ONE STEP AT A TIME: ADVANCES IN OSTEOARTHRITIS

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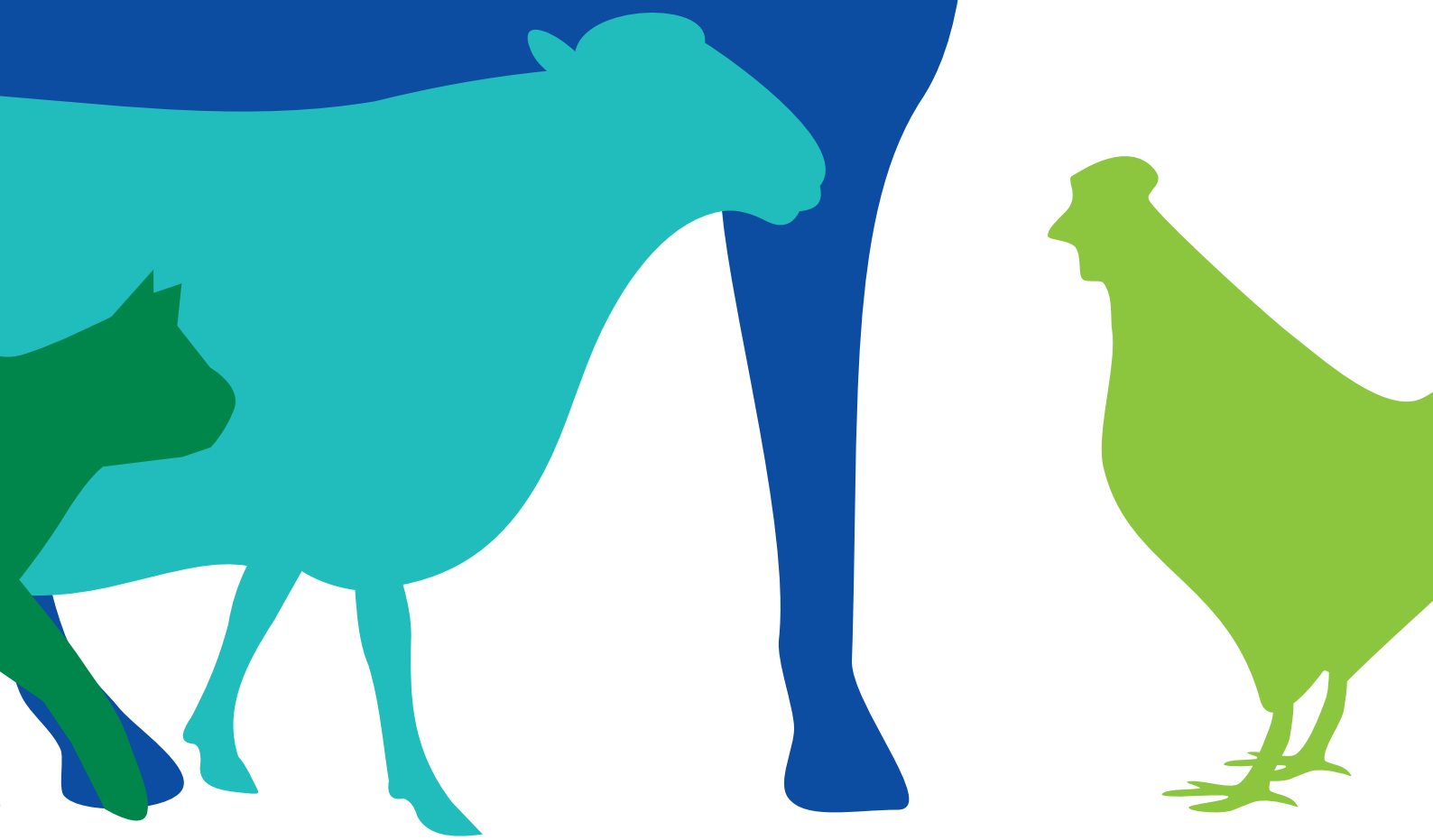

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ONE STEP AT A TIME: ADVANCES IN OSTEOARTHRITIS

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EDITORIAL
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Editorial: One Step at a Time: Advances in Osteoarthritis

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Editorial on the Research Topic

One Step at a Time: Advances in Osteoarthritis

Osteoarthritis (OA) is the most common form of arthritis[1] and the fastest growing cause of disability worldwide (1, 2). OA is a degenerative and low-grade inflammatory disease that affects humans, companion and captive animals (3). For many years OA was considered a non-inflammatory disease of articular cartilage in elderly individuals and studied in the context of aging (4–6). However, despite the fact that aging is the primary risk factor for OA (7), there is emerging evidence to suggest that OA is a low-grade inflammatory disease that affects the whole joint (8), impacts younger people (9) and involves innate immunity (10, 11). Loss of articular cartilage has long been considered a hallmark of OA (12). However, all the connective tissues that make up a synovial joint have been shown to be altered during the development and progression of OA (6). Cartilage, synovium, subchondral bone, and the crosstalk between these tissues are central components of OA development (13). However, emerging evidence suggests that other tissues such as the meniscus, cruciate ligament, and infrapatellar fat pad, also known as Hoffa's fat pad, are involved in knee OA (14–19).

Low-grade inflammation (20, 21) and metabolic alterations (22–24) occur as individuals age and joint tissue accumulate damage and undergo senescence over time (25, 26), but this does not mean that OA is simply a "wear and tear" disease. Indeed, describing OA as "wear and tear" has hampered research progress and drug development (27, 28). The interplay among diverse cells and their microenvironment which becomes increasingly inflammatory as the disease progresses, as well as the diverse biomechanical and molecular factors of different joints (29, 30) are all active co-conspirators in making this disease so complex and difficult to treat. Although age, obesity, and a previous history of joint trauma are important risk factors for the development of OA (31–33), there is not a single common risk factor defining the origin of the disease, making it even more difficult to define, diagnose, and manage (34). In fact, even when the origin is similar, progression is usually not predictable because of individual variation in metabolic status, fitness level, lifestyle choices, and pain tolerance. To complicate matters even further, there are underlying disease endotypes (35) and emerging phenotypes (36), which add to the complexity of OA. However, the advent of OA endotypes and phenotypes have created new opportunities for stratification and subtyping, allowing us to develop targeted treatments, for example, new therapies for the inflammatory phenotype of the disease (37). There is currently no cure or treatment for OA

[1] https://www.cdc.gov/arthritis/basics/osteoarthritis.htm.

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making effective management extremely challenging (38, 39). This is especially important for an increasingly younger group of individuals who have to live with OA and joint pain for more years and suffer disability and a limited range of motion for most of their lifetime (40). To date, most treatments focus on palliative relief of symptoms. Non-steroidal anti-inflammatory drugs (NSAIDs) are often effective for relief of signs of musculoskeletal pain, however systemic adverse side-effects remain a major concern (41, 42). Furthermore, NSAIDs are not suitable for use in patients with cardiovascular co-morbidities (43, 44) and especially those with chronic asthma (45). Targeted treatment via intra-articular administration of corticosteroids is also palliative but can have undesired consequences for joint tissues (46), although experimental conditions can alter conclusions about the true importance of these effects (47, 48). Results of studies regarding effects of orthobiologic treatments for OA are promising but long-term outcomes remain unknown and require larger and more comprehensive clinical trials with appropriate endpoints.

Therefore, it is paramount that the OA research community continues to strive toward a better mechanistic understanding of the disease. This is the only strategy that can reveal novel therapeutic targets and facilitate the development of earlier diagnostics so that the disease can be identified and managed as early as possible to minimize structural and symptomatic progression. The ultimate goal should be to halt and reverse the progression of OA, but in the absence of such treatments any preventive strategy that can provide effective management such that the joint is not only protected, but that it also has the capability to heal to the best of its ability would support the concept of joint health maintenance (49).

OA is not specific to just one species as veterinary and human patients alike are afflicted by this naturally-occurring disease. This provides opportunity for both; for instance, there is evidence that research in dogs can reliably predict treatment efficacy in humans and vice versa. Across many classes of anti-inflammatory and analgesic compounds in which there have been studies in companion animal chronic pain conditions and the same conditions in humans, analogous results have been seen (3). This has helped advance the knowledge base about the disease process because important comparisons can be made between species. There are numerous animal models of OA and although many can advance our knowledge of disease mechanisms and the pathophysiology of pain (50), none of the small animal models are as relevant to humans as the spontaneously occurring canine and equine models of OA. Indeed, because of the complexity of OA, such spontaneous models of naturally-occurring disease may offer the best way forward for elucidation of pathophysiologic mechanisms and discovery of treatments (51). Nevertheless, the multitude of spontaneous and induced animal models of OA have allowed both physicians and veterinarians to understand the underlying mechanisms and develop strategies that can help decrease the morbidity of the disease for their patients and advance clinical trials of new OA treatments (52).

Much of what we know about OA comes from large-scale epidemiological studies (53–56), especially studies that were conducted in large cohorts such as the Framingham Study (57, 58). Many investigators have designed and conducted clinical trials to examine the effects of physical activity and inactivity on synovial joint health and OA symptoms (59). In humans obesity and lack of physical activity are major contributors to the development of OA (57). There is no convincing published evidence in humans that exercises such as running contributes to OA (60, 61). Recent clinical research suggests that weight loss, physical activity, and increasing muscle mass and strength are the only effective strategies for reducing pain and enhancing mobility in subjects with OA (62). The only way to slow down the pain and progression of OA appears to be physical exercise, avoiding obesity and maintaining a healthy weight (63–69). A recent systematic review of the published literature on studies of the senolytic effects of exercise and physical activity on senescent cells under various states in both human and animal models suggests that exercise has senolytic properties (70). There is also emerging evidence that exercise can support the immune system and generate immune cells through its actions on bone (71). Therefore, these recent observations may explain the beneficial impacts that patients with OA see when they exercise. Reduced levels of physical activity can accelerate the development of OA (72). Therefore, the physical activity that has been recommended for human patients with OA can also be recommended for companion animals, who need it as much as we do.

It is important to pause and reflect on where we are going, which areas we should focus on in the future and which topics might greater effort in this field. The study of extracellular vesicles (ECVs) in OA has become a whole new area (73–75). Epigenetics, epigenomics and the study of microRNAs are also rapidly expanding and thriving areas of research in the OA field (76, 77). Advances in analytical platforms such as omics technologies for deep phenotyping (28, 78), biomarkers (79, 80), structural and functional imaging (81, 82) and artificial intelligence (83, 84) are also having an impact on our understanding of OA. Continuing technological advances that impact clinical and laboratory diagnostics are making it possible to investigate this disease in ways that were impossible to do just a few decades ago. This includes, but is not limited to, learning more about the basic biology of joint tissues and how they can be stimulated to repair or be replaced, how the disease can be diagnosed earlier using biological or imaging biomarkers, as well as identifying and testing various therapeutic targets.

The authors strongly believe that future advances in OA research require multidisciplinary and interdisciplinary collaboration and a genuine "One Health," "One Medicine" approach to OA, with closer interaction between veterinarians, human clinicians and bioscience researchers (85).

This Research Topic comprises 4 review articles and 6 original research publications from a number of OA researchers. Taken together, these articles are geared toward the advancement of our understanding, diagnosis, and treatment of OA by researching multiple intra-articular tissues including synovial membrane, articular cartilage, subchondral bone, and fat. Multiple technologies are used in the original research articles to determine changes that occur with disease and/or treatment using novel *in vitro* methods, animals with naturally-occurring

OA or post-traumatic OA models that have translational relevance to human OA.

REVIEW ARTICLES

Regenerative medicine and cell-based therapies are promising areas of research and development in many disease areas, including OA. These therapies have the potential to provide symptomatic relief while also potentiating repair. In this Research Topic, 2 reviews are presented on this area as it relates to veterinary medicine. Bogers reviews the known mechanism of action of mesenchymal stem cells, and the blood derived products autologous conditioned serum (ACS) and platelet-rich plasma (PRP) for canine and equine OA patients. The review explores current preclinical and clinical efficacy in joint disease in the context of the processing type and study design, as well as the regulatory aspects that need to be considered when administering cell-based therapies. Garbin and Morris focus their review on ACS and autologous protein solution hemoderivatives that produce high concentrations of Interleukin-1 receptor antagonist (IL-1Ra) and other cytokines and growth factors that can modulate OA effects and progression. The review compares and contrasts them with each other focusing on the clinical and modulatory effects as well as the limitations of use in equine OA patients.

Knowledge of the interplay of other articular tissues besides articular cartilage and synovium on the onset and progression of OA continues to expand with the rapid and frequent advancement of imaging technology such as computed tomography (CT) and magnetic resonance imaging (MRI). For instance, changes to intra-articular fat pads have increasingly been highlighted in MRIs of OA patients. Labusca and Zugun-Eloae discuss the role of intra-articular adipose tissue, such as Hoffa's fat pad in the knee, in the homeostasis of synovial joints and the pathogenesis of joint pathologies such as OA. They state that even though the structure is similar to subcutaneous adipose tissue, the molecular regulation of intra-articular adipose tissue is different, and they propose that articular fat pads are an active component of the joint with multiple functions and important roles in the maintenance of tissue homeostasis. Subchondral bone changes have also been highlighted in multiple species with OA by advanced imaging. Stewart and Kawcak review the current understanding of the role of subchondral bone in OA. They explore the importance of studying the osteochondral unit and the relationship between subchondral bone and OA across veterinary species, but their focus is on equine, arguably one of the most suitable biomechanical models for the study of OA. They detail recent progress in advanced imaging for the diagnosis of early disease and provide thoughts on treatment and prevention.

ORIGINAL RESEARCH ARTICLES

In vitro Studies

Byron and Trahan evaluated a novel *in vitro* co-culture system for investigating new and existing OA treatments. This co-culture system was comprised of equine osteochondral and synovial explants and they compared it to traditional equine articular cartilage explants. Both systems were stimulated with Interleukin-1beta (IL-1beta) and their results suggest similar outcomes with some important differences between culture systems in their response to inflammatory stimuli. In particular, the co-culture system was able to significantly dampen the increase in the degradative enzyme matrix metalloproteinase-13 compared to the cartilage explant. Since the synovium is a key contributor to a joint environment, these findings suggest that the co-culture system may be more relevant to *in vivo* physiology than traditional *in vitro* articular cartilage explants alone.

Concentrations of PRP are subject to variability from the patient itself as well as the processing method. Therefore, Gilbertie et al. set out to examine the effect of a pooled allogenic platelet-rich plasma lysate (PRP-L) as an alternative therapy that might decrease variability since preparations of PRP-L are acellular, contain high concentrations of growth factors and cytokines, and can be stored for immediate use. The effects of PRP-L were evaluated on equine synoviocytes and chondrocytes challenged with pro-inflammatory mediators *in vitro*, in a model that mimics the inflammatory micro-environment in the OA joint. The results showed a protective effect of PRP-L mostly through an increase in anti-inflammatory cytokines rather than a reduction of pro-inflammatory mediators, demonstrating a need for further studies on the use of pooled PRP-L for the treatment of OA.

Models of OA (Equine Naturally-Occurring and/or Post-traumatic)

In synoviocytes and chondrocytes galectins are potent regulators of cell adhesion, growth, and apoptosis, however the role of galectins-1 and-3 are unknown in OA. Therefore, Reesink et al. set out to investigate these galectins in naturally-occurring OA as well as in a post-traumatic osteochondral injury model in horses. They demonstrated that both are present in healthy synovial fluid, and that concentrations increase after osteochondral fragmentation. Furthermore, galectin-3 staining was found around healthy superficial chondrocytes whereas galectin-1 staining was limited to chondrons and injured cartilage. Their work demonstrates a possible role in OA, but further research is needed.

Macrophage phenotypes in synovium from healthy and OA joints are poorly characterized. Menarim et al. set out to compare the patterns of activation of M1-like and M2-like macrophage phenotypes in healthy and naturally-occurring OA equine joints with comparison to histology and cytokine/chemokine profiles in synovial fluid. Their results demonstrated that all macrophage markers were proportionate to the degree of synovial inflammation, with minimal difference between OA and normal joints. They emphasized that equine synovial macrophages are in a hybrid state of activation that display a regulatory response that targets the resolution of inflammation, but point out that further study is needed to see if this homeostatic response can be maximized.

Response to Therapeutics

While intra-articular administration of corticosteroids, hyaluronan, or a combination of both have been commonly used in equines and humans, they are not used commonly in

canines. Alves et al. sought to determine if a single dose of Triamcinolone Acetonide, hyaluronan, or a combination of both would be safe and effective at decreasing pain in working police dogs with naturally-occurring hip OA. The results demonstrated that all 3 treatment arms were safe and effective, but the combination was the only one that demonstrated significant improvement on multiple visual pain scales. Future studies are needed with larger sample sizes, different doses, and administration frequency.

Cannabidiol (CBD) is a non-competitive antagonist of cannabinoid receptors with potential immunomodulatory, anti-hyperalgesic, anti-nociceptive, and anti-inflammatory actions. Gamble et al. carried out a clinical study to determine the basic pharmacokinetics, safety, and analgesic efficacy of a CBD-based oil in dogs with radiographic OA. Their study suggests that CBD is bioavailable, with no observed side effects. They noted that 2 mg/kg of CBD twice daily was well-tolerated and appeared to increase physical activity in dogs with OA. The long-term effects of CBD still need to be determined.

We have enjoyed editing this Research Topic for Frontiers in Veterinary Science and sincerely hope that readers will enjoy reading these significant contributions that remind us of the crucial importance of interdisciplinary collaboration between those working on OA in human medicine and their counterparts in veterinary medicine. Future progress will be significantly enhanced if these communities communicated and collaborated more effectively.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Comparison of the Effects of Interleukin-1 on Equine Articular Cartilage Explants and Cocultures of Osteochondral and Synovial Explants

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Osteoarthritis (OA) is a ubiquitous disease affecting many horses. The disease causes chronic pain and decreased performance for patients and great cost to owners for diagnosis and treatment. The most common treatments include systemic non-steroidal anti-inflammatory drugs and intra-articular injection of corticosteroids. There is excellent support for the palliative pain relief these treatments provide; however, they do not arrest progression and may in some instances hasten advancement of disease. Orthobiologic treatments have been investigated as potential OA treatments that may not only ameliorate pain but also prevent or reverse pathologic articular tissue changes. Clinical protocols for intra-articular use of such treatments have not been optimized; the high cost of *in vivo* research and concerns over humane use of research animals may be preventing discovery. The objective of this study was to evaluate a novel *in vitro* articular coculture system for future use in OA treatment research. Concentrations and fold increases in various markers of inflammation (prostaglandin E_2 and tumor necrosis factor-alpha), degradative enzyme activity [matrix metalloproteinase-13 (MMP-13)], cartilage and bone metabolism (bone alkaline phosphatase and dimethyl-methylene blue), and cell death (lactate dehydrogenase) were compared between IL-1-stimulated equine articular cartilage explant cultures and cocultures comprised of osteochondral and synovial explants (OCS). Results suggested that there are differences in responses of culture systems to inflammatory stimulation. In particular, the IL-1-induced fold changes in MMP-13 concentration were significantly different between OCS and cartilage explant culture systems after 96 h. These differences may be relevant to responses of joints to inflammation *in vivo* and could be important to the biological relevance of *in vitro* research findings.

Keywords: articular coculture, prostaglandin E2, bone alkaline phosphatase, matrix metalloproteinase-13, tumor necrosis factor-alpha, osteoarthritis, horse

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INTRODUCTION

Osteoarthritis (OA)-related joint pain affects a large proportion of the horse population resulting in chronic pain, decreased mobility, decreased performance, reduced quality of life, and high owner expense (1, 2). Common remedies for OA have included systemic administration of non-steroidal anti-inflammatory drugs (NSAIDs) and intra-articular injection of corticosteroids. However, these

treatments are only palliative and do not modify the progression of OA. Furthermore, long-term NSAID use carries potentially serious side effects and corticosteroids may cause negative sequelae in articular cartilage (3). Therefore, orthobiologics (commonly termed regenerative therapies) have been used as potentially safer and more efficacious alternatives.

Orthobiologic techniques available for use in domestic animals include platelet-rich plasma, autologous conditioned serum [also known as IL-1 receptor antagonist protein (IL-1ra)], and autologous or allogeneic stem cells. Such treatments can improve function of equine joints (4–6). However, minimal beneficial effects may be found *in vitro* (7), and mechanisms of action remain unknown. In addition, clinical protocols for the use of orthobiologic treatments are currently not optimized. Therefore, there is a need for further research to refine clinical use of such therapies.

