF, et al: Evaluation of a sensor-based system of motion analysis for detection and quantification of forelimb and hind limb lameness in horses. Am J Vet Res 65:665-670, 2004.

51. Keegan KG, Dent EV, Wilson DA, et al: Repeatability of subjective evaluation of lameness in horses. Equine Vet J 42:92-97, 2010.

52. Strober W: Trypan blue exclusion test of cell viability. Curr Protoc Immunol 111: A3.B.1-A3.B.3, 2015.

53. Menarim BC, Gillis KH, Oliver A, et al: Inflamed synovial fluid induces a homeostatic response in bone marrow mononuclear cells in vitro: Implications for joint therapy. FASEB J 34:4430-4444, 2020.

54. Niemelä TM, Tulamo R-M, Hielm-Björkman AK: A randomised, double-blinded, placebo-controlled clinical study on intra-articular hyaluronan treatment in equine lameness originating from the metacarpophalangeal joint. BMC Vet Res 12:60, 2016.

55. Gaustad G, Dolvik NI, Larsen S: Comparison of intra-articular injection of 2 ml of 0.9% NaCl solution with rest alone for treatment of horses with traumatic arthritis. Am J Vet Res 60:1117-1121, 1999.

56. Kraus VB, McDaniel G, Huebner JL, et al: Direct in vivo evidence of activated macrophages in human osteoarthritis. Osteoarthritis Cartilage 24:1613-1621, 2016.

57. Kurowska-Stolarska M, Alivernini S: Synovial tissue macrophages: friend or foe? RMD Open 3:e000527, 2017.

58. Gordon S, Plüddemann A, Martinez Estrada F: Macrophage heterogeneity in tissues: phenotypic diversity and functions. Immunol Rev 262:36-55, 2014.

59. Bapat S, Hubbard D, Munjal A, et al: Pros and cons of mouse models for studying osteoarthritis. Clin Transl Med 7:36, 2018.

CHAPTER 3: CONCLUSIONS AND FUTURE DIRECTIONS

Synovial inflammation is responsible for the initiation, perpetuation, and progression of OA. Macrophages play a pivotal role in driving synovial inflammation, but also in maintaining joint health. It is critical to understand that mediators involved in inciting inflammation are also necessary to induce the synthesis of signaling pathways aimed at mediating endogenous resolution of the inflammatory process. As such, macrophages are responsible for inciting as well as resolving inflammation. In the acute phase of inflammation, macrophages recruit other immune cells to the injury site to fight aggressors and clear tissue debris but importantly to orchestrate repair of the damaged tissue through cellular and paracrine effects. If inflammation is not resolved, then chronic inflammation ultimately leads to the pathologic cartilage and bone changes characteristic of OA. Once tissue repair has been achieved, macrophages coordinate inflammation resolution, eventually promoting homeostasis.

Because macrophages are involved in both inflammation and health, they have been described as having inflammatory or suppressive/regenerative phenotypes. However, the idea of macrophage polarization is not as straightforward as once postulated. Macrophage phenotypes *in vivo* seem to exhibit a continuum of marker expression and exist in a hybrid state – producing both pro- and anti-inflammatory cytokines that are all essential for resolution of inflammation.

Macrophage progenitors derived from bone marrow (BMNC) are innately biased toward an anti-inflammatory response when injected into an inflamed environment such as an OA-affected joint. Our study, combined with recent *in vivo* and *in vitro* studies performed by Menarim et al suggest that intra-articular administration of exogenous macrophage progenitors derived from bone marrow can effectively improve clinical lameness and induce a shift within

the joint toward repair and homeostasis. The horses in our clinical trial had objective as well as subjective improvement in lameness, synovial effusion, and quality of life. Treated horses were able to find new vocations or compete at new levels that were not thought possible prior to therapy. Owners, trainers, and veterinarians involved in this project have commented on the positive impact that our treatment has made. Although it has been challenging to summarize our results in a way that clearly conveys our subjective observations, the results of this small pilot study are really exciting and show great promise. One future direction would be to create patient-side kits prepared ahead for the end-user to efficiently harvest and isolate BMNC. It would be interesting to perform trials similar to one performed with mesenchymal stem cells and assess the minimal effect amount of bone marrow required to isolate an effective number of BMNC. Although our work was performed in horses, our results translate to human OA as well. As such, our findings lay the ground work for larger clinical trials and have the potential to benefit thousands of people and horses.

Moreover, our study offers a novel way to treat OA and provides evidence to support the notion that some inflammation is beneficial and actually required for the resolution of joint inflammation. Blocking inflammatory mechanisms with intra-articular corticosteroids may provide symptomatic relief but does not favor recovery of joint homeostasis, which is fundamentally dependent on pro-inflammatory mediators. Therefore, inhibition of inflammation in OA therapies warrants additional research. An alternative way of thinking about the treatment of OA is to stimulate endogenous resolution of inflammation by increasing the innate homeostatic mechanisms of the joint, largely attributable to macrophages. This study and along with the preceding ones by Dr. Menarim provide an exciting basis for future development of this new way of approaching the management of OA.