Although directly relevant to clinical application of treatments, use of live animal models is expensive, numbers of experimental subjects in studies may be insufficient to detect differences among groups (i.e., low statistical power), and there are welfare concerns over humane use of animals in research. The vast majority of rheumatology research in human and veterinary fields has been conducted with *in vitro* models including cells of only a single tissue type, cartilage. Cartilage damage has long been considered the hallmark of OA. However, molecular crosstalk between cartilage and subchondral bone cells is an important component of OA progression (8, 9). In addition, synoviocytes are important moderators of articular cartilage damage (10). *In vitro* models should account for this close relationship among articular tissues. There is a need for a physiologic *in vitro* model that can be used for the testing of potential OA treatments while reducing the use of live animals in research.

Coculture of articular tissues has been previously investigated, and results suggest that inclusion of multiple cell or tissue types changes molecular responses that may be more physiologic. Loss of glycosaminoglycans (GAGs) from cartilage, increase in expression of degradative enzymes, and decrease in expression of aggrecan in response to stimulation with IL-1 are partially abrogated by inclusion of synoviocytes in cartilage explant cultures (11). Coculture of bovine cartilage and subchondral bone improves chondrocyte survival compared with culture of cartilage alone (12). Coculture of canine articular cartilage and synovium seems to mimic responses of normal and osteoarthritic joints to stimuli (13, 14). Bovine chondrocyte expression patterns are altered when cartilage explants are cocultured with synovial explants (15). The cytokine profile of cocultured human cartilage and synovial explants obtained from patients with OA more closely represents the *in vivo* profile of osteoarthritic joints than monoculture of either tissue alone (16).

Despite the importance of cartilage, synovium, and subchondral bone in OA and data indicating inclusion of multiple articular tissue types in cultures results in more physiologic responses, coculture of cartilage, subchondral bone, and synovium has not been evaluated. The purpose of this study was to compare IL-1-induced expression of select metabolic markers in cultures containing cartilage explants alone versus cultures containing osteochondral and synovial explants (OCS). We hypothesized that changes in expression would differ between culture types. Results are expected to be useful in development of an *in vitro* culture model that more closely mimics *in vivo* articular responses to inflammatory stimulation than culture of single articular tissues alone.

MATERIALS AND METHODS

Samples

Articular tissue samples (synovium, osteochondral explants, and cartilage explants) were collected from femoropatellar joints of five horses without clinical or gross evidence of degenerative joint disease that died as a result of causes unrelated to this study. Tissues from horses with synovial effusion, history of lameness attributable to stifle joints, or with gross signs of degenerative joint disease (hyaline cartilage erosion, score lines, discoloration, or fibrillation) were not used (17). No experiments were performed on animals prior to euthanasia. Use of cadaver tissues was in accordance with an approved IACUC protocol (number 14-259).

Collection of Samples and Articular Tissue Culture

Immediately after death or euthanasia (*via* IV injection of an overdose of pentobarbital), samples of synovium, osteochondral explants, and cartilage explants were aseptically collected from femoropatellar joints of horses. Synovial tissue samples without fibrous joint capsule were collected with a biopsy punch (diameter, 6 mm; Integra Miltex, Plainsboro NJ, USA) from the dorsolateral aspect of the joint. Then, osteochondral explants (diameter, 7.9 mm; cartilage depth, approximately 2 mm; subchondral bone depth, approximately 4 mm) were collected from the axial aspect of the lateral trochlear ridge with a coring reamer (TEKTON Hollow Punch, Michigan Industrial Tools, Grand Rapids, MI, USA). Cartilage explants without subchondral bone (diameter, 7.9 mm) were also collected with a coring reamer (TEKTON Hollow Punch, Michigan Industrial Tools, Grand Rapids, MI, USA) from the axial aspect of the lateral trochlear ridge. Tissue samples were incubated for 1 h at 25°C in physiologic saline (0.9% NaCl) solution containing 1% penicillin and streptomycin (Thermo Fisher Scientific, Waltham, MA USA). Then, articular tissues were transferred to 12-well coculture plates (Transwell, Corning Life Sciences, Tewksbury MA, USA; well diameter, 12 mm) with polyester membranes (thickness, 10 μm; pore size, 3 μm). For each OCS coculture well, two synovial tissue samples were placed in the bottoms of plate wells and one osteochondral explant was suspended in well inserts. The ratio of synovium to osteochondral explants was determined on the basis of articular synovium and cartilage surface area ratios in mammals (18). For cartilage only cultures, one cartilage explant was placed in each well without other articular tissues. Articular tissue samples were incubated at 37°C with 95% relative humidity and 5% carbon dioxide in Dulbecco's Modified Eagle Medium containing 1% ascorbate-2-phosphate, 1% insulin–transferrin–selenium, 1% penicillin and streptomycin, and 50 μg/mL L-proline (2.8 mL of medium/well; Corning Life Sciences, Tewksbury MA, USA).

Each treatment group was cultured with duplicate samples. Tissues were allowed to equilibrate in culture for 48 h prior to initiation of treatments. Groups included cocultures (OCS) with and without IL-1 [10 ng/mL (11, 19); rhIL-1β, R&D Systems, Minneapolis, MN, USA] and cartilage explants with and without IL-1. Media were replenished and collected at 48 and 96 h. Samples were stored at −80°C until analysis. Sample storage times were 6–10 months and all samples were analyzed concurrently. Assays included prostaglandin E_2 (PGE_2), tumor necrosis factor-alpha (TNF-alpha), matrix metalloproteinase-13 (MMP-13), dimethyl-methylene blue (DMMB), bone alkaline phosphatase (BAP), and lactate dehydrogenase (LDH).

PGE_2 Assay

The concentration of PGE_2 in spent media was determined by use of a commercial colorimetric assay (R&D Systems, Minneapolis, MN, USA) following the directions of the manufacturers. Briefly, media (dilution, 1:25) were incubated in assay buffer containing primary anti-PGE_2 antibody for 1 h at 25°C. Then, 50 µL of horseradish peroxidase-conjuated PGE_2 solution were added to each well and incubated for 2 h at 25°C. Assay wells were washed four times, and 200 µL of a solution containing hydrogen peroxide and tetramethylbenzidine were added to each well. Plates were incubated for 30 min at 25°C. A stop solution (100 µL) of 2 N sulfuric acid was added to each well. Absorbance was measured at 450 nm (Molecular Devices SpectraMax M5, Sunnyvale, CA, USA) and PGE_2 concentrations determined by comparison to a standard curve with 4-parameter logistic regression.

TNF-Alpha Assay

The concentration of TNF-alpha in media was determined with a commercial assay (Thermo Scientific, Waltham, MA, USA) in accordance with the manufacturer's instructions. Briefly, plate wells were coated with anti-TNF-alpha antibody and 100 µL of media (dilution, 1:2) were added to each well. Plates were incubated for 1 h at 25°C and then washed three times. Anti-equine TNF-alpha detection antibody was added to each well (100 µL/well) and plates were incubated for 1 h at 25°C. Wells were washed three times and 100 µL of a Streptavidin-horseradish peroxidase solution were added to each well. Plates were incubated for 30 min at 25°C. Wells were washed three times, 100 µL of a substrate solution were added to each well, and plates were incubated for 20 min in the dark at 25°C. The reaction was stopped by the addition of 100 µL of a 0.16 M sulfuric acid to each well. Optical density was measured at 450 nm (Molecular Devices SpectraMax M5, Sunnyvale, CA, USA) and TNF-alpha concentrations were determined by comparison with a standard curve.

MMP-13 Assay

Stored media were assayed to detect MMP-13 with a commercially available kit (RayBiotech, Norcross, GA, USA) in accordance with the instructions of the manufacturer. Briefly, 100 µL of prepared standard and test media was incubated at 25°C for 2.5 h in assay wells coated with anti-MMP-13 antibody. Wells were washed four times with the supplied buffer and incubated at 25°C for 1 h with 100 µL of biotinylated anti-MMP-13 antibody. Wells were washed four times and incubated at 25°C for 45 min with 100 µL of Streptavidin solution. After washing four times, plated were incubated for 30 min at 25°C with 100 µL of 3,3,5,5′-tetramethylbenzidine solution and then the reaction was stopped by the addition of 0.2 M sulfuric acid. Optical density was measured immediately at 450 nm (Molecular Devices SpectraMax M5, Sunnyvale, CA, USA) and MMP-13 concentrations were determined *via* comparison with a standard curve and 4-parameter logistic regression.

DMMB Assay

Media were digested in papain (0.5 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) at 65°C for 4 h. The 1,9-dimethylmethylene blue assay (Sigma-Aldrich, St. Louis, MO, USA) was performed on digested media (dilution, 1:4) by use of the direct spectrophotometric method to measure the total GAG content in the spent media (20). Optical density was measured at 525 nm (Molecular Devices SpectraMax M5, Sunnyvale, CA, USA). Results were compared with a chondroitin sulfate standard curve to determine GAG concentrations.

BAP Assay

Media were assayed to determine BAP concentrations with a commercially available kit (Quidel, San Diego, CA, USA) in accordance with the manufacturer's instructions. Briefly, 125 µL of supplied assay buffer and 20 µL of sample media (dilution, 1:2) were added to plate wells precoated with anti-BAP antibody and incubated for 3 h at 25 C. Wells were washed four times and 150 µL of a 2-amino-2-methyl-1-propanol substrate solution were added to each well. Plates were incubated for 30 min at 25°C. The reaction was stopped by the addition of 100 µL of 0.5 N NaOH and optical density determined with a plate reader at 405 nm (Molecular Devices SpectraMax M5, Sunnyvale, CA, USA). Concentrations of BAP were determined *via* comparison with a standard curve generated with standard reagents supplied by the manufacturer.

LDH Assay

Concentrations of LDH in media were determined with a commercially available assay (Roche, Basel, Switzerland). Briefly, 100 µL of sample media was incubated with 100 µL of reaction mixture containing diaphorase/NAD+, iodotetrazolium chloride, and sodium lactate in 96-well plates in the dark at 25°C for 30 min. Formazan was quantified as a measure of LDH activity by measuring absorbance at 492 nm on an automated microplate reader (Molecular Devices SpectraMax M5, Sunnyvale, CA, USA). Concentrations of LDH were determined by 4-parameter logistic regression.

Data Analysis

Normality was assessed with probability plots. Concentrations of biomarkers were compared between positive and negative conditions (i.e., with and without IL-1β, respectively) within each combination of culture type group (OCS and cartilage) and time point (48 versus 96 h) using Friedman's chi-square with horse as a blocking factor (SAS/STAT, SAS Institute, Cary, NC, USA). A logarithmic (base e) transformation was applied to the fold

changes before any downstream analyses. Effects of culture type and time on the log fold changes were assessed using mixed model analysis of variance. Where appropriate *P*-values were adjusted for multiple comparisons using Bonferroni's procedure. The linear model specified culture group, time, and interaction between group and time as fixed effects. Denominator degrees of freedom for the fixed effects were approximated using the Kenward–Roger method. Horse identification was specified as the random effect. Within the specified interaction, the following comparisons were extracted: (1) time point 48 versus time point 96 for each group and (2) OCS versus cartilage at each time point. For all analysis of variance models, residuals were inspected to verify that the errors followed a normal distribution with constant variance. Values of $P < 0.05$ were considered significant.

RESULTS

PGE_2

Stimulation of OCS explant cultures with IL-1 resulted in a mean 8.4- and 1.6-fold increase in the media PGE2 concentration at 48 and 96 h, respectively (**Figure 1**). Stimulation of cartilage explant cultures with IL-1 resulted in a 2.6- and 3.0-fold increase in the PGE2 concentration at 48 and 96 h, respectively. The IL-1-stimulated OCS explant culture, PGE2 concentration was significantly ($P = 0.03$) higher than the concentration for unstimulated OCS explants at 48 h. The IL-1-stimulated cartilage explant culture PGE2 concentration was significantly ($P = 0.03$) higher than the concentration for unstimulated cartilage explants at 96 h. Differences between IL-1-stimulated and unstimulated culture PGE2 concentrations were not significantly different for cartilage at 48 h and OCS cultures at 96 h. Comparisons of fold changes in PGE2 concentrations between IL-1 stimulated and unstimulated explants were not significantly different between culture types at 48 and 96 h or between 48 and 96 h times for each culture type.

Tumor Necrosis Factor-Alpha

Stimulation of OCS explant cultures with IL-1 resulted in a mean 1.1-fold increase in the media TNF-alpha concentration at 48 h and a 1.3-fold decrease in TNF-alpha concentration at 96 h (**Figure 2**). Stimulation of cartilage explant cultures with IL-1 resulted in a 2.9- and 2.7-fold increase in the TNF-alpha concentration at 48 and 96 h, respectively. However, differences between IL-1-stimulated and unstimulated culture TNF-alpha concentrations were not significantly different for cartilage or OCS explant cultures at 48 or 96 h. At 48 h, the fold increase in TNF-alpha concentration between IL-1-stimulated and unstimulated cultures was significantly ($P = 0.04$) greater for cartilage versus OCS cultures. Comparisons of fold changes in TNF-alpha concentrations between IL-1 stimulated and unstimulated explants were not significantly different between culture types at 96 h or between 48 and 96 h times for each culture type.

Matrix Metalloproteinase-13

Stimulation of OCS explant cultures with IL-1 resulted in a mean 8.4- and 3.6-fold increase in the media MMP-13 concentration at 48 and 96 h, respectively (**Figure 3**). Stimulation of cartilage explant cultures with IL-1 resulted in a 74- and 26-fold increase in the MMP-13 concentration at 48 and 96 h, respectively. The IL-1-stimulated OCS explant culture MMP-13 concentration was significantly ($P = 0.03$) higher than the concentration for unstimulated OCS explants at 48 h but was not significantly different at 96 h. The IL-1-stimulated cartilage explant culture MMP-13 concentration was significantly ($P = 0.03$) higher than

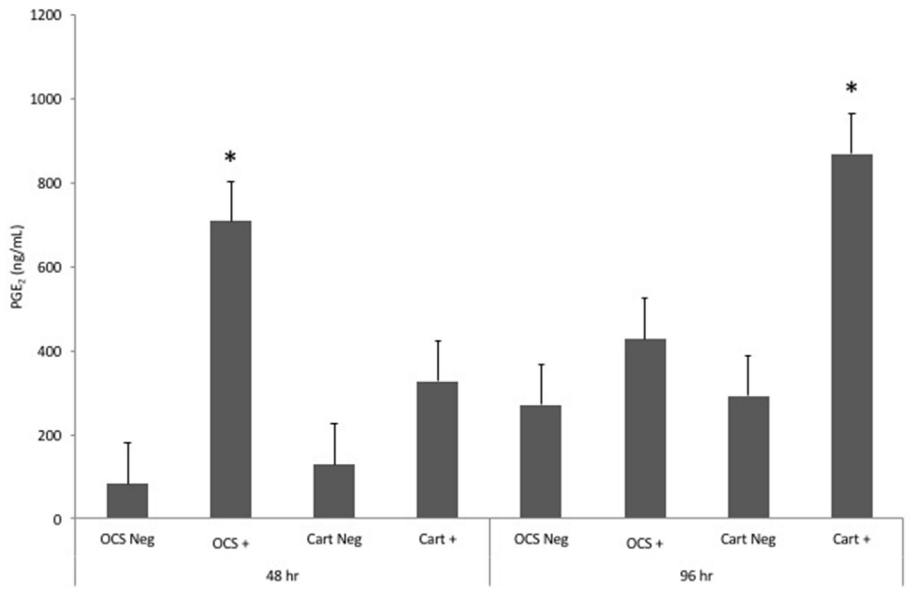

FIGURE 1 | Mean ± SE concentrations of PGE_2 in media samples of cultures containing osteochondral and synovial explants (OCS) or cartilage explants alone (Cart) that were unstimulated (Neg) or stimulated with IL-1 (10 ng/mL; +) at 48 and 96 h after initiation of treatments. *Within a culture type and time, concentration for IL-1-stimulated culture is significantly different from than that for the unstimulated culture.

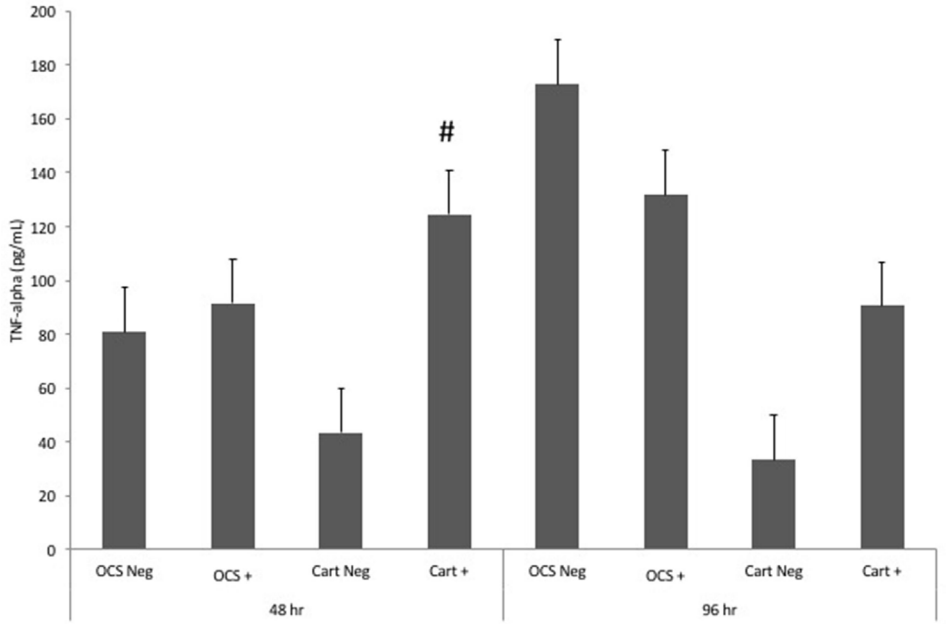

FIGURE 2 | Mean ± SE concentrations of tumor necrosis factor-alpha (TNF-alpha) in media samples of cultures containing osteochondral and synovial explants (OCS) or cartilage explants alone (Cart) that were unstimulated (Neg) or stimulated with IL-1 (10 ng/mL; +) at 48 and 96 h after initiation of treatments. #Within a time, the fold increase in TNF-alpha concentration between unstimulated and stimulated cultures is significantly different between culture types.

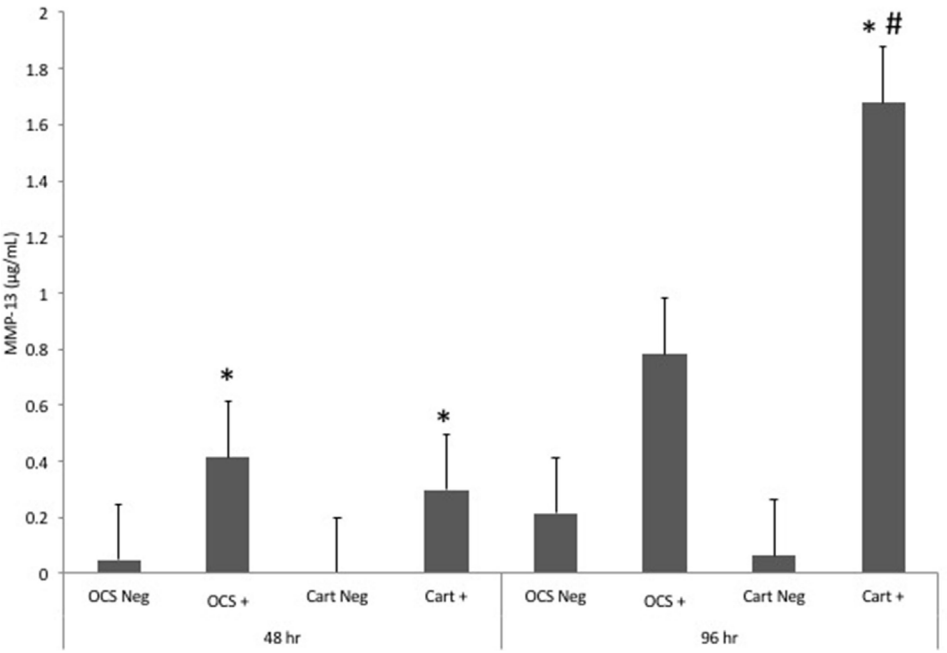

FIGURE 3 | Mean ± SE concentrations of matrix metalloproteinase-13 (MMP-13) in media samples of cultures containing osteochondral and synovial explants (OCS) or cartilage explants alone (Cart) that were unstimulated (Neg) or stimulated with IL-1 (10 ng/mL; +) at 48 and 96 h after initiation of treatments.

the concentration for unstimulated cartilage explants at 48 and 96 h. At 96 h, the fold increase in MMP-13 concentration between IL-1-stimulated and unstimulated cultures was significantly ($P = 0.02$) greater for cartilage versus OCS cultures. Comparisons of fold changes in MMP-13 concentrations between IL-1 stimulated and unstimulated explants were not significantly different between culture types at 48 h or between 48 and 96 h times for each culture type.

Dimethyl-Methylene Blue

Stimulation of OCS explant cultures with IL-1 resulted in a mean 1.7- and 1.3-fold increase in the media GAG concentration at 48 and 96 h, respectively (**Figure 4**). Stimulation of cartilage explant cultures with IL-1 resulted in a 2.1- and 2.3-fold increase in the GAG concentration at 48 and 96 h, respectively. The IL-1-stimulated cartilage explant culture GAG concentration was significantly ($P = 0.03$) higher than the concentration for unstimulated cartilage explants at 48 h but was not significantly different for cartilage explants at 96 h or for OCS explants at either 48 or 96 h times. Comparisons of fold changes in GAG concentrations between IL-1-stimulated and -unstimulated explants were not significantly different between culture types at 48 or 96 h times or between 48 and 96 h times for each culture type.

Bone Alkaline Phosphatase

Stimulation of OCS explant cultures with IL-1 resulted in a mean 5.6- and 3.2-fold decrease in the media BAP concentration at 48 and 96 h, respectively (**Figure 5**). Stimulation of cartilage explant cultures with IL-1 resulted in a 14.1- and 24.3-fold decrease in the BAP concentration at 48 and 96 h, respectively. The IL-1-stimulated cartilage explant culture BAP concentration was significantly lower than the concentration for unstimulated cartilage explants at 48 and 96 h ($P = 0.03$ and 0.04, respectively). The IL-1-stimulated OCS explant BAP concentration was significantly ($P = 0.03$) lower than the concentration for unstimulated cartilage explants at 48 h but was not significantly different at 96 h. Comparisons of fold changes in BAP concentrations between IL-1 stimulated and unstimulated explants were not significantly different between culture types at 48 and 96 h, although the values of P were nearly significant ($P = 0.055$ and 0.051, respectively). Comparisons of fold changes were not significant between 48 and 96 h times for each culture type.

Lactate Dehydrogenase

Concentrations of LDH were not significantly different between IL-1-stimulated and unstimulated explants for either culture type at 48 or 96 h (**Figure 6**). Likewise, no significant differences in fold change comparisons were found.

DISCUSSION

This study was conducted to compare responses of various cell and tissue metabolic markers to IL-1 stimulation in monoculture (cartilage explants only) and coculture (OCS) systems. These included markers of inflammation (PGE_2 and TNF-alpha), extracellular matrix degradation (MMP-13 and DMMB assays), bone metabolism (BAP), and cell viability (LDH). Results suggested that there are differences in responses of culture systems to inflammatory stimulation. In particular, the IL-1-induced fold changes in MMP-13 concentration were significantly and substantially different between OCS and cartilage explant culture systems. These differences may be relevant to responses of joints to inflammation *in vivo* and could be important to the biological relevance of *in vitro* research findings.

In response to IL-1 stimulation, both OCS and cartilage explant cultures had an increase in PGE_2 concentration. The increase was greatest and statistically significant at 48 h for OCS cultures and

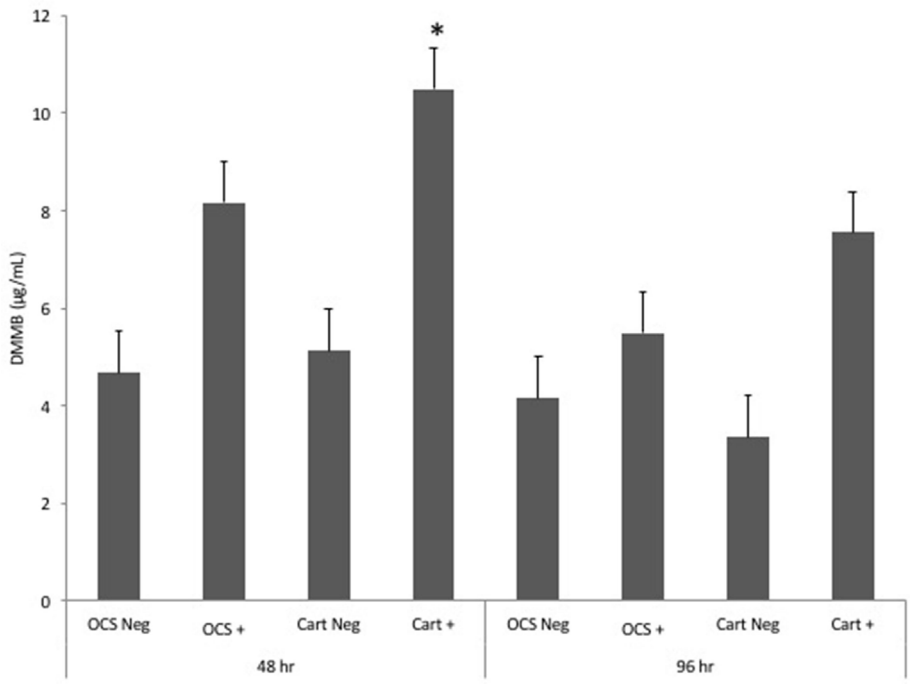

FIGURE 4 | Mean ± SE concentrations of dimethyl-methylene blue (DMMB) in media samples of cultures containing osteochondral and synovial explants (OCS) or cartilage explants alone (Cart) that were unstimulated (Neg) or stimulated with IL-1 (10 ng/mL; +) at 48 and 96 h after initiation of treatments.

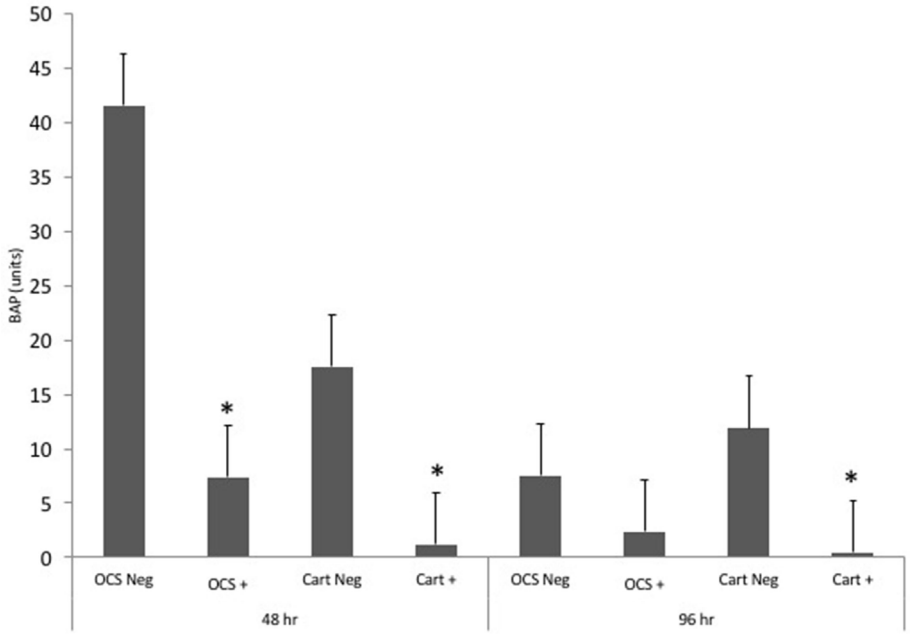

FIGURE 5 | Mean ± SE concentrations of bone alkaline phosphatase (BAP) in media samples of cultures containing osteochondral and synovial explants (OCS) or cartilage explants alone (Cart) that were unstimulated (Neg) or stimulated with IL-1 (10 ng/mL; +) at 48 and 96 h after initiation of treatments.

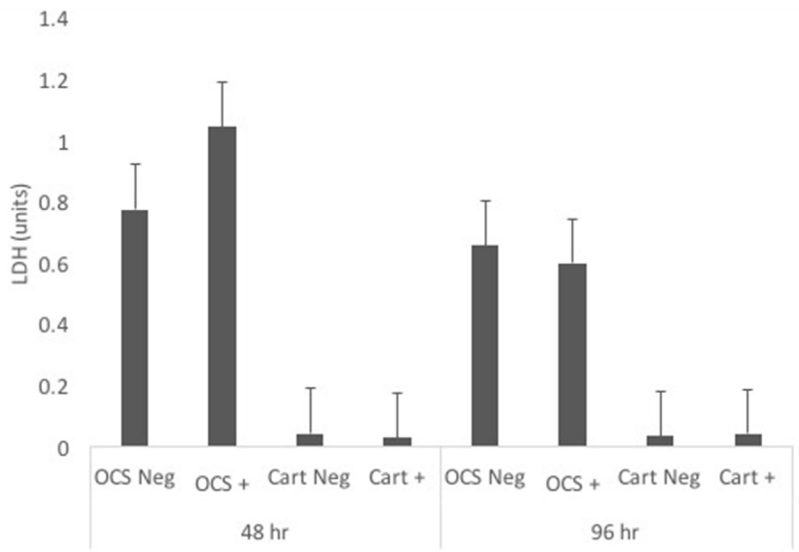

FIGURE 6 | Mean ± SE concentrations of lactate dehydrogenase (LDH) in media samples of cultures containing osteochondral and synovial explants (OCS) or cartilage explants alone (Cart) that were unstimulated (Neg) or stimulated with IL-1 (10 ng/mL; +) at 48 and 96 h after initiation of treatments.

at 96 h for cartilage explant cultures. This finding may indicate temporal differences in PGE_2 responses for these culture systems. However, the magnitude of the increase in PGE_2 concentration was not significantly different between culture types at 48 or 96 h. Also, the magnitude of the increase in PGE_2 concentration was similar at each time point for OCS and cartilage cultures. These findings suggest that, while there may be temporal differences in PGE_2 expression between cartilage explant monocultures and articular tissue cocultures, the responses are overall similar. To our knowledge, no other studies have compared the PGE_2 expression responses between cartilage explant cultures and articular tissue cocultures. Although an explanation for temporal differences in PGE_2 expression between culture types is not known, we believe it is due to enhanced expression of anti-inflammatory cytokines in OCS cultures. Other investigators have shown that synovial tissue produces IL-1ra, but cartilage explants do not (16). This

would lead to reduction in IL-1 response in cocultures over time, which is consistent with our finding of lower PGE_2 expression at 96 h in the OCS group.

Stimulation of cartilage explants with IL-1 resulted in a significant increase in TNF-alpha expression at 48 h, whereas stimulation of OCS explants did not result in a significant change in expression at either time point evaluated. Although the response of both culture systems was modest, there was a significant difference in the magnitude of the IL-1-induced increase in TNF-alpha expression between cartilage and OCS explant cultures at 48 h. The modest increase in expression of TNF-alpha in these culture systems is not unexpected. Human cartilage and synovial tissue obtained from osteoarthritic joints have low expression of TNF-alpha when culture alone or together in a coculture system (16). Other authors found that synovial fluid concentrations of TNF-alpha do not increase in joints with various types of damage (21) or in carpal joints with pathologic changes related to OA (22). However, findings of another study (23) indicate TNF-alpha concentrations increase in joints with osteochondrosis dissecans or acute trauma. On the basis of these results, it seems that the TNF-alpha response to inflammation and joint damage is variable. Our results indicated a mild decrease in TNF-alpha for OCS cultures at 96 h; this result was not significant and the difference is likely attributable to variability in response among horses and modest protein expression. The differences in findings may be attributable to characteristics of inflammation and trauma or to the articular tissues (cartilage, synovium, or subchondral bone) involved. Further research is warranted to determine the contributions of each tissue type to articular expression of TNF-alpha.

Of the biomarkers evaluated in this study, the response of MMP-13 expression to IL-1 stimulation was the greatest in both types of cultures. Both cartilage and OCS explant cultures substantially increased MMP-13 expression in response to IL-1. In particular, cartilage explant cultures exposed to IL-1 had very high expression of MMP-13 protein. The magnitude of the MMP-13 response to IL-1 was significantly greater for cartilage explants compared with OCS explants at 96 h. This finding indicates a substantial difference between these culture systems in the inflammation-induced expression of MMP-13. The inclusion of synovium and subchondral bone in culture seemed to partially abrogate the increase in MMP-13. Although we did not determine the individual contributions of synovium and subchondral bone to this result, this difference in response seems to be biologically relevant. Other authors (15) found that coculture of cartilage with synovial tissue alters expression of MMP-13. In another study (11), responses of cartilage explants were compared with those of cartilage and synovium cocultures; results indicated no significant differences between these groups in expression of MMP-13 mRNA after 96 h of exposure to IL-1. In contrast to our results, other authors reported that general matrix metalloproteinase activity is enhanced by coculture of synovium with cartilage explants (16). Inclusion of subchondral bone in the OCS group of our study may have downregulated MMP-13. This difference in results between the present study and that other study suggest that the tissue composition of *in vitro* culture systems can have a large effect on expression of MMP-13. Unfortunately, the design of our study does not allow differentiation of the effects of each individual tissue type. In light of this, further investigation seems warranted to determine similarities between of *in vitro* coculture systems and *in vivo* responses of joints.

Loss of extracellular matrix GAG into culture media indirectly indicates activities of certain degradative enzymes. Results of other studies indicate the effects of coculture on loss of cartilage GAG are variable. Coculture of equine cartilage and synovium protects against IL-1-induced loss of GAG from cartilage explants (11). However, coculture of human synovium with cartilage obtained from osteoarthritic joints results in a decrease in GAG production compared with monocultures of cartilage alone (16). Coculture of cartilage and synovium did not have a significant effect on release of GAG into culture media in either of those studies. Likewise, results of the present study did not indicate a significant effect of synovial and subchondral bone coculture with cartilage on IL-1-induced release of GAG into media. These findings suggest that coculture of osteoarthritic cartilage with other articular tissues has an effect on extracellular matrix GAG content, which is primarily attributable to changes in GAG production, but the effects on cultures in acute inflammatory conditions are variable.

Bone alkaline phosphatase has been used as a biomarker of bone turnover in humans and horses (24, 25). Exposure of rabbit chondrocytes to IL-1 dramatically decreases production of BAP (26). Interleukin-1 decreases bone formation in adult rats (27). The BAP expression of human osteoblasts decreases after exposure to IL-1 (28). Other authors found that IL-1 increases BAP expression (29). Although results of the present study did not indicate significant differences between culture types with regard to IL-1-induced changes in BAP expression, these results were very nearly significant. This suggests that inclusion of multiple articular tissue types in culture may have an effect on BAP expression, as would be expected considering molecular crosstalk between bone and cartilage is an important component of OA (30). The decrease in BAP expression after IL-1 exposure in this study was somewhat unexpected, considering synovial fluid levels in horses increase after joint injury. Other authors found that synovial fluid concentrations of BAP are higher in equine carpal joints with osteochondral injury than in normal carpal joints (24); however, metacarpophalangeal joints with and without injury did not significantly differ in that study. Results of another study of racehorses differed (31); BAP concentrations in fetlock joints of Thoroughbred racehorses with injury were significantly higher than in uninjured joints. Likewise, other authors have found signficantly higher BAP concentrations in carpal and fetlock joints of horses with cartilage damage compared with contralateral joints (32). We used articular tissues obtained from femoropatellar joints of horses. There are differences in BAP expression among joints (24). Prior studies evaluating equine articular BAP concentrations have primarily evaluated distal joints. Expression of BAP in the femoropatellar joint may differ from other joints because of differences in anatomic location and biomechanical forces (primarily shear rather than compression).

No significant differences were detected in LDH concentrations between untimulated and IL-1 stimulated cultures or in

fold changes between culture types at 48 or 96 h. This finding indicates minimal cytotoxicity in cartilage explant and OCS cocultures. These results were similar to results of another study in which human OA cartilage was cultured with or without synovium (16); minimal cytotoxicity in cultures up to 21 days was detected *via* LDH release in that study. In another study, coculture of bovine synovial fibroblasts with chondrocytes protected against cell membrane damage secondary reactive oxygen species exposure (33).

Both subchondral bone (9) and synovial (10) cells are important in the progression of OA. In addition, molecular crosstalk between cartilage and subchondral bone is an important contributor to the pathogenesis of OA (30). Accordingly, the coculture system investigated in this study was intended to account for physiologic responses of all major articular tissues. In contrast, traditional *in vitro* models of joint disease only include chondrocytes or cartilage explants; results of such studies may not be directly applicable to joints in living animals. Other authors have investigated use of engineered articular cocultures comprised of osteogenic and chondrogenic mesenchymal stem cells (34) or chondrocytes and macrophages (35) in scaffolds to mimic *in vivo* responses. While these approaches may account for interactions among articular cells, they require additional processing of tissues and do not replicate native interactions between cells and the extracellular matrix.

This study had several limitations. The low ($n = 5$) number of horses included may have precluded detection of small differences among groups. In addition, horses of various ages and breeds were included, which may have contributed to high variability in responses among tissues from these animals. Responses of tissues to inflammatory stimulation was only investigated at 48 and 96 h times. There may be temporal differences in molecular responses that were not detected at these time points. Also, other investigators have maintained articular cocultures for substantially longer times (21 days) (16), which may be more relevant to long-term *in vivo* joint tissue responses. Another potential limitation is the use of IL-1 for induction of an inflammation to mimic an articular OA environment. Naturally occurring OA involves upregulation of multiple inflammatory cytokines. However, IL-1 known to be a major component of the inflammatory response in osteoarthritic joints of horses and is a well-established method for *in vitro* joint disease testing (36–38).

This study was conducted to compare responses of a novel *in vitro* articular coculture system with that of another *in vitro* model of joint physiology (cartilage explant monoculture). Results indicated overall similarity in outcomes. However, there were some notable differences that are likely attributable to molecular interplay between tissue types. Future OA research may benefit from the use of coculture systems, and findings may be more relevant to *in vivo* physiology. However, further research is needed to compare *in vitro* molecular responses with those of joints in horses. Validation of *in vitro* coculture systems would be valuable for testing of orthobiologic and other treatments prior to application in living animals with OA.

AUTHOR CONTRIBUTIONS

CB conceived of the study design, conducted experiments, analyzed data, and wrote and revised the manuscript. RT conducted experiments, analyzed data, and revised the manuscript.

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The Unexplored Role of Intra-articular Adipose Tissue in the Homeostasis and Pathology of Articular Joints

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Intra-articular adipose tissue deposits known as articular fat pads (AFPs) are described to exist within synovial joints. Their assumed role in normal joint biomechanics is increasingly objectivized by means of advanced methods of functional imaging. AFPs possess structural similarity with body subcutaneous white adipose tissue (WAT), however, seems to be regulated by independent metabolic loops. AFP dimension are conserved during extreme WAT states: obesity, metabolic syndrome, lipodystrophy, and cachexia. Hoffa fat pad (HFP) in the knee is increasingly recognized as a major player in pathological joint states such as anterior knee pain and osteoarthritis. HFP contains numerous population of mesenchymal and endothelial progenitors; however, the possible role of mature adipocytes in the maintenance of stem cell niche is unknown. We propose that AFP is an active component of the joint organ with multifunctional roles in the maintenance of joint homeostasis. Endowed with a rich network of sensitive nervous fibbers, AFPs may act as a proprioceptive organ. Adipokines and growth factors released by AFP-resident mature adipocytes could participate in the maintenance of progenitor stem cell niche as well as in local immune regulation. AFP metabolism may be locally controlled, correlated with but independent of WAT homeostasis. The identification of AFP role in normal joint turnover and its possible implication in pathological states could deliver diagnostic and therapeutic targets. Drug and/or cell therapies that restore AFP structure and function could become the next step in the design of disease modifying therapies for disabling joint conditions such as osteoarthritis and inflammatory arthritis.

Keywords: intra-articular fat pad, Hoffa fat pad, white adipose tissue, cellular therapies, osteoarthritis

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INTRODUCTION

White adipose tissue (WAT) is increasingly recognized as a multifunctional, metabolically active organ (1). The evolutionary conserved attribute of storing excess energy as lipid deposits is coupled with WAT role in controlling metabolic balance as a body-wide distributed endocrine organ (2). By accumulating nutrients deposits, mature WAT resident cells—the adipocytes—fulfill a basic life function necessary to provide energy during periods of high caloric demands. However, far from being mere inert warehouses, the adipocytes not only store triacylglycerol but secrete as well regulatory bioactive molecules such as adipokines (leptin, adiponectin, resistin, adipsin, and

visfatin), cytokines (IL-6 and TNF-α), and acylation-stimulating protein. Such bioactive molecules have local, peripheral, and central effects in controlling nutrient intake, energy storage, and expenditure (3). The multifactorial role WAT posses in coordinating and executing a diversity of organismal functions is reflected by its heterogeneous cellularity. WAT is composed not only of adipocytes but harbors a variety of blood cells, immune resident and endothelial elements, pericytes, and adipose precursor cells as well as mesenchymal progenitor cells. Various enzymatic or mechanical methods can be used to separate the so called stromal vascular fraction (SVF)—a mixture of mononuclear elements among which adipose derived mesenchymal stromal cells (ADSCs).

Mature adipocytes together with WAT resident macrophages are active players in local and systemic immune response by releasing pro-inflammatory cytokines and adipokines that orchestrate local and central pathways of the innate immune system (4). The endothelial cells, pericytes together with adipose precursor cells are responsive of the angiogenetic and expansive capabilities WAT displays (5). In the last decades, WAT has been recognized as a reservoir of ADSCs and sought as convenient, easy accessible, source for cellular therapies (6). Complex physiological roles of WAT in lipid and glucose metabolism, coagulation, appetite regulation, angiogenesis, body weight control, and reproduction have been well described and documented [for review, see Ref. (2)].

White adipose tissue is widely distributed in almost the entire body subcutaneous region, in organs and hollow viscera of the abdominal cavity, in mediastinum as well as in several muscle groups functioning as a thermal insulator and shock absorber. Its role in mechanical protection has been related to the lax extracellular matrix (ECM) structure and to the important capability to recover from mechanical deformation (7).

White adipose tissue structure and function varies with body distribution. Subcutaneous fat presents distinct cellular and secretory profile compared to visceral fat (8). Regions where fat deposits might serve a mere mechanical role—soles, palms, and periarticular deposits—seem to have a particular genetic profile as they are conserved in some forms of congenital generalized lipodystrophy (9).

However, despite WAT heterogeneity in structure and function, its responsiveness appears to be closely correlated by similar, if not the same, neuroendocrine and biochemical pathways. Shared biochemical profile during pathological states obesity and metabolic syndrome at one pole and lipodystrophy and cachexia at the opposite pole draws the picture of a body-wide organ functioning as an organismal network with possible site-specific adaptive particularities.

ARTICULAR FAT PAD (AFP) IS A MULTIFUNCTIONAL TISSUE WITHIN THE NORMAL JOINT ORGAN

An interesting and potentially important WAT location has been, surprisingly, largely neglected. AFPs have been mainly mentioned in the context of pathological joint states (such as knee pain and osteoarthritis—OA) (10) or as a source of progenitor and stem cells (11). Their potential roles in maintaining homeostasis in normal joints remains unexplored. It remains obscure if AFP function and metabolic profile is correlated with systemic WAT normal and pathological states or is regulated by potential separate mechanism connected or not to the biomechanical function within the joint.

This paper will introduce the hypothesis of AFP as an internal homeostatic joint regulator, in possible relation but distinctive from body WAT function and balance. We propose that AFP is a specialized tissue of the joint organ contributing to its homeostasis by releasing bioactive molecules implicated in cell and ECM growth, turnover, and repair. AFP biomechanical role and its function in joint homeostasis are intertwined and might be locally regulated and systemically coordinated. AFP might act by converting information about joint biomechanics (alignment, axis, and dynamics) into biochemical cues that contributes to regulating the homeostasis of all articular tissues.

THE ANATOMY—AFP, UBIQUITOUS PRESENCE IN SYNOVIAL AND NON-SYNOVIAL JOINTS

Commonly, intra and periarticular fat deposits are included within the category of joint adjacent supportive structures together with menisci and ligaments. Such structures, described to be heterogeneously present in some joints (hip, knee) are thought to contribute to joint stability and to function as a shock absorber (12).

Possibly the largest AFP in humans, the infrapatellar fat pad of the knee joint, known as Hoffa's fat pad (HFP) (13) is one of the three fat pads of the knee joint interposed between the capsular layer and the synovium, described as intra-articular (intracapsular) but extra synovial structures. HFP is delimited superiorly by the inferior pole of the patella, inferiorly by the tibial bone, intermeniscal ligament, meniscal horns, and infrapatellar bursa, anteriorly by the patellar tendon while posteriorly is bounded by the femoral condyles and the intercondylar notch. It occupies the entire anterior part of the joint in all knee positions (14). Macroscopically, HFP is composed of a fibrous scaffold filled with fat lobules containing as well a number of septae such as the infrapatellar plica (IPP) (also known as the ligamentum mucosum) (15). A clinically relevant classification of HFP variants based on the presence or the absence of IPP has been proposed (Class I, HFP constrained, IPP present; and, Class II, FP unconstrained, no IPP), suggesting class I HFP might function as an intra-articular ligament involved in joint stability (16). Similarly with WAT, HFP cellularity consist of mature adipocytes but include as well fibroblasts, macrophages, and leukocytes within a lax network of conjunctive tissue and a rich network of blood vessels. As a particularity, the presence of peptidergic C-fibers, nerve fibers suggests HFP role as a sensory organ. HFP has anatomical, histological, and imagistic characteristic that distinguish it from underlying synovial tissue (**Figures 1** and **2**) (17). HFP is sought to act as a deformable space filler that adapts to the changing articular contours during joint movement and to facilitate synovial joint distribution. HFP is seen as a contributor to anterior knee pain probably due to an impingement mechanism after

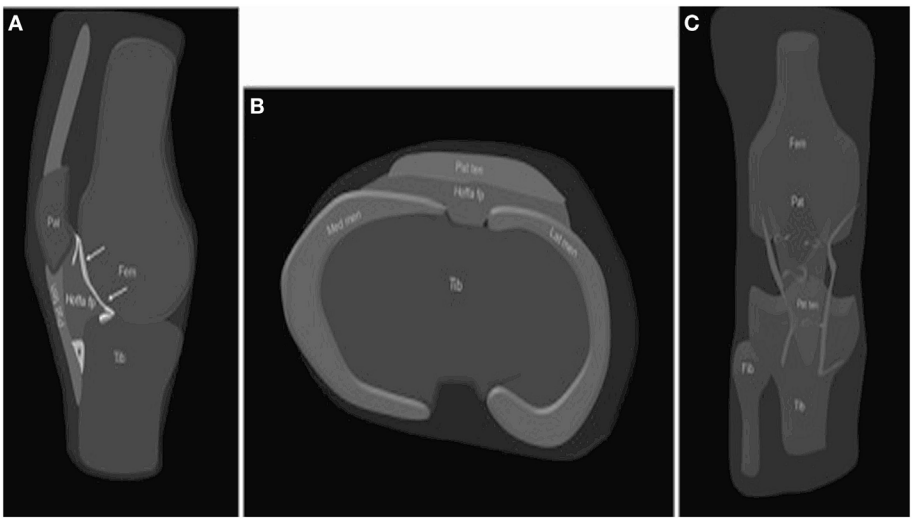

FIGURE 1 | Anatomy of the HFP. The HFP (*Hoffa fp*) is limited anteriorly by the patellar tendon (PT) (*Pat ten*) and the joint capsule, superiorly by the inferior pole of the patella (*Pat*) **(A)**, inferiorly by the proximal tibia (*Tib*) and the deep infrapatellar bursa (*asterisk*), and posteriorly by the synovium (*arrows*) and femur (*Fem*). It is attached directly to the anterior horns of the menisci (*Med men*, *Lat men*) **(B)**. Normal vascular supply consists of two vertical arteries, posterior and parallel to the lateral edges of the PT **(C)**. Courtesy of Draghi et al. from Insights into Imaging 2016 (15).

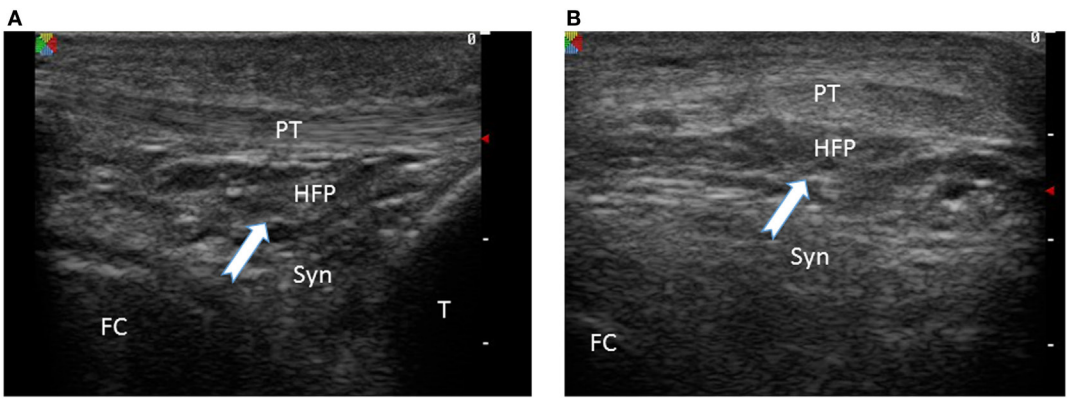

FIGURE 2 | Ultrasound image of infrapatellar fat pad of a normal knee joint White arrow indicates the fat tissue with distinct ultrasound features compared to the underlying patellar tendon (PT) and synovial tissue (Syn). HFP, Hoffa fat pad; FC, femoral condyle; T, tibia. **(A)** Sagittal view, **(B)** coronal view.

joint trauma (18) and as a source of inflammation and disease progression in knee OA (10). By far the most known, HFP is not, however, the sole AFP.

In the hip joint, a relatively recently identified structure placed at the anterior head–neck junction of the upper femur has AFP characteristics and is sought to be a source of femoroacetabular impingement (19). Fat pads opposite the olecranon, coronoid, and radial fosse as well as fibroadipose meniscoids in the non-articular waists of the trochlear notch and into the posterolateral aspect of the radiohumeral joint were described in 28 normal adult cadaveric elbows and proposed to be starting points in arthrofibrosis (20).

Articular fat pads were described to be present superior and inferior to lumbar facet joints from which fat-filled synovial folds project between the articular surfaces the superior being intracapsular between the ligamentum flavum and the lamina while the inferior remains extracapsular lying on the back of the lamina below and communicates with the joint through a hole in the inferior capsule. Lumbar facets AFPs can be identified by computer tomography scans. Lumbar facets AFPs enlargement is sought to be associated with degenerative changes and capsular laxity of the facet joints (21).

Intermetacarpal fat pads have been described to be adipose structures located between the heads of the second, third, fourth, and fifth metacarpal bones, filling the spaces between the palmar fascia and its deep expansions. They are proposed to act as protection from shear forces during gripping, to protect neurovascular finger bundles, and to contribute to neurological symptoms when inflamed or injured (22).

The development of modern imagistic such as ultrasound and arthroscopy made possible not only the identification of previously unknown AFPs location but enabled as well direct

visualization, dynamic assessment, and quantification of their biomechanical role within the joint.

THE PHYSIOLOGY—AFP—SIMPLE CUSHION OR MULTIFUNCTIONAL REGULATOR?

It is noteworthy to mention that historically, "synovial fat pads" were regarded as the sources of synovial fluid (SF). AFPs were denominated harversian glands and thought to produce substances that "oil" the joint surface. Currently, the name is kept only in regard with the acetabular floor AFP (known as harversian fat). The attributed physiological role in joint lubrication was only later replaced by an assumed mechanical filler-cushioning function.

The existence of physiological particularities of the intra-articular deposits distinguishing this tissue from widespread subcutaneous fat was suspected by anatomists as late as the middle of the twentieth century. Despite the structural similarity to body-wide WAT, AFPs persist even during advanced states of malnutrition, when all other deposits are depleted. This observation raised the hypothesis that such tissue might possess yet unexplored particular ultrastructural and secretory features of potential importance for joint function (23).

However, the study of fat pat deposits seem to have fallen into oblivion until late 1990s when sports physicians and physiotherapists become interested in its involvement in producing (especially knee) joint pain. Reports related almost, if not completely to HFP, are stressing out the involvement of its rich nervous network and abundant substance P fibers in producing anterior knee pain in various pathological states (18, 19, 24). Noteworthy, until not too long ago, AFP biomechanical role was simple assumption. Very few biomechanical studies were conducted to address directly the role of adipose structures within or adjacent to joints. Indirect evidence points toward inflammatory reactivity of HFP during patellar mal tracking correlated with trochlear morphology and patella alignment (25). Moreover, static imaging or cadaveric studies make it difficult to assess the dynamics of articular structures. With the development of high resolution ultrasound probes, fat pad kinesiology within the knee joint and the mechanism of impingement could be explored (26). Kager's fat pad located in Kager's triangle between the Achilles tendon, the superior cortex of the calcaneus, and flexor hallucis longus muscle and tendon has been reported to perform important biomechanical functions that are crucial for the maintenance of ankle posterior tendons as well as ankle joint. Using high-resolution dynamic ultrasound and electromyogram, Kager fat was shown to lubricate the subtendinous region, to reduce the pressure change within the Achilles tendon enthesis, and to remove debris from within the retro calcaneal bursa (27). Due to advances in functional imaging, the assumed role of AFP and its participation to the joint biomechanics is increasingly documented. Its importance in the normal functioning of several joints is increasingly acknowledged. Little is known, however, about the possible participation of AFP in joint mechanical balance. Could it be possible that similar to other musculoskeletal structures, AFP possess a proprioceptive role contributing to dynamic alignment of the structures around the joint? Further studies about the presence of such proprioceptive receptors within AFP could elucidate this question.

POSSIBLE LINKS BETWEEN ADIPOSE TISSUE METABOLISM AND JOINT FUNCTION

Recent years have broadened understanding about the role of local and systemic hormonal balance in maintaining joint health (28). With increasing understanding about the WAT function as an endocrine organ, a growing list of adipokines with pleiotropic local and systemic actions are investigated in relation to musculoskeletal tissue biology (29). Adipokines have been implicated in a bidirectional bone—energy metabolism interplay, in the central regulation of bone mass as well as in the fatty bone marrow metabolism (30). Leptin levels are correlated with WAT mass, functioning as a food intake and energy consumption regulator. Leptin has been demonstrated to play crucial roles in influencing prenatal development and postnatal growth as well as in modulating systemic immune response. In humans, leptin deficiency or resistance is implicated in the pathogeny of obesity, metabolic syndrome, diabetes, and infertility. Leptin is expressed not only by adipocytes but as well by osteoblasts and chondrocytes and contribute to regulating chondrocyte differentiation and matrix maturation during enchondral bone formation (31). Human and murine chondrocytes express leptin and adiponectin both *in vivo* and *in vitro* (32) in normal conditions. Cultured and native normal human chondrocytes express leptin receptor b, shown to modulate expression of Frizzled-1 and Frizzled-7 in a possible cross talk with canonical Wnt signaling pathway that could be implicated in cartilage homeostasis (33). Not surprisingly, HFP express leptin however to date, the majority of existent data result from investigating pathological joint states (see below). Mainly studied in relation to joint degenerative processes. Leptin is known to stimulate inflammatory cytokine production (such as interleukine β Ilβ), to induce expression of matrix degradative peptides (such as matrix metalloproteinases—MMPs) and to activate nitric oxide synthase. Leptin facilitates the activation of macrophages, neutrophils, dendritic cells, and natural killer cells contributing to establishing an inflammatory milieu within OA joints (34). Little is known about the mechanisms of leptin production by AFP and its levels in normal joints. SF leptin levels have been shown to fluctuate in correlation to body mass index (BMI) as well as with knee OA stages (35) however not correlated with plasma leptin levels (36) suggesting an independent regulatory mechanism within the joint. Moreover, existent basic science studies on normal osteoblast and chondrocyte development and metabolic homeostasis points toward an independent intra-articular regulatory mechanism of leptin levels that could have AFP as central point. Another well-studied adipokine, adiponectin, possess divergent roles in metabolism and musculoskeletal biology, being implicated in bone loss and inflammation-mediated matrix degradation (29). Human normal chondrocytes express

functional adiponectin receptors that under specific stimulation were shown to express pro-inflammatory cytokines and nitric oxide synthase type II (37). Adiponectin SF levels were found, however, to be lower in female subjects with OA compared to plasma levels while higher levels could be recorded in rheumatoid arthritis (RA) compared to OA joints. Adiponectin could have anti-inflammatory role in RA by counteracting the pro-inflammatory role of tumor necrosis factor alpha (TNF-α), mechanism not reproduced in OA patients. While the multifaceted role of adiponectin in RA progression still needs to be clarified, to date there is no evidence that intra-articular and serum levels are correlated or that the protein can cross the capsular barrier to enter the joint. Intra-articular adiponectin was found to be released by synovial tissue, HFP, and even by osteophytes (38). Here again, there is a scarcity of data collected from normal joints. If adiponectin intervene in joint homeostasis or its release is solely an adaptive mechanism triggered by the presence of inflammatory mediators, needs to be further elucidated. Other adipokines such as resistin and visfatin were shown to be present in SF or plasma of RA or/and OA patients mainly in correlation with increased pro-inflammatory cytokines levels TNFα or interleukin 6. Their role as possible mediators of destructive joint inflammation is under investigation (39).

Adipokines are involved in normal joint development and possibly participate to adult bone and cartilage homeostasis. As resulting from epidemiological studies, intra-articular levels in diseased joints do not correlate with plasma serum levels. Due to obvious ethical limitations, very few information exist to profile the adipokine levels in normal joints in subjects with various BMI values. It is yet unknown if intra-articular adipokines in normal and pathological joints are produced by local elements or originate in WAT with different location (such as subcutaneous fat). In the absence of relevant information, AFP contribution to the homeostasis of the normal joints remains unknown. Conversely, AFP involvement in pathological joint conditions is increasingly stressed out and suggests a local regulatory mechanism in close dialog with the well-known inflammatory milieu that characterizes such diseases. The interplay between AFP biomechanical and secretory function may serve as a turning point between joint dynamics, axis, and alignment and the control of local metabolism, under the influence but possible distinct from body nutritional status. Deciphering the cross talk between AFP as a bio mechanic sensory organ that responds by modulating joint organ turnover and controlling local inflammation could contribute to increased understanding of joint functioning.

AFP—A POSSIBLE ROLE IN MAINTAINING ARTICULAR STEM CELL NICHE

Tissue niches are known to control site-specific stem cell function, governing their transition from quiescence to proliferation and maturation. Mature adipocytes were shown to contribute to the maintenance of stem cell niche in various locations and to generate niches for other cell types. Mature adipocytes within bone marrow were shown to inhibit hematopoetic stem cell engraftment (40). Conversely, adipocytes were proved to upregulate the branching and development of mammary gland epithelium (41). Adipocyte precursors may promote muscle differentiation since interaction between muscle cells and adipogenic PDGFR alpha(+) mesenchymal progenitors has a considerable positive impact on muscle turnover (42). Mature adipocytes were shown to be necessary and sufficient for the activation of skin epithelial stem cells (43).

HFP was shown to represent a rich source of ADSCS or perivascular stem cells with superior chondrogenic potential compared to the subcutaneous fat pad (44). HFP-derived stem cells from diseased knee states maintain their chondrogenic potential *in vitro* suggesting a conserved cartilage progenitor pool might exist within the tissue (45).

The mechanism by which HFP contributes to controlling the decision of intra-articular stem cells of various origin to entering cell cycle and differentiation remains to be established. Local release of growth factors (GFs) that trigger stem cell activation or an indirect immune-mediated contribution could be involved. Indeed IL-10-producing type 1 regulatory T cells (Tregs) were shown to modulate the activity of mice MSCs in a mice model of RA (46). Further *in vivo* studies are needed to confirm the interplay between local Tregs and mesenchymal progenitor in normal joint states and disease as well as the possible influence of intra-articular mature adipocytes in maintaining this balance.

HOFFA FAT PAD—AN ACTIVE PLAYER IN KNEE PAIN AND OSTEOARTHRITIS

By far the most investigated AFP, HFP begins to develop in humans starting with the 11th gestational week from the mesenchymal tissue below the patella, between the cruciate and the patellar ligaments (38). Its structure is very similar with subcutaneous WAT, however, does not fluctuate quantitatively with caloric intake, persisting even in severe cases of malnutrition and do not increase with BMI in obese subjects. Noteworthy, impaired functionality of HFP mature adipose cells with decreased adipose-related markers PPARγ together with increased fibrosis and macrophage infiltration could be demonstrated in obese compared to lean patients during late-stage OA of the knee (47). HFP has been implicated in a direct manner in occurrence of persistent anterior knee pain during local trauma or impingement syndrome. Its involvement in the development and progression of joint degenerative diseases is proposed to be multifactorial. Sensory tissue innervation and the contribution to increasing immune cell amount and activity within the joint are doubled by the role of pro-inflammatory adipokines are proposed as mechanisms of knee joint degeneration.

HFP is a very sensitive structure due to the presence of peptidergic C-fibers, nerve fibers staining positive for substance P (48) that are implicated in the development of knee pain after repetitive trauma in athletes and in painful knee OA (49). Moreover, substance P-induced Hoffa pad vasodilatation and immune cell extravasation could be the mechanisms of fat pad edema documented in patients with Lyme arthritis (50). The disturbed balance between substance P fibbers and sympathetic nerve fibers releasing anti-inflammatory cytokines and endogenous

opioids was implicated in RA knee pain or in painful total knee arthroplasty. Besides their role in neuropathic sensitization, P fibers could have a direct pro-inflammatory effect that ignite and maintain OA development (51).

As it is the case with synovial tissue, HFP is the stage of immune cells invasion during OA and RA that triggers production of pro-inflammatory and pro fibrotic cytokines from local activated macrophages. After the initiation of joint degradation, cartilage breakdown molecules could activate monocytes and trigger innate immunity mechanisms (52). HFP-resident macrophages could produce various GFs, cytokines, and enzymes having as effect osteophyte formation, cartilage breakdown by MMPs activity, joint effusion by vasodilation, and perturbed subchondral bone metabolism.

The role of WAT produced adipokines in the initiation and aggravation of inflammatory processes at systemic level as well as within the joint is well established. Leptin, adiponectin, and resistin were reportedly found in SF of OA and RA patients at concentrations that differ from blood levels. Such pro-inflammatory mediators could be produced by HFP by an independent locally regulatory mechanism that is not correlated with body fat and nutritional status.

Pertaining to the largest joint in the body, HFP contribution to knee pain and pathological conditions is increasingly recognized. If, however, there is a connection between biomechanical joint misbalance and HFP function in contributing and sustaining joint inflammatory milieu, has not yet been established. Dynamic biomechanical studies could elucidate if potential HFP function as a proprioceptive sensor correlates with its secretory role and contributes to both joint organ maintenance. Its perturbed functional states could generate targets for complex joint re-balancing.

WAT AND ITS EXTREMES—IMPLICATION FOR AFP FUNCTIONING

Obesity and metabolic syndrome are currently recognized to generate systemic and peripheral pro- inflammatory status. Moreover, OA has been proposed to be a metabolic disease associated with the chronic low-grade inflammation that defines obesity and metabolic syndrome and with the impaired cartilage homeostasis in the context of lipid and glucose abnormalities (53). Far less is known about systemic and local WAT metabolism and pathways that are involved in involuntary adipose tissue loss—lipodystrophy and cachexia.

Lipodystrophy is defined as the acquired or genetically induced partial or complete loss of metabolically active WAT. Age or disease-associated cachexia are wasting syndromes associating severe fat and muscle loss (54). As a common denominator, all pathological systemic involuntary WAT loss cannot be reversed by nutrition. Many of the metabolic impairments associated with obesity and metabolic syndrome are shared between the two extremes. Insulin resistance, glucose intolerance, and systemic inflammation are common findings in both excess and waste of systemic WAT (55). The particularities of joint metabolism during extreme adipose tissue loss are less understood. Disabling decrease in joint and body mobility is generally attributed to severe muscle loss and disturbed energy metabolism. During RA, progressive stage of the disease is associated with progressive fat and muscle waste, reduced joint mobility correlated with increased levels of intra-articular and systemic pro-inflammatory cytokines. Despite a theoretically adequate diet, TNF-α and IL-1 β were found to increase resting energy expenditure (REE) and to alter body composition in RA patients (56). Cancer-associated systemic inflammation, indicated by the production of C reactive protein and fibrinogen, was associated with increased muscle catabolism, hypothalamic-driven anorexia, and increase in REE in cachectic patients (57). There are currently no available data to characterize the metabolic activity of AFP during extreme WAT pathological states. Description of an eventual independent AFP regulatory mechanism and/or its fluctuations in relation to systemic WAT has the potential to generate therapeutic targets for degenerative and inflammatory joint diseases such as OA and RA. Interestingly enough, both waste syndromes and diseases of excess WAT, regardless of their origin, are reported to benefit physical activity. Various regimens of exercise therapy are among the very few effective therapeutic interventions in cachexia, age-related lipodystrophy but as well in obesity, metabolic syndrome, and diabetes mellitus (54, 58). Recently, muscle mitochondrial activity and exercise-driven fibroblast growth factor 2 release were found to significantly reduce muscle mass and WAT loss in aging mice, linking muscle metabolism to both muscle and WAT maintenance during senescence (59). Moreover, diet and exercise are known to be efficient for the prevention and treatment of OA including in the non-weight-bearing joints (60).

Articular fat pad deposits remain quantitatively unmodified during extreme WAT states as a possible mechanism for preserving the joint homeostasis and hence enabling the body to remain mobile and to interact with the surroundings. In turn, preservation of mobility and the capability to engage in physical activities are mandatory for self-adjusting the equilibrium in multiple hierarchical systems inside and outside the body. AFP acting as a biomechanical sensor could adjust joint organ homeostasis by means of physical activity. Systemic WAT extremes are prevented and/or adjusted by mobility and so is the organism ability to interact within its ecosystem. Obviously, at the extremes, obesity and advanced wasting states overcome articular joint capability to self-adjustment by imposing severe external restrictions in mobility. Morbid obesity but as well lipodystrophy and cachexia mechanically restrict joint movements either by excessive body weight or by muscle wasting. Systemic or local inflammatory status and insulin resistance overcome the capability of AFP to maintain intra-articular homeostasis. Here, complex intervention that addresses both systemic conditions and intra-articular AFP impairment are needed.

Recently, intra-articular therapies using SVF and/or adipose tissue administration in OA joints are reporting favorable results in the management of knee OA (61, 62); however, their mechanism of action remains unknown. It is possible that such therapies act by recovering the HFP structural and functional balance that in turn contributes to restoring cellular turnover in several joint compartments and rehabilitate the metabolic and immune joint microenvironment. Intra-articular cell therapies could prove a

disease modifying procedure to stop degradative processes during OA and RA.

We propose that AFP is an active component of the joint organ with multifunctional roles in maintenance of joint homeostasis. AFP rich network of sensitive nervous fibbers could act as a sensory organ possible involved in proprioception having role in acquiring information about joint axis, stability, and dynamics. Endocrine and paracrine secretion of adipokines and GFs AFP mature adipocytes could participate to joint organ turnover being involved in the maintenance of progenitor stem cell niche, cell renewal, and differentiation as well as local immune regulation. Local immune residents such as macrophage and Tregs are involved in balancing cellular growth and respond to pathological stimuli by controlling joint organ inflammatory status. While correlating with body-wide WAT status, AFP could possess genetic particularities as well as an independent mechanism of local control. Genetic and metabolic profiling of AFT could possibly result in description of molecular particularities that define distinct disease phenotypes. A metabolic-based classification of OA could result in predicting therapeutic response to existent preventive and therapeutic methods (63).

Further studies are needed to assess the biomechanical and molecular particularities of AFP in normal and diseased joints during normal and extreme WAT conditions. Cellular components as well as sensory fibers and ECM should be the subject of comparative investigation in both normal and pathological joint states as well as during normal and WAT pathological states. Using omics technologies at the single-cell level, complete AFP genetic and epigenetic profiling could be performed potentially deriving targets for future therapies. *In vivo* monitoring of AFP function (biomechanics, endocrine and paracrine release, and immune modulation) in animal models could elucidate its role within normal joint organ functioning and discriminate the contribution to the occurrence and progression of diseases. Bioinformatics analysis and computational modeling could identify currently unknown pathways involved in AFP functioning, eventually identifying AFP as an internal homeostatic system that connects joint biomechanics with structural maintenance mechanisms, correlated with systemic WAT but independently regulated. Cell therapies that aim to restore AFP structure and function could become the next step in delivering disease modifying therapies for disabling joint conditions such as OA and RA. Intra-articular therapies using adipose tissue derivatives might act by triggering AFP secretory and/or biomechanical role in regenerating joints structure and function.

CONCLUSION

The presence and biomechanical importance AFP deposits are increasingly revealed due to the use of new and improved advanced dynamic imaging. Historically thought to possess a role in joint physiology by assumed production of joint lubricants, AFP metabolic role has been largely disregarded. WAT is increasingly recognized as an important endocrine organ with impact in body homeostasis. The similarity between WAT and articular fad pad regarding structure, cellularity, and composition invites to the reconsideration of its role in the maintenance of normal joint homeostasis. Methods that are designed to locally restore the functionality of the intra-articular adipose tissue could represent an effective modality to re balance joint homeostasis, improve joint function, and restore body mobility. This will derive important consequences for the treatment not solely for joint diseases but for extreme WAT misbalances—obesity, metabolic syndrome, age, and disease-associated wasting.

AUTHOR CONTRIBUTIONS

LL contributed to formulating and launching the hypothesis presented in this manuscript FZ-E contributed to gathering literature data and orienting manuscript writing.

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REVIEW
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Cell-Based Therapies for Joint Disease in Veterinary Medicine: What We Have Learned and What We Need to Know

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Biological cell-based therapies for the treatment of joint disease in veterinary patients include autologous-conditioned serum, platelet-rich plasma, and expanded or non-expanded mesenchymal stem cell products. This narrative review outlines the processing and known mechanism of action of these therapies and reviews current preclinical and clinical efficacy in joint disease in the context of the processing type and study design. The significance of variation for biological activity and consequently regulatory approval is also discussed. There is significant variation in study outcomes for canine and equine cell-based products derived from whole blood or stem cell sources such as adipose and bone marrow. Variation can be attributed to altering bio-composition due to factors including preparation technique and source. In addition, study design factors like selection of cases with early vs. late stage osteoarthritis (OA), or with intra-articular soft tissue injury, influence outcome variation. In this under-regulated field, variation raises concerns for product safety, consistency, and efficacy. Cell-based therapies used for OA meet the Food and Drug Administration's (FDA's) definition of a drug; however, researchers must consider their approach to veterinary cell-based research to meet future regulatory demands. This review explains the USA's FDA guidelines as an example pathway for cell-based therapies to demonstrate safety, effectiveness, and manufacturing consistency. An understanding of the variation in production consistency, effectiveness, and regulatory concerns is essential for practitioners and researchers to determine what products are indicated for the treatment of joint disease and tactics to improve the quality of future research.

Keywords: mesenchymal stem cells, osteoarthritis, platelet-rich plasma, autologous-conditioned serum, cell-based therapies, Food and Drug Administration regulation, autologous conditioned plasma

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INTRODUCTION

Regenerative medicine focuses on therapies that regrow, repair, or replace damaged cells or organs (1). Cell-based therapies derived from tissues such as blood, bone marrow, and adipose tissue are a cornerstone of regenerative medicine. These products contain enhanced quantities of biological response modifiers, which are normally produced in the body at low levels and include stem cells, anti-inflammatory cytokines, growth factors, or a combination (2–4). The potential to both relieve symptoms of disease and repair damaged tissue have led to investigation of cell-based therapy for a wide range of human and animal orthopedic disease. As of February 2018, the U.S. National Institutes of Health had 57 active or recruiting clinical trials investigating cell-based therapies for osteoarthritis (OA) alone (5).

Osteoarthritis is an irreversible, complex disease that involves all tissues of the joint in a cycle of inflammation and tissue degradation (6). OA affects over 80% of horses >15 years of age and up to 2/3 of Thoroughbred racehorses, making it one of the highest causes of wastage and loss of use in this population (7, 8). Treatment in these populations has traditionally been intra-articular corticosteroid therapy, supplemented with polysulfated glycosaminoglycans, glucosamine and chondroitin sulfate, or hyaluronic acid (9). However, traditional pharmacological therapies decrease symptoms as opposed to modifying or reversing the disease process. Although some pharmaceuticals have been classified as disease-modifying osteoarthritic drugs (DMOADs) on initial clinical and preclinical trials, subsequent meta-analysis has shown insufficient levels of disease-modifying effects in humans (10). As a result, optimism is high for cell-based therapies that alter the inflammatory cycle of the disease, regenerate damaged tissues or, ideally, both.

Veterinary medicine's commercial environment and the perceived benefits of cell-based therapies as delivering disease-modifying and reparative effects, as well as pharmaceutical restrictions in equine athletes (11–13), has led to widespread use of cell-based therapies in horses and dogs for OA **Table 1**. Commonly used cell-based products include autologous-conditioned serum (ACS), platelet-rich plasma (PRP), and expanded or non-expanded mesenchymal stem cell (MSC) products. Quality of published literature and practitioner understanding about safety, efficacy, and consistency of these products varies. Due to funding constraints, many studies have a low number of animals, a lack of control groups or have not progressed beyond pilot data. In addition, variability derived from factors including individual donor and processing method challenges our ability to draw meaningful conclusions (14–20).

Researchers, practitioners, and regulatory agencies are understanding that collective and regulated data collection will help to overcome challenges associated with product variability and study limitations (35). Guidelines set in the USA by the Food and Drug Administration (FDA) are an example of how government-led regulation could force the industry to prove product efficacy, manufacturing quality, and safety before commercialization. The guidelines are controversial given inevitability that following this process will slow, or even stall, the transition of cell-based products from experimental to clinical use. However, the impact on research quality and informed practitioner use will no-doubt drive forward development of OA cell-based therapies that do meet current optimism. Until that time, we can only assess the efficacy of biological therapies used in equine and canine OA in light of the disease environment, product variation, and legislative recommendations. Understanding clinical and experimental findings in light of these elements is essential for practitioners and researchers to determine what products could be indicated for treatment of joint disease and to highlight areas of future research.

CELL-BASED THERAPIES CURRENTLY USED IN DOGS AND HORSES FOR JOINT DISEASE

Cell-based therapies investigated in horses and dogs include blood-derived products such as ACS, autologous protein solution (APS), and PRP, as well as products containing MSCs such as

TABLE 1 | Regenerative medicine products used in the dog and horse for OA.

Category	Description	Examples of US based veterinary suppliers/products	Effects in OA
Autologous-conditioned serum	Autologous blood product that increases anti-inflammatory cytokines including interleukin-1 receptor antagonist	IRAP (Dechra/Orthokine); IRAP II (Arthrex); MediVet; Biologics; EC-ACS (Vetlinebio)	Improved lameness, synovial thickness, and cartilage fibrillation (21, 22)
Platelet-rich plasma (PRP)	Autologous blood product that contains growth factors including IGF-1 and PDGF	MediVet; VetStem; Osteokine (Dechra); Arthrex ACP; V-Pet (Pall Life Sciences); PRPKits.com; DrPRP USA; RegenKit-BCT (RegenLab); E-Pet (V-Care); V-PET (Nupsala)	Variable response to intra-articular injection in horses, some show reduction in lameness and joint effusion (23–25). In dogs has a pain-relieving effect that is slower onset but similar effect compared with corticosteroid injection (26, 27)
Autologous protein solution	Autologous blood product that contains both growth factors and ant-inflammatory cytokines via a 2 step process	Pro-Stride; N-Stride	Reduced clinical signs of pain and lameness in dogs at 12 weeks (28) and horses at 14 days and 12 weeks *via* client assessment (29)
Adipose-derived stromal vascular fraction	Digest of autologous adipose tissue that contains ~1–2% of CFU-fibroblasts	VetStem (Biopharma)	Subjectively less effective than cultured bone marrow-derived stem cells when compared with placebo for experimental OA in horses (30). Functional improvements in naturally occurring and induced canine OA, with some evidence of improvement when paired with PRP (31, 32)
Mesenchymal stem cells (MSCs)	Autologous or allogeneic plastic adherent cells that are commonly isolated from bone marrow or fat. Capable of differentiating into osteogenic, chondrogenic, or adipogenic cell lines	Variable—stem cell therapy may be offered by comparative orthopedic research laboratories	Bone marrow-derived MSCs showed no significant effects for naturally occurring OA; however, it can improve return to work of horses with intra-articular soft tissue injury (33). Canine studies using adipose-derived MSCs show improved functional outcomes, their effect may be complemented when PRP is used as a vehicle (34)

adipose-derived stromal fraction, bone marrow aspirate concentrate (BMAC), cultured adipose-derived stem cells [adipose-derived MSC (AdMSC)], and cultured bone marrow-derived stem cells (BMSCs). All cell-based products are multimodal, containing multiple and combinations of growth factors, cytokines, and cells. The combination of factors may trigger an anabolic, chemotactic, inflammatory, anti-inflammatory, or immune-mediated response. This review describes how the complex nature of the composition and biological effect of cell-based products is further influenced by product source, donor variation, preparation technique, storage, injection vehicle, and characteristics of the disease environment in our veterinary patients.

Inherent variation in cell-based products and variation in study size and quality in veterinary species leaves veterinary researchers and practitioners to piece together currently available species-specific evidence, or reference human literature, to make clinically relevant decisions. Due to a lack of robust clinical data, *in vitro* data that highlight mechanisms of action cannot be overlooked. In addition, there are few studies that directly compare different cell-based therapies for OA (30, 36–38), and foundational information such as optimum processing and storage methods, as well as safe and effective doses and dosing regimens, are inconclusive (21, 22, 39, 40). Therefore, critical analysis of available information as well as a thorough understanding of key therapeutic elements of cell-based therapies is essential for the practitioner and researcher alike.

MSC PRODUCTS

Stem cells are adult or embryonic in origin. Adult stem cells do not exhibit telomerase activity, a marker of stem cell self-renewal in embryonic stem cells, so undergo senescence in 30–40 population doublings. However, this gives them clinical advantages such as reduced tumorogenicity when used *in vivo* (41, 42). MSCs have regenerative, anti-inflammatory, immunomodulatory, and trophic functions (43). As a result of the multifaceted nature of stem cell function, they are being investigated in the treatment of a wide range of diseases, with promise to aid in cartilage regeneration as well as amelioration of inflammation during OA. The beneficial effects of MSCs for intra-articular soft tissue injury was first demonstrated by Murphy et al. (44), in a caprine menisectomy model of OA. There was regeneration of the medial meniscus, which subsequently ameliorated OA development. Since that time, MSCs have been used successfully for the treatment of intra-articular soft tissue injury in horses and dogs (45, 46), as well as for cartilage regeneration (47, 48). However, there have been more variable outcomes when used for primary OA (30, 49). As with other cell-based therapies, there are many sources of variation that need to be considered before treatment of patients with intra-articular MSCs, or before conducting or reviewing clinical or preclinical research. Such factors include stem cell source, collection, and propagation techniques, effects of shipping and transportation, as well as what vehicle and what needle size will be used for injection **Figure 1**.

Considerations for Collection, Propagation, and Administration of MSCs

Bone marrow and adipose tissue are the most common tissues harvested for MSCs in veterinary species. These tissues are either processed as a point-of-care product or shipped to a laboratory to process the tissue and expand MSCs in culture for 2–4 weeks

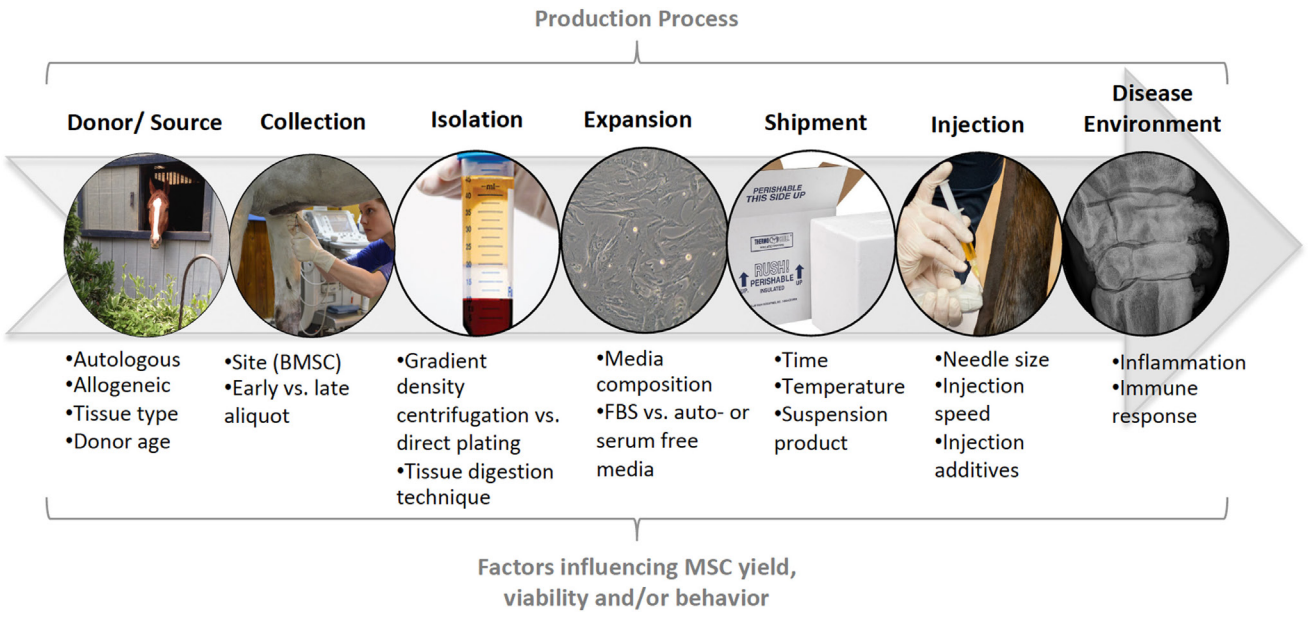

FIGURE 1 | Summary of the production process for mesenchymal stem cells (MSCs). At each stage in the production process (top), specific factors may influence the yield, viability, and/or behavior of MSCs in veterinary patients. These factors need to be considered during clinical use of MSCs and controlled for, or described, in preclinical and clinical research.

before shipment back to the end-user. Point-of-care products can be used within hours of tissue collection. BMAC is bone marrow aspirate that is collected with an anti-coagulant then gradient-density centrifugation removes red blood cells, granulocytes, immature myeloid precursors, and platelets. The resulting cells are mononuclear cells, comprising approximately 13% of the total nucleated cells in horses (50). Within this number, there are MSCs, which have been found to be approximately 0.001–0.01% of mononuclear cells (51). Adipose-derived stromal vascular fraction is a similar gradient-density centrifugation product derived from adipose tissue. Like BMAC, there are low numbers of CFU fibroblasts, the MSC fraction proposed to be active in the product, for example, canine point-of-care adipose preparations are reported to contain 1.72% of CFU fibroblasts (52, 53). The low fraction of MSCs are a point of controversy in these products, with some arguing that marketing products as a stem cell source is misleading. However, proponents of the products suggest that favorable clinical results may be related to paracrine or immunomodulatory effects rather than providing a direct MSC source. These arguments are supported by findings that freshly isolated cells from equine BMAC do not undergo trilineage differentiation (54), but do induce endogenous MSC proliferation, chemotaxis, and paracrine response (55). Differences between therapeutic activity of CFU-fibroblasts and other cytokines or growth factors in point-of-care products and expanded MSCs, as well as effective or bio-equivalent doses of each are currently unknown.

Mesenchymal stem cells are harvested from the end-user (autologous) or harvested from one animal and used in another of the same species (allogeneic). Veterinary medicine most commonly uses autologous MSCs because the perceived risk of immune rejection is lower. However, findings that allogeneic MSCs can decrease proliferation of T cells, alter the phenotype of macrophages, and cause reduction in inflammatory cytokines horses in a similar manner to autologous MSCs challenge this perception (56–59). Allogeneic MSCs are desirable because they can be used "off the shelf" as opposed to waiting for MSCs to be expanded from autologous tissue. For this reason, allogeneic adipose- and bone marrow-derived stem cells are available for horses and dogs in Australia (60), and recently Harman et al. (61) completed an FDA-registered clinical trial for the treatment of canine OA with allogeneic AdMSCs. However, several smaller *in vivo* studies in horses have found transient inflammation lasting 24–48 h after allogeneic MSCs have been injected into joints (36, 62–64). Similar transient inflammation may be elicited with autologous MSCs on the initial injection (58, 62). A limitation of studies that investigate single intra-articular doses of allogeneic MSCs in a small group of horses or dogs is that they may give a false representation of the safety of these products. *In vitro* studies show that certain inflammatory conditions cause equine MSCs to express MHC II, which induces immune detection in unmatched recipients (65, 66). Such alloantibody production is induced after intravenous, intraocular, and intraarterial injection of allogeneic BMSCs and AdMSCs in horses (67). It is unknown if the alloantibody response to intra-articular injection is similar; however, repeat intra-articular injection 4 weeks after treatment with the same allogeneic cell line increased synovial fluid cell counts, total protein, and lameness in horses for 24 h (64). These findings indicate that even if authors such as Harman et al. (61) conduct studies in a prospective, controlled and double-blinded manner, if measurements are taken after the acute period and are based on a single injection, safety of these products may be falsely represented. Specifically, the results cannot be extrapolated to repeat use of allogeneic cell lines and missing evaluation in the initial 24–48 h would fail to detect initial transient inflammation or pain.

Bone marrow-derived MSCs and AdMSCs are the most common culture-expanded MSCs used in veterinary patients, with BMSCs dominating equine and AdMSCs dominating canine veterinary medicine. In horses, BMSCs are harvested from the sternum or ileum with a 10- to 11-G Jamshidi needle (68–70). Although cardiac puncture is a potential danger with sternal collection, harvest from the 5th sternebra avoids iatrogenic trauma to the apex of the heart and the harvest site can be confirmed with ultrasound (69). Neither MSC viability, density, nor proliferation are different between bone marrow aspirates obtained from the sternum or ileum of young horses (2–5 years old) (71); however, in middle-aged horses (13 years old), sternal samples have a greater density of MSCs than ileal samples (72). Therefore, the sternum is most commonly chosen as the harvest site in middle-aged to older horses. In addition, the highest yield of cells occurs in the initial 5 mL collected, so collection of large volumes of bone marrow is not necessary (71). A higher yield of cells can be achieved by advancing the needle into the sternum 5 mm three times to harvest from four sites, rather than harvesting from one site (73). However, the benefit of this technique is negated after the first passage in culture, so the technique is most relevant for point-of-care preparations. AdMSCs are collected from adjacent to the tail head in horses (74) and harvest from the falciform ligament eases collection in dogs compared with harvest from peritoneal fat (75). The same sites for tissue collection are used for point-of-care systems.

Once collected, adipose or bone marrow tissue samples are shipped to a laboratory for expansion in culture. Laboratories vary slightly in methods of isolation and culture of MSCs; however, strict aseptic technique as well as quality control measures increase safety and consistency of the product (35). In addition, demonstrating Good Manufacturing Practice will be an essential part of ensuring quality and consistency of products seeking regulatory approval (35, 76, 77). In general, bone marrow-derived mononuclear cells are isolated from bone marrow aspirate *via* gradient-density centrifugation as described for BMAC, then plated onto adherent plastic where they undergo population doublings until there are a sufficient number of cells (68, 78, 79). An alternative approach is to transfer neat bone marrow into adherent plastic tissue culture flasks and culture with growth medium with the disadvantage of reducing the density of colony-forming units (54). Both techniques rely on the inherent property of MSCs to adhere to plastic (80). Adipose tissue is mechanically and enzymatically digested before centrifugation to separate the cellular fraction from the adipose fraction before expansion in culture. Comparison of equine AdMSC and BMSC cultures shows that AdMSCs are able to undergo more population doublings (81, 82), which has also been described in humans (83).

In veterinary species, MSCs are often expanded using cell culture media containing fetal bovine serum (FBS), which raises concerns for immunogenicity, consistency in bio-composition from batch to batch and downstream effects on MSC function. FBS proteins cause antibody production in humans, despite washing MSCs before implantation (84) and 89% of horses had antibody production after systemic injection of MSCs (67). Xenoproteins may cause adverse effects upon repeat injection, even if the MSCs are autologous. For example, equine BMSCs cultured in FBS caused an inflammatory reaction upon repeat intra-articular injection, whereas BMSCs cultured for 2 days in serum-free media did not (64). In an attempt to overcome antigenicity of xenoproteins, some laboratories culture MSCs in serum-free media, autologous platelet lysate, or autologous serum for at least 48 h before harvest. Of these options, serum-free media has the most consistent bio-composition, given the wide variation in growth factors and cytokines described for blood-derived products (refer to the next section). However, Clark et al. (85) found that serum-free media can cause alterations in the ability of equine BMSCs and canine AdMSCs to cause an immunomodulatory response, which may affect the therapeutic efficacy of serum-free cultured MSCs. Immunomodulatory properties of canine and equine MSCs cultured in platelet lysate or autologous serum have not been investigated; however, media containing platelet lysate or FBS caused similar proliferation and viability for equine umbilical cord, bone marrow, and adipose-derived MSCs if the additives did not exceed 30% of the culture media (86, 87). A caveat is that platelet lysate media has reduced ability to isolate cells and a tendency to induce adipogenesis after 4 days so short-term use is recommended (86). Despite current clinical use of media additives from platelet lysate, to autologous serum, to serum-free culture, the effects of media additives on MSC function are largely unknown for veterinary species. Given large variation in cytokines and growth factors from equine and canine blood-derived products, it is likely that additives have varying effects on MSCs with unknown effects on their eventual therapeutic efficacy (14–20).

Following culture, expanded MSCs are shipped from a laboratory to the end-user. However, the shipping time, temperature, and suspension product can influence cell viability (39, 88). Although there are not enough studies to draw a consensus about the best protocol for shipping equine and canine MSCs, time of transport limits MSC viability so administration should be within 12–24 h of cell harvest (39, 40, 88). While one study found cell mortality of 30–40% after 12 h (39), other studies have found 8–10% cell mortality at 24 h (40, 88, 89). Given that a 10% or less reduction in cell viability is found in studies with refrigeration at 4–8°C, this is the most commonly applied shipping condition. For short-term (12–24 h) shipment, no significant effects on cell viability have been found between suspension products (39, 88). However, some blood or bone marrow-derived suspension products rapidly increase cell mortality rates after 24 h (88). In addition, there are conflicting findings when shipping temperatures are directly compared (39, 40). Therefore, laboratories should conduct their own quality control tests to find the best shipping protocols and packaging for their products.

Addition of other intra-articular medications, as well as needle-size selection may influence MSC viability. Just under half (46%) of equine practitioners add adjunctive antimicrobials to intra-articular medications (9). However, addition of high levels of antibiotics such as aminoglycosides, enrofloxacin, and ceftiofur compromises MSC viability (90, 91). Although the antimicrobial concentrations tested were supraphysiological for systemically administered antimicrobials, both gentamicin and amikacin at doses used for intra-articular injection caused >95% equine BMSC death within 2 h *in vitro* (91). In addition, injection through small gauge needles reduces the viability and proliferative potential of equine MSCs (88, 92). In an effort to optimize cell viability, MSCs should not be injected concurrently with antibiotics, and implantation needles 20 G or greater should be used for intra-articular injection. It is unknown if canine MSCs require a different needle gauge for injection. However, injection of human MSCs through 25 G needles did not affect viability, suggesting either species differences or confounding factors such as injection speed or needle length, which may influence sheer stress on cells (93, 94). Some horse and dog clinical studies for OA suspended MSCs in PRP (34, 37, 95). However, suspension of cultured MSCs or point-of-care cell-based products with blood-derived products such as PRP, ACS, or autologous serum adds complexity and variation to joint therapies in veterinary medicine because of potential for products to interact and also because it is unclear what substance is having the primary therapeutic effect. Dahr et al. (96) found alterations of equine BMSC proliferation and differentiation when exposed to PRP, so addition of these substances could affect therapeutic activity of injected MSCs. Both *in vitro* and *in vivo* studies need to be performed to understand the biological effect of combining products before widespread use of specific combinations.

Stem Cells for Cartilage Resurfacing

The use of MSCs for cartilage resurfacing of equine osteochondral defects has been investigated; however, stem cells are often coupled with a scaffold that also contributes to cartilage healing (47, 97, 98). For example, AdMSCs in fibrin glue reduced joint inflammation and improved histological and functional quality of repair tissue, but these were compared with no treatment controls resulting in significant differences despite a small sample size (97). By contrast, when compared with autologous platelet-enriched fibrin scaffold alone, addition of BMSCs did not alter biomechanical properties of cartilage at 1 year. In fact, grafts with BMSCs had increased bone edema and some horses had ectopic bone formation (98). This example highlights the need for controlling for scaffold when performing cartilage-resurfacing studies, but also highlights potential adverse effects that may occur upon differentiation of MSCs. In comparison, BMAC that contains a low number of MSC-like cells and also contains other trophic factors has been used as an alternative to culture-expanded MSCs for cartilage resurfacing (50, 54). Likely trophic and chemotactic properties improved integration, collagen, and proteoglycan content of healed tissue at 8 months compared with microfracture alone (50). Using cell-based products as anabolic and trophic stimulators may replace microfracture and improve healing by allowing continued integrity

of the subchondral bone plate. Recently, Chu et al. (54) treated osteochondral defects with BMAC, finding a similar appearance to microfracture-treated defects on arthroscopy after 1 year, but there was subchondral bone fissure and void formation in the microfracture group. Resurfacing studies have also found that treatment effects occur early, or are delayed. For example, BMSC implantation in a fibrin gel glue improved histological cartilage defect healing, collagen type II, and proteoglycan content 30 days after surgery; however, there was no prolonged benefit at 8 months (47). In comparison, intra-articular scaffold-free BMSCs injected 30 days after creating an osteochondral defect and performing microfracture improved tissue repair, quality, and firmness at 6–12 m (48). The success of the second approach may be due to the trophic or anabolic effects of MSCs on already forming fibrocartilage.

Intra-Articular Stem Cell Injection for OA

Scaffold-free intra-articular injection of MSCs has been investigated in both naturally occurring and experimental equine OA; however, the number of published clinical trials is limited compared with current commercial use **Table 2**. Results have been variable; which may be an indicator of the degree of inflammatory environment that varies significantly between models or naturally occurring disease, follow-up time, MSC dose and source, as well as inter-observer differences in subjective outcome parameters. Experimental *in vivo* models of equine OA and synovitis include the carpal osteochondral fragment (COF) model and the LPS-induced synovitis model with characterized levels of inflammation, cartilage matrix degradation, and lameness (99–101). The COF model mimics post-traumatic OA by creating an osteochondral fragment arthroscopically followed by exercise (102). The pathological response produces low levels of inflammation, as to be expected with naturally occurring OA (102). By contrast, acute joint inflammation can be induced by intra-articular injection of a low dose of LPS (103), which causes a transient synovitis that lasts for up to 72 h and horses recover without lasting deleterious effects (100, 103). Investigation and treatment of animals with naturally occurring OA is also a source of treatment–response information. However, there is more variation in naturally occurring OA because duration and severity of disease varies, different joints are affected, and patient signalment varies compared with a group of pre-selected experimental animals.

Models with different inflammation severity are a problem for ascertaining the treatment efficacy of MSCs because the stem cell niche induces stem cells into an anti-inflammatory phenotype. Anti-inflammatory induction of MSCs has been termed "cytokine licensing" because IFNγ and also TNFα, IL-1β, and IL-17 induce MSCs into an anti-inflammatory state (104). MSCs enhance production of anti-inflammatory and immunomodulatory factors such as TSG-6, IL-6, and PGE_2 at higher levels of inflammation (105–110). This principle of MSC anti-inflammatory biology may explain variation between results of studies that use models with different severity of joint inflammation. The equine studies demonstrate that MSCs exposed to non-inflamed, healthy joints cause transient inflammation, evident as synovitis and increased total protein, total nucleated cell count, and inflammatory cytokines for 24–48 h (36, 58, 62, 63). By contrast, MSCs used in the most severe model for intra-articular inflammation, LPS-induced synovitis, reduced total nucleated cell count compared with LPS alone, essentially modulating the inflammatory response to LPS (63). MSC treatment has a variable effect in studies with variable or low intra-articular inflammation, as is the case with naturally occurring OA or the COF model. For example, BMSC treatment did not result in appreciable levels of reduced inflammation aside from reduction in PGE_2 in the COF model (30). In addition, the ability of horses to return to similar athletic activities varied upon treatment of 165 horses with naturally occurring OA, which likely paralleled the variation in disease stage and joint inflammation in the population (95). Studies that investigate MSCs for OA need to be cognizant of the effect of disease stage and inflammation on the anti-inflammatory effects of MSCs.

Studies that investigate MSCs for the treatment of naturally occurring OA or articular injury in horses have limitations that need consideration before concluding about treatment efficacy. Such limitations include lack of objective outcome measures, variation in joints treated and lack of control groups. Two equine studies report improved lameness results for primary OA; however, this was a delayed response with no degree of improvement reported in one study (49), and the other lacked control groups with highly variable results and study design that

TABLE 2 | Clinical trials using culture-expanded mesenchymal stem cells (MSCs) for OA in horses.

Disease	Stem cell type	Dose	Vehicle	Control	Results	Reference
OA—Tarso-metatarsal joint	Auto-adipose-derived MSC	5×10^6	Saline	Betamethasone No treatment	No change in lameness at 30 days but reduced at 60 days. 180 days improvement remained in MSC group but not betamethasone group. Decreased neutrophil count at 90 days in MSC and betamethasone compared with pre-injection	Nicpoń et al. (49)
OA—Stifle, fetlock, pastern, coffin joints	Allo-peripheral blood MSCs With or without chondrogenic induction	Not stated	Platelet-rich plasma	None	1.8% (of 165 horses) synovitis in first week, improved return to work at 18 weeks compared with 6 weeks, chondrogenic MSCs resulted in higher return to work in distal limb joints but not stifle joints	Broeckx et al. (95)
OA—due to meniscal, ligament, cartilage injury	Auto-BMSC + arthroscopy	15–20×10^6	Autologous serum/5% DMSO + HA	Results compared with previous literature	Unilateral affected horses 45% return to previous work, 23% return to work, 32% failure to return to work. In comparison to previous studies without MSCs, more meniscal injuries returned to work/previous level of work. 3/33 horses had acute joint inflammation after MSC injection	Ferris et al. (33)

included multiple different joints (95). The ability to control for specific joint analyzed is likely important given that Broeckx et al. (95) found that the 1.8% of horses that developed synovitis all had treatment of their metacarpophalangeal (fetlock) joints. Improved results have also been shown with intra-articular injection of BMSCs after stifle arthroscopy (33). However, positive outcomes may be due to the large number of concurrent intra-articular soft tissue injuries seen in the stifle, given that both equine BMSCs and AdMSCs cause healing of meniscal lesions with fibrocartilage *in vivo* (111). A cornerstone study by Murphy et al. (44) previously demonstrated that MSC-mediated meniscal and intra-articular soft tissue injury repair and subsequent return of joint stability can be a confounder in MSC studies that use instability models of OA. This can be extrapolated to the patient with naturally occurring injury. Therefore, clinical equine studies need to specify presence and degree of intra-articular soft tissue injury especially in the stifle joint. In addition, joints treated need to be defined as having or not having intra-articular soft tissue structures, for example, the palmar intercarpal ligaments in the middle carpal joints of horses and cruciate ligaments or menisci in the stifle. Overall, the functional outcomes for horses (lameness) with primary OA seem to be less consistent than those observed in other species, which may be due to the high standard of pain relief needed for horses to return to athletic use. Given the variation of efficacy found in the literature to date, further controlled studies are needed for cases of primary OA with subjective and objective functional outcome assessment to assess efficacy.

Cranial cruciate transection and/or menisectomy induce joint instability in experimental *in vivo* canine models of OA (112). As discussed, interpretation of the efficacy of MSCs is complicated in models that rely on instability to induce OA due to potential regeneration of these soft tissue structures. As a result, studies that use instability models will not be discussed. There are, however, a significant number of studies that show improved functional outcomes after treatment with AdMSCs for naturally occurring canine coxofemoral, cubital, and scapulohumeral joint OA (34, 38, 52, 53, 61, 113). The majority of clinical canine studies for primary OA are placebo-controlled, blinded, and randomized. Additionally, larger study sizes compared with equine help to account for variation in naturally occurring disease (38, 52, 53, 61). Improved functional outcome has been reported using adipose-derived stromal vascular fraction and cultured AdMSCs for the treatment of naturally occurring cubital and coxofemoral joint OA (31, 34, 38, 52, 53, 113). These therapies have shown large effect size on lameness measured by subjective grading scale, pain on manipulation and range of motion (52, 53), improved objective lameness measurements (31, 34), and overall client satisfaction with treatment (34, 38, 52, 53). A main limitation of the larger prospective, placebo-controlled studies is that objective lameness measures, such as force plate are not used (52, 53, 61). In addition, no studies compare intra-articular MSC injection to intra-articular or parenteral pharmaceuticals, which are the current standard of care in veterinary medicine. Another variable that can affect interpretation of the therapeutic efficacy of MSCs is that canine OA MSC studies vary significantly in their preparation of MSCs and the vehicle for injection ranges from hyaluronic acid, to PRP, to saline (34, 38, 52, 53, 113). Experimental studies suggest that both these factors can influence clinical outcome due to cell–vehicle interaction (32).

BLOOD-DERIVED PRODUCTS

Autologous blood-derived products include ACS and PRP. The concentration of platelets, presence of leukocytes and activation method, or a combination of these factors (114, 115) can further subdivide PRP products. Both ACS and PRP can be produced using kits, and defined as drugs due to the final product's interaction with the body (116). Both ACS and PRP vary in cytokine and anabolic factor levels between and within preparation types because they are influenced by patient factors and preparation method **Figure 2**. These variations, as well as differing protocols used for timing and dose of intra-articular injection, make it difficult to extrapolate efficacy for the treatment of joint disease.

Autologous-Conditioned Serum

Autologous-conditioned serum is produced by incubating whole blood with borosilicate glass beads. It was investigated as a biological treatment for OA due to increased concentration of interleukin-1 receptor antagonist (IL-1Ra), a protein that is a competitive antagonist of the main inflammatory cytokine of OA IL-1β, as well as increased presence of anti-inflammatory cytokines IL-10 and IL-4 (117). The alteration in the cytokine profile of serum is thought to be due to the interaction of leukocytes with borosilicate glass beads during incubation. ACS preparation techniques used for veterinary applications include IRAP I™ (Dechra Veterinary Products/Orthokine) and IRAP II™ (Arthrex).

Levels of anti-inflammatory and inflammatory cytokines, as well as growth factors have been investigated for IRAP I™ and IRAP II™ treatment of equine and canine blood. Both techniques increase IL-1Ra and IL-10 levels as well as growth factors IGF-1 and TGF-β1 in equine blood (16). In canine blood, IRAP I™ (20) and IRAP II™ (19) cause significantly increased IL-1ra at levels comparable to equine and human products, but no significant differences were found for other growth factors and anti-inflammatory cytokines when investigated for IRAP I™ (20). The main limitation of such studies to-date is that absolute levels, or ratios of anti-inflammatory and inflammatory cytokines have not been linked to biological or therapeutic efficacy. When equine and canine studies found that pro-inflammatory cytokines IL-1β and TNFα were also increased with ACS preparation in horses (15, 16) and dogs (20), it was suggested that the ratio of anti-inflammatory to pro-inflammatory cytokines may be important for therapeutic effect (16). However, this has not been directly shown in veterinary species. Short (<72 h) clearance time of ACS cytokines *in vivo*, coupled with minimal effects of ACS-derived TNFα on cartilage metabolism in humans may support the counter argument that cytokine profiles of ACS are of little therapeutic consequence (15). Despite lack of evidence as to the therapeutic consequence of cytokine composition, it is clear that preparation technique (15–17, 20) and individual variation (18, 19) alter the bioactive composition of equine and canine ACS. IRAP II™ has a higher IL-1Ra:IL-1 ratio than IRAP I™ in horses (16). In addition, horses that have undergone surgical stress produce ACS

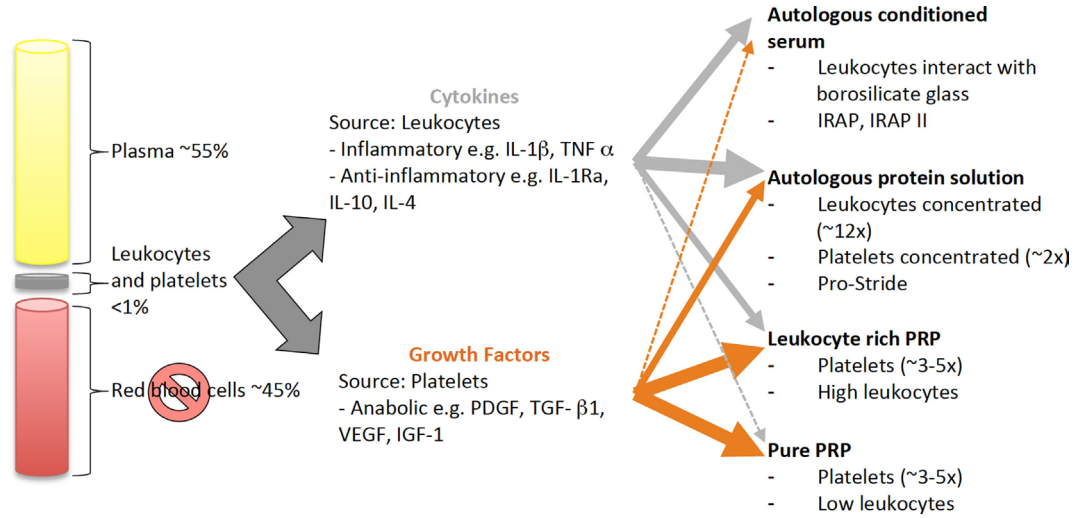

FIGURE 2 | Schematic of the source and distribution of cytokines and growth factors in blood-derived cell-based products. Cytokines and growth factors are derived from leukocytes and platelets, respectively. All blood-derived cell-based products have a combination of cytokines and growth factors, which vary in amount depending on how the products are produced. Leukocytes and platelets can be concentrated by centrifugation or filtration techniques or, the cytokines and growth factors are released during the production process, e.g., by interaction with borosilicate glass and during clot formation.

with reduced IL-1Ra and TGF-β1 levels that are further decreased at high levels of systemic inflammation (18); therefore, collection of blood before induction of surgical stress may be important to optimize the IL-1Ra:IL-1 ratio.

Clinical results for the treatment of equine OA with ACS have been promising; however, the precise mechanisms of action remain incompletely understood due to the action of multiple-bioactive factors in the product and few *in vitro* studies linking composition to therapeutic effect. Treatment of the COF model of equine OA with IRAP I™ injected four times at weekly intervals found decreased lameness scores, synovial thickness, and cartilage fibrillation compared with saline-treated controls to 70 days after OA induction (21). The injection frequency of ACS is likely important. Horses with arthroscopically defined naturally occurring OA treated with three injections of IRAP II™ at 2-day intervals had significantly lower levels of IL-1β, biomarkers of cartilage degradation, and IL-1Ra 42 days after treatment initiation compared with horses injected at 7-day intervals (22). Despite clinical improvements, *in vitro* studies have not demonstrated chondroprotective effects and it is likely that mitigation of inflammation is mainly responsible for reduction in lameness and cartilage degradation. Although ACS (IRAP II™) increased IL-1Ra and IGF-1 in equine cartilage explants treated with IL-1β, there was no significant difference in MMP-3 production and proteoglycan loss or synthesis between ACS and serum-treated samples suggesting minimal beneficial effects of ACS on cartilage matrix metabolism (17). Taken together, these results suggest that ACS predominantly acts as a mild anti-inflammatory agent in the joint. While the benefits of reduced inflammation during OA are clear, the benefit of ACS over pharmacological anti-inflammatories such as triamcinolone acetate are not because preclinical and clinical veterinary studies have not included positive control corticosteroid groups. In addition, the effects on articular cartilage are unlikely sufficient to support DMOAD effects at this time.

A variant of ACS called APS, Pro-Stride™ (Biomet Biologics), has been gaining clinical popularity because the product does not require an incubation period and has been investigated using a single intra-articular injection in horses and dogs. A bench-top centrifuge firstly isolates white blood cells, platelets, and plasma proteins, then they are further concentrated in a second centrifugation step (29). Pro-Stride™-treated equine blood resulted in a leukocyte count 12 times and platelet count 1.6 times higher than whole blood (29). The increased leukocytes result in elevation of anti-inflammatory cytokines such as IL-1Ra, IL-10, and soluble TNF receptor 1 (29). The same study compared one intra-articular injection of Pro-Stride™ to saline control in horses with naturally occurring OA. The APS group had significantly more horses that were sound or had improved by approximately one AAEP lameness grade at 7 and 14 days. However, such favorable outcomes occurred in horses with no radiographic signs of moderate-to-severe osteophytes, subchondral sclerosis, or joint space narrowing (29). The advantage of this study was that it was performed in horses with naturally occurring OA; however, the follow-up period for objective lameness and biochemical data was short (14 days) and joint type varied. In addition, outcome was significantly linked to stage of OA and a non-significant trend for the APS group to have lower radiographic evidence of disease and reduced synovial inflammation pre-injection, may have influenced outcome. Wanstrath et al. (28) also demonstrated a positive effect on canine lameness and pain scores with a single Pro-Stride™ injection compared with saline-treated controls. Both studies exhibited transient synovitis in the initial period after injection, which is likely due to the high leukocyte content of the products. High leukocyte content increases inflammatory cytokine content for other biologics such as PRP (118). However,

in both studies, the levels of inflammatory cytokines such as IL-1β and TNFα in the product were not investigated. In addition, effects on cartilage matrix metabolism are unknown so DMOAD effects cannot be claimed. Further studies that compare APS to traditional pharmacologic drugs and indentify effects on disease progression are needed before widespread use and disease-modifying claims for APS.

Although there are relatively few studies in horses and dogs regarding intra-articular use of ACS and APS, they all show evidence of mild symptom or inflammation-relieving effects. The promise to additionally provide regenerative or disease-modifying effects is yet to be realized and their potency compared with traditional symptom-modifying OA drugs, such as corticosteroids, has not been investigated. However, predominantly in equine sports medicine, clinicians are faced with insulin resistant patients, those with previous laminitic episodes, or competition medication rules that prevent them using intra-articular corticosteroids (11–13). Autologous anti-inflammatory biologics have a niche to treat such patients and, as a result, will continue to have clinical utility. Major areas of investigation that lag behind clinical misconception and opinion are their efficacy to have disease-modifying effects, the relevance of biological composition, comparison to traditional pharmacological anti-inflammatory drugs, and their ability to have long-term intra-articular presence or therapeutic effects.

Platelet-Rich Plasma

Platelet-rich plasma is the plasma portion of the patient's own blood that has an increased concentration of platelets through centrifugation or filtration steps. Alpha granules in the concentrated platelets are the source of both growth factors and cytokines. They release primarily PDGF and TGF-β1, but also VEGF and IGF-1 (119) when they are activated by the disease environment, before injection through the use of $CaCl_2$, thrombin, or a combination, or platelets are lysed during freeze–thaw cycles. It must be noted that PRP is perhaps the most variable of the blood-derived cell-based products because it has been shown to vary in the number of platelets, white blood cells, activation technique, and fibrin content depending on what preparation technique is used **Table 3** (114, 115, 120, 121). Furthermore, within PRP types and individuals, the concentration of platelets and leukocytes can vary. For example, horses given NSAIDs had increased platelet concentrations and the leukocyte concentration was elevated by dehydration and sampling at night (122).

TABLE 3 | Average platelet and leukocyte counts reported for commercially available platelet-rich plasma systems in the horse.

	Platelets/μL (fold Δ)[a]	Leukocyte/μL (fold Δ)[a]	Reference
Pall corporation	542,000 (3.2)	13,000 (1.9)	Textor and Tablin (123)
E-Pet/V-pet[b]	533,300 (3.8)	11,000 (1.8)	Hessel et al. (121)
	550,000 (~4)	–	Mirza et al. (24)
Harvest	513,000 (5.54)	6,910 (NC)	McCarrel and Fortier (124)
SmartPrep2	725,000 (4.2)	14,800 (~2)	Kisiday et al. (125)
Arthrex ACP	276,000 (1.6)	30 (~0.005)	Kisiday et al. (125)
	183,000 (1.3)	600 (0.1)	Hessel et al. (121)
Arthrex Angel	320,300 (2.3)	9,100 (1.5)	Hessel et al. (121)
Biomet GPS III	761,000 (5.4)	40,600 (6.7)	Hessel et al. (121)

[a]*Fold Δ is over whole equine blood, NC = no change, – represents data not available.*
[b]*Final platelet diluent in E-Pet/V-Pet system is hypertonic saline, not plasma.*

There is growing controversy over how concentrated platelets need to be for positive therapeutic effect. In some cases, optimum platelet concentrations are quoted in product manufacturer websites with no stated reference, which disregards the differences in platelet activity between species or over-represents the understanding we have of these products in horses and dogs (126). Both the structure and mechanism of degranulation differ between human and equine platelets, making it difficult to draw direct comparisons between species (124, 127). The minimum platelet concentration that defines human PRP is >1 million platelets per μL (128), which is approximately two to six times more concentrated than whole blood. PRP is occasionally referred to as autologous platelet concentrate; however, this should be reserved for platelets that are maximally concentrated rather than increased above baseline. There are no minimum platelet concentrations or fold increase over systemic platelet count defined for equine or canine PRP. While there are no studies currently investigating the effect of platelet concentration on therapeutic efficacy for OA, horses that had tendinopathy treated with >750,000 platelets per μL (approximately five times baseline), returned to work in 3 months compared with 8 months for those treated with less concentrated PRP (129). However, more is not better in regard to platelet concentration. Boswell et al. (118) found an apparent concentration/benefit plateau where tendon metabolism decreased at high platelet concentrations in a linear manner, although the specific platelet count at the plateau point was not defined. Despite controversy over the exact fold increase in platelet count, specific guidelines for equine or canine PRP have not been set and may differ between tendinopathy and OA.

Available systems to make PRP for horses and dogs concentrate platelets to varying degrees, which influences growth factor levels (120, 121). Growth factor levels are directly correlated to platelet concentration in horses and dogs; however, it is unclear how growth factor levels influence OA or cartilage metabolism in these species (118, 120, 121). Human studies show that TGF-β1 and IGF-1 stimulate extracellular matrix synthesis from chondrocytes (130, 131) and IGF-1 decreases synovial inflammation (132). However, high physiological levels of TGF-β1 have undesirable effects on the synovium in mice including increased leukocyte infiltration, synovial fibrosis, and osteophyte formation (133). It is unclear if these effects are related to a high concentration of a single growth factor, and if the growth factor mileu in PRP would cause similar results. Platelet-derived growth factor stimulation of human synoviocytes causes production of hyaluronic acid, which may be a source of indirect anti-inflammatory activity and enhance joint lubrication (134). It is likely that PRP's mechanism of pain modulation or anti-inflammatory activity is multimodal and its efficacy could be related to the stage of OA. No differences were found between people treated with PRP or hyaluronic acid (135) unless cartilage degeneration was present, where there was a trend for improved pain and motion in the pure PRP (PPRP) group vs. the hyaluronic acid group (135, 136). Optimum platelet concentration and the effects of growth factor

levels in equine and canine OA are currently unknown, leaving us to extrapolate from human or species-specific tendon research. This approach is less than ideal given both species- and disease-specific differences.

Perhaps the most important source of variation when considering PRP for intra-articular use is the leukocyte content, which has been related to the degree of catabolic signaling induced by collagen matrix in horses (118). Liquid-phase PRP used for intra-articular injection in veterinary patients can be defined as PPRP, which is leukocyte-reduced over whole blood, or leukocyte and platelet-rich plasma (LPRP). PPRP is termed as such to denote a more uniform ("pure") presence of platelets vs. other cellular components; however, it is impossible for all leukocytes to be removed during PRP processing so the term "leukocyte-reduced PRP" is occasionally, and more correctly, used to describe this PRP subtype. A potential limitation of PRP is that inflammatory cytokines including IL-1β, IL-6, and IL-8 have been found using different preparation techniques with human blood (137, 138). Inflammatory cytokines are related to leukocyte content and can be reduced by leukocyte depletion (138). Both leukocyte content and PRP concentration are likely important for the biological effect on synovium, cartilage, and meniscus (125, 139, 140). IL-1ra was increased in LPRP compared with PPRP, and LPRP used at low concentrations as a gel (which had the confounding effect of reducing LPRP concentration) had the greatest anti-inflammatory and anabolic effects on synovial and cartilage explants (139, 140). However, increased to maximal concentration of leukocytes may have a detrimental effect on both cartilage and meniscal metabolism (125), so LPRP should be used cautiously in joints and if so, used at low concentrations, which are yet to be defined.

Pure PRP use for equine OA has shown some success in clinical studies; however, compared with ACS, there are less controlled studies, study sizes are extremely small, and production and activation techniques of PPRP offer inherent variation. PPRP improved lameness and effusion scores in a pilot study of 4 horses that was maximal 2 months after injection and persisted for 8 months (23), and PPRP with lysis of platelets *via* freeze–thaw improved lameness associated with distal interphalangeal joint OA compared with a saline control in 10 horses (25). In addition to the small number of horses, there was either little or incomplete analysis of the growth factor and cytokine profiles of the products tested. There is high variability associated with preparation system (139), platelet activation (141), and individual horse factors (119) that could affect the clinical response to PPRP treatment in horses and require a greater study size to achieve results that can extrapolate to larger populations. As discussed above, both platelet and leukocyte content vary the growth factor and cytokine content of PRP and could affect therapeutic efficacy.

Perhaps the largest source of variation in therapeutic response and largest concern for safety lies with activation method. PRP is most commonly administered in its non-activated state; however, activation *via* bovine thrombin or calcium chloride can be used in an attempt to enhance degranulation of platelets and subsequently growth factor release. When bovine thrombin-activated PPRP was injected into healthy metacarpo-/metatarsophalangeal joints in horses, there were higher levels of growth factors released, but it caused joint effusion and generalized distal limb soft tissue swelling (123) with increased synovial fluid TNFα and IL-6 (141). The authors guarded against the safety of bovine thrombin due to the apparent inflammatory reaction to this xenogeneic protein. They recommended the use of non-activated or calcium chloride-activated PRP for intra-articular use, which had no adverse reactions. No controlled clinical investigation of PPRP for OA treatment in horses has been performed, and so-far clinical improvement after injection is variable (24, 25). In addition, a positive response to intra-articular anesthesia does not ensure reduction of lameness after PPRP injection (24). Further research to ascertain the efficacy of PPRP products derived from various systems needs to be performed before widespread use for OA.

In contrast to ACS, more research has been performed for the intra-articular use of PRP clinically in dogs. In dogs with OA, a single intra-articular LPRP treatment (3-fold increase in platelet count, 1.8-fold increase in leukocytes) decreased objective and subjective lameness and comfort scores compared with baseline or placebo controls (26), and pain-relieving effects were not significantly different from traditional intra-articular therapy of corticosteroid and hyaluronic acid (27). It must be noted that in these studies the leukocyte count was significantly higher (1.8 times systemic), constituting LPRP (26), or the leukocyte and platelet counts were not reported (27). The difference between PRP and traditional therapy demonstrated in dogs is that maximum pain-relieving response is seen at approximately 1 week with traditional therapy, but is most prominent after 6 weeks with PRP therapy (27). A slow onset of maximum therapeutic response was also seen when PRP was combined with AdMSCs (34) and was observed in a small number of horses (23). In these studies, PRP was used in a non-activated state. The lag in therapeutic response may be due to the gradual release of growth factors when platelets are allowed to be activated by the disease environment, shown experimentally over 4 days in an equine tendon explant model (124) and over 9 days in healthy equine joints (123). Experimental canine models using PRP suggest that reduction in synovitis as well as reduced collagen break down and matrix metalloproteinase activity could be responsible for the positive therapeutic response (142, 143). If similar disease-modifying effects could be shown in naturally occurring OA, PRP may reach DMOAD status in the future. However, PRP will need to overcome significant challenges associated with the variation discussed to prove that it is consistent and effective for the treatment of OA in veterinary species.

Summary

It is clear that both blood-derived cell-based products and MSCs have a complicated pathway from harvest to the end-user with scope for variations that make cell-based products different even within the same category. Variation transfers to the patient and is compounded for MSCs because they react to the specific disease environment encountered, making results between and within studies variable. The variation between veterinary cell-based studies could allow researchers to determine favorable protocols, but will not allow consistent and safe cell-based products to be produced. If veterinarians want effective, consistent cell-based products, research needs to be shared and the quality adequate to

select protocols that allow similar production techniques, shipping and injection methods, and standardized outcome parameters. This approach would allow some uniformity between studies and allow meta-data analysis to produce meaningful conclusions. As the variation of veterinary cell-based research stands, synthesis of study results to draw meaningful conclusions is difficult, if not impossible. Regulatory bodies recognize variation in veterinary cell-based products and the potential risk to our patients. The FDA has been a world-leader in publication of guidelines that aim to get researchers and clinicians recording their protocols, quality control measures, and treatment results. Using the regulatory pathways that make other drugs safe and effective is certainly the preferred pathway for veterinary cell-based products to take from a clinical standpoint. From a biological standpoint, inherent variation in source and donor will make regulatory pathways challenging. Therefore, a solid understanding of underlying cell biology is imperative for researchers, clinicians, and regulatory agents.

REGULATORY ASPECTS OF CELL-BASED THERAPIES

Regulatory and ethical aspects of stem cell therapy are topics of global discussion. However, even for human stem cell research and treatment, regulatory control varies internationally, from creation of national stem cell banks and regenerative medicine select committees in Britain, to minimal regulation or forum for data collection in other countries. Overall, most European countries, as well as Asia-Pacific have some bioethics legislation for the use and acquirement of human stem cells (144). In contrast, there is little regulation for the collection or use of animal stem cells for research or clinical purposes. In the USA, the governing body for both human and animal food and drugs is the FDA. Specifically, the FDA's Center for Veterinary Medicine (CVM), controls approval of animal drugs. The CVM has been the only legislative body to-date to formally publish specific definitions and recommendations for veterinary cell-based products, in a direct response to the growing use of cell-based products in animals clinically (76, 77). The guidance not only foreshadows where enforceable legislation will lead but is also an example of how other regulatory bodies may provide a framework for researchers and clinicians to record manufacturing processes and clinical results. These steps are expected to promote achievement of product consistency, safety, and efficacy to enhance the welfare of our patients.

How Does the FDA Regulate Cell-Based Products?

Stem cell-based products, as well as those derived from whole blood like ACS and PRP are defined by the FDA as "cell-based products" as they contain, consist of, or are derived from cells. There are many types of cell-based products currently being marketed to the veterinary industry including stem cells, ACS, PRP, and in-clinic kits used to produce these products. FDA guidelines define cell-based products as an animal drug because they are intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease and are articles intended to affect the structure or any function of the body [21 U.S.C. 321 Section 201(g)(1)(B) & (C)]. FDA guidance states that manufacturers of cell-based products meeting the definition of a new animal drug are subject to the same statutory and regulatory requirements as manufacturers of other new animal drugs. Therefore, cell-based products are required to go through pre-market review of experimental data to ensure that the product is safe, effective, and high quality before marketing of the product (145, 146). Currently, the FDA does not define specific *in vitro* or *in vivo* models that are needed or accepted for experimental data on veterinary species. Rather, the focus for regulation is on clinical trials using client-owned animals with naturally occurring disease. This process is regulated by the FDA's CVM, who published guidance regarding the regulation of cell-based products for animal use in 2015 (76, 77). The FDA recommends directly contacting them if researchers are considering pre-market review of experimental data, which may be done before data collection to ensure adequately detailed results.

There are currently no animal cell-based products that are FDA approved and can be legally marketed (145, 146).

How Do FDA Guidelines Affect Cell-Based Therapies?

As discussed, MSCs have been used for the treatment of intra-articular soft tissue injury (33, 45) and cartilage regeneration (47, 48) in veterinary species. In June 2015, the FDA released guidelines for the veterinary industry on cell-based products (76, 77). The guidelines defined and categorized types of cell therapies to clarify what products require an approved New Animal Drug Application (NADA) before legal marketing. The guidelines classified products from other species (xenogeneic), other individuals of the same species (allogeneic), and those from the same individual (autologous). Autologous cell therapies were divided into two categories: type I and type II. Type I are autologous cell-based therapies that are more than minimally manipulated (have processing that alters their relevant biological characteristics, such as expansion, addition, or purification of a cell-based factor); intended for non-homologous use (replacement of recipient tissue with a cell or tissue that does not perform the same basic function in the recipient as it did in the donor); intended for use in a food producing animal; dependent on the metabolic activity of its living cells for effect; or combined with other articles, drugs, or devices. Examples of autologous type I include any stem cells expanded in culture or cell-based products derived from fat or bone marrow used for cartilage repair. By contrast, type II autologous cells are minimally manipulated (for example, centrifugation); intended for homologous use; intended for use in non-food producing animals; and are not combined with other articles, drugs, or devices. An example of type II would be isolated non-expanded chondrocytes used to fill an articular cartilage defect.

The Pre-Market Review Process

If an investigator, manufacturer or practitioner has a xenogeneic, allogeneic, or type I autologous cell-based therapy that they intend to market or investigate in client-owned animals, it is recommended that they contact FDA to discuss the appropriate

pathway for their product. The suggested route for pre-market approval of cell-based products is through the NADA pathway. The requirements for approval of a NADA include, in part, demonstration of safety, effectiveness, and manufacturing quality (76, 77). The regulations also provide a pathway for investigational use allowing for the conduct of research to gather information necessary to demonstrate safety and effectiveness. Investigational use of cell-based products in client-owned animals may be conducted under a clinical investigational exemption. The clinical investigational exemption contains a number of conditions including items such as prior notice of shipment, or delivery of the investigational product, and reporting of study information and adverse events to an Investigational New Animal Drug (INAD) file. The investigational exemption also prohibits marketing or commercializing the investigational product. Investigational use of cell-based products intended solely for *in vitro* studies or laboratory research animals (non-client-owned animals) may be conducted without establishing an INAD file.

It is recommended to contact the FDA's CVM if you are currently manufacturing or intending to manufacture or use a cell-based product for use in client-owned animals so that the correct steps are taken. An explanation of how the guidelines could impact institutions currently marketing and manufacturing cell-based products, as well as contact information for the FDA's CVM, can be found in the FDA's letter to veterinary schools (76, 77). Currently, no FDA involvement is needed for use of these products solely in research or laboratory animals (animals that are not client-owned).

Cell-Based Products and the Regulatory Future

The regulatory standards for autologous cell and tissue-derived products in veterinary medicine are now a reality for investigators, manufacturers, and sole practitioners in the USA. It is likely that tighter regulatory standards will spread globally. Although creating a record with a regulatory body, like an INAD file, may seem like an administrative burden, the process has key benefits to the industry by obligating us to collect and record data on our patients. This opportunity to combine data on multiple patients treated with experimental cell-based products will likely secure a future for safe, effective products in our veterinary species. Since the FDA's 2015 guidelines, both academia and industry in the USA have moved toward involving the FDA to ensure a head-start in the future regulatory and competitive environment. Compared to pharmaceuticals, cell-based products have inherent variation as the levels of cytokines, growth factors, stem cell activity, or other biological response modifiers vary with multiple factors that include individual, diurnal variation, environmental stress, and processing procedures (16, 18, 147–149). Therefore, manufacturers will be faced with the challenge of proving that a product's strength, quality, and purity are maintained from batch to batch

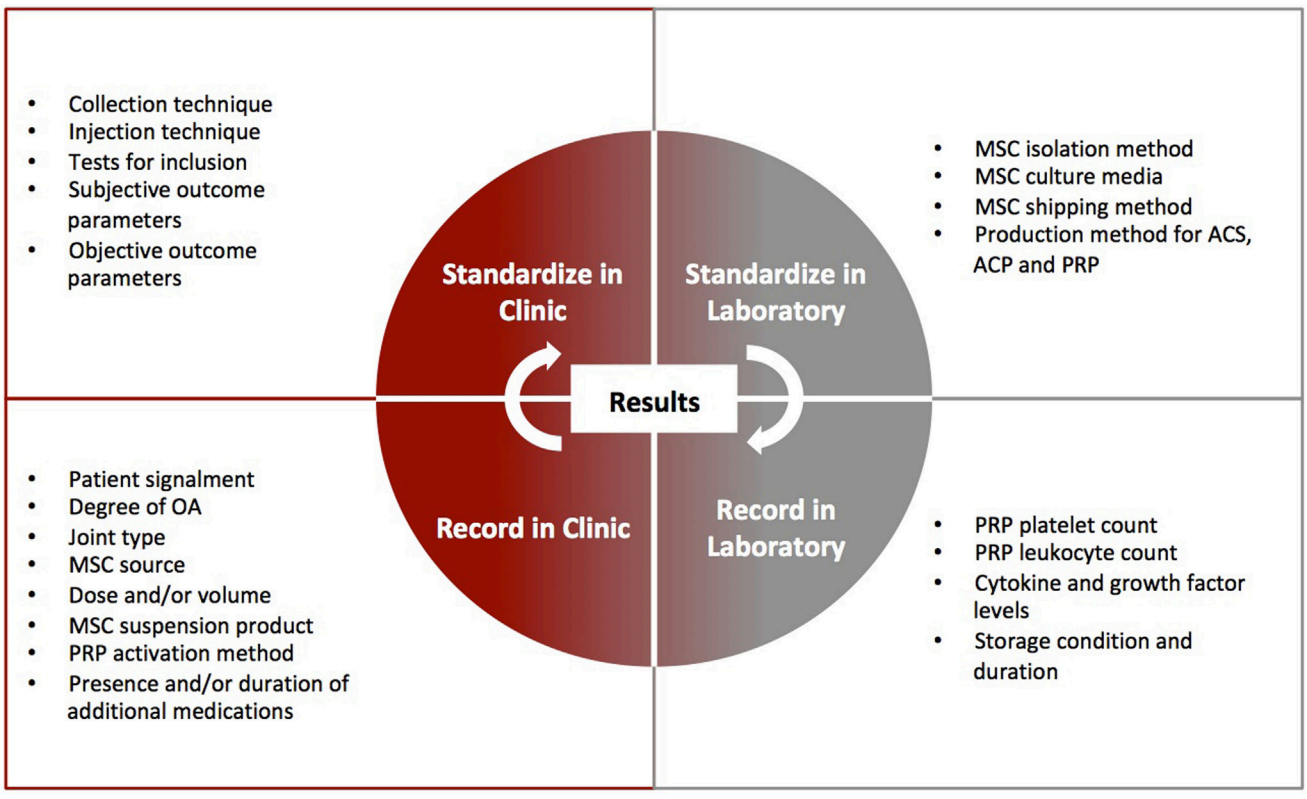

FIGURE 3 | Outline of clinical and laboratory parameters that can be standardized or recorded to enhance interpretation of clinical trial results for cell-based therapy. Standardization and recording for cell-based therapies will be imperative in regulatory approval pathways.

to demonstrate efficacy. Overcoming this limitation of cell-based products will require a solid understanding of the cellular and molecular biology behind the manufacture and use of cell-based products, as well as ensuring that regulatory reviewers understand the inherent variation. Regulators, scientists, and industry will need to work together to understand the critical parameters impacting the safety and effectiveness of these products, and to set appropriate standards for approval of cell-based products.

CONCLUSION AND PERSPECTIVE

Review of the veterinary cell-based literature to-date emphasizes that as we learn more from published findings, the scope for variation within and between cell-based therapies grows. Effectively, what we have learned is that it is impossible to draw finite conclusions from current data. The difficulties are inherent to the field because variation can occur during multiple stages from harvest to therapeutic effect, such as the source, manufacturing processes, shipping techniques, administration techniques, and disease environment. In addition, variation is complex within the formulation of each cell-based product because of the multiple-bioactive factors that can be affected, and the influence that different components may have on each other and the joint environment. Examples described in this review included PRP, where the amount of platelets and leukocytes may affect how the product performs in an intra-articular environment, or MSCs that can be affected by the degree of inflammation during OA and synovitis. Even investigation of levels of bioactive components can seem futile when it is unclear what components have the largest influence on therapeutic response. Deciphering true therapeutic response from clinical variation is complicated by studies that have low numbers, use different joints, stages of disease, vary between experimental and naturally occurring OA, or have different outcome parameters. Unfortunately, these issues are inherent to veterinary research due to reduced opportunities for funding and the high expense of cell-based as well as large-animal research.

Proving safety, consistency, and effectiveness of cell-based products is the best way to protect our patients and ensure longevity of the field. It is clear that the current variable approach to preclinical and clinical research does not allow clear conclusions about any of these essential facets of our cell-based therapeutics. Regulatory agencies like the FDA have recognized this and are influencing veterinary cell-based product manufacturers to pre-plan and record data in an attempt to standardize clinical research. However, only a fraction of veterinarians and researchers using or investigating cell-based products will be influenced by regulation. To move the field of veterinary cell-based therapies forward, the solution is less about what we need to know and more about what we need to do. Practitioners and researchers must collaborate globally, mimicking a regulatory body, if safe, effective, and consistent cell-based products are desired.

Through already formed professional bodies, veterinarians and veterinary researchers need to create a cell-based therapy forum. Ideally, action is needed to create uniformity between studies that include standardized preparation methods and transport conditions for MSCs, enforced reporting of platelet and leukocyte composition and activation technique in PRP, resolution of what inflammatory and anti-inflammatory cytokines are important to report for ACS or ACP, MSC studies with standardized cell culture media and suspension product, analysis by joint type and disease stage in clinical studies, performance of dose–response studies, appropriate and standardized experimental models in the absence of naturally occurring OA and recommend a standardized set of outcome parameters for clinical trials within each species **Figure 3**. Such uniformity will enable more direct comparisons between studies, as well as pooling of data for meta-analysis so that we can draw conclusions about symptomatic and disease-modifying effectiveness of cell-based therapies, which is what we need to know. A counter argument is that the variability seen so-far has allowed discovery of novel approaches to cell-based therapy, and that the expense of veterinary studies contrasts to reduced animal-specific funding compared with human medicine. However, variability is a direct challenge for cell-based products in their pathway to become safe, effective therapeutics. Unless we can prove consistency, cell-based products may not endure regulatory processes. A practical solution to funding constraints is for practitioners to form alliances with veterinary researchers. This will allow them to treat patients with cell-based therapies in a pre-defined manner and record outcomes that can be analyzed rather than the current trend, whereby many treatments on client-owned animals are not recorded as part of research. In the USA, the FDA has encouraged record keeping in the form of an INAD file. Globally, professional bodies could provide a forum for collaboration as well as provide access for recording and analysis of results. As our collaboration and understanding of the effect of cell-based therapies on OA improves, so too will the transition of cell-based therapies from variable but promising therapeutics to consistent and effective drugs for OA.

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Pooled Platelet-Rich Plasma Lysate Therapy Increases Synoviocyte Proliferation and Hyaluronic Acid Production While Protecting Chondrocytes From Synoviocyte-Derived Inflammatory Mediators

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Platelet-rich plasma (PRP) preparations are being used with moderate success to treat osteoarthritis (OA) in humans and in veterinary species. Such preparations are hindered, however, by being autologous in nature and subject to tremendous patient and processing variability. For this reason, there has been increasing interest in the use of platelet lysate preparations instead of traditional PRP. Platelet lysate preparations are acellular, thereby reducing concerns over immunogenicity, and contain high concentrations of growth factors and cytokines. In addition, platelet lysate preparations can be stored frozen for readily available use. The purpose of this study was to evaluate the effects of a pooled allogeneic platelet-rich plasma lysate (PRP-L) preparation on equine synoviocytes and chondrocytes challenged with inflammatory mediators *in-vitro* to mimic the OA joint environment. Our hypothesis was that PRP-L treatment of inflamed synoviocytes would protect chondrocytes challenged with synoviocyte conditioned media by reducing synoviocyte pro-inflammatory cytokine production while increasing synoviocyte anti-inflammatory cytokine production. Synoviocytes were stimulated with either interleukin-1β (IL-1β) or lipopolysaccharide (LPS) for 24 h followed by no treatment or treatment with platelet-poor plasma lysate (PPP-L) or PRP-L for 48 h. Synoviocyte growth was evaluated at the end of the treatment period and synoviocyte conditioned media was assessed for concentrations of hyaluronic acid (HA), IL-1β, tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6). Chondrocytes were then challenged for 48 h with synoviocyte conditioned media from each stimulation and treatment group and examined for gene expression of collagen types I (COL1A1), II (COL2A1), and III (COL3A1), aggrecan (ACAN), lubricin (PRG4), and matrix metallopeptidase 3 (MMP-3) and 13 (MMP-13). Treatment of inflamed synoviocytes with PRP-L resulted in increased synoviocyte growth and increased synoviocyte HA and IL-6 production. Challenge of

chondrocytes with conditioned media from PRP-L treated synoviocytes resulted in increased collagen type II and aggrecan gene expression as well as decreased MMP-13 gene expression. The results of this study support continued investigation into the use of pooled PRP-L for the treatment of osteoarthritis and warrant further *in-vitro* studies to discern the mechanisms of action of PRP-L.

Keywords: platelet-rich plasma lysate, osteoarthritis, IL-1β, LPS, HA, collagen type II, aggrecan, MMP-13

INTRODUCTION

Intra-articular injections of autologous platelet-rich plasma (PRP) are commonly used to treat osteoarthritis (OA) in humans and veterinary species, including horses and dogs (1–16). There is tremendous variability, however, in the composition of PRP generated based on the systemic health and hydration status, sex, and age of the patient, the quality of the venipuncture technique, the system or processing methods used, and whether or not the PRP is activated prior to injection (4, 17–21). The end result of such variability is large differences in platelet concentration, and therefore, growth factor and cytokine concentrations as well as leukocyte concentration within the products being used (4, 17–21). While classification systems have been put in place to define leukocyte-poor and leukocyte-rich PRPs (22–25), there is still controversy over which preparation is most efficacious for the treatment of OA and other musculoskeletal diseases (1, 4, 26–30). In addition, the optimal concentration of platelets within these preparations has yet to be elucidated (4, 26).

There has been increasing interest in the use of platelet lysate (PL) instead of PRP as PL is an acellular preparation containing high concentrations of growth factors and cytokines (31–48). The acellular nature of PL is important because it has the potential to be used in an allogeneic manner with further processing to remove immunoglobulins and also because it can be quality tested and then stored frozen to have available for immediate patient use (31–48). Furthermore, the use of pooled PL, or PL generated from multiple healthy donors, is being explored to capitalize on the natural variability that exists between individuals and the growth factors and cytokines that are released from their platelets upon lysis. This concept of optimal pooled PL has been investigated both for the use of PL as a non-immunogenic serum substitute for cell culture as well as for the use of PL as a clinical treatment (34, 41, 49).

A recent study evaluating the ability of equine PL preparations to modulate the innate immune responses of equine monocytes found interesting results when comparing data obtained from six individual PL preparations to data obtained from a pooled PL preparation created from those same six PL preparations (41). Notably, while none of the six individual PL preparations lead to significantly reduced tumor necrosis alpha (TNF-α) production from monocytes compared to fetal bovine serum (FBS), the pooled PL preparation did. Similarly, the pooled PL preparation dramatically reduced the variability observed in individual PL preparations for monocyte production of interleukin-1β (IL-1β) and interleukin-10 (IL-10). Lastly, the pooled PL preparation in this study was found to significantly decrease the production of both TNF-α and IL-1β by lipopolysaccharide (LPS) stimulated monocytes compared to controls (41). These results suggest that pooled PL preparations reduce variability and increase efficacy compared to individual PL preparations and that pooled PL preparations should be further examined as a means to suppress inflammation (41).

The aim of this study was to examine the effects of a pooled allogeneic platelet-rich plasma lysate (PRP-L) preparation on equine synoviocytes and chondrocytes challenged with inflammatory mediators *in-vitro* to mimic the OA joint environment. Our hypothesis was that PRP-L treatment of inflamed synoviocytes would protect chondrocytes challenged with synoviocyte conditioned media by reducing synoviocyte pro-inflammatory cytokine production and increasing synoviocyte anti-inflammatory cytokine production. In particular, we expected chondrocytes challenged with conditioned media from IL-1β or LPS stimulated synoviocytes treated with PRP-L to have increased gene expression of collagen type II and decreased gene expression of MMP-3 and MMP-13 compared to conditioned media from non-treated or platelet-poor plasma lysate (PPP-L) treated synoviocytes.

MATERIALS AND METHODS

Study Design

A schematic of the study design is shown in **Figure 1**. The Institutional Animal Care and Use Committee of North Carolina State University approved the use of horses in these studies.

Platelet-Poor Plasma Lysate and Platelet-Rich Plasma Lysate Preparation

Whole blood was collected via jugular venipuncture from 6 healthy horses in our closed research herd into four 60 mL syringes containing 6 mL of acid citrate dextrose (ACD) each for a total volume of 240 mL per horse. These horses included 3 geldings and 3 nonparous mares between the ages of 6 and 19 years. Routine automated complete blood counts and platelet counts were performed on each whole blood sample. Erythrocytes were allowed to settle for 30 min in the syringes and the layer above the erythrocytes containing the leukocytes, platelets, and plasma (approximately 120 mL) was then transferred to a 50 mL conical tube and centrifuged at 250 g for 15 min. From each conical, the supernatant above the leukocyte pellet containing the platelets and plasma was then harvested and centrifuged at 1,500 g for 15 min. From this spin, the supernatant containing the platelet-poor plasma (PPP) was removed and saved. The platelet pellet was then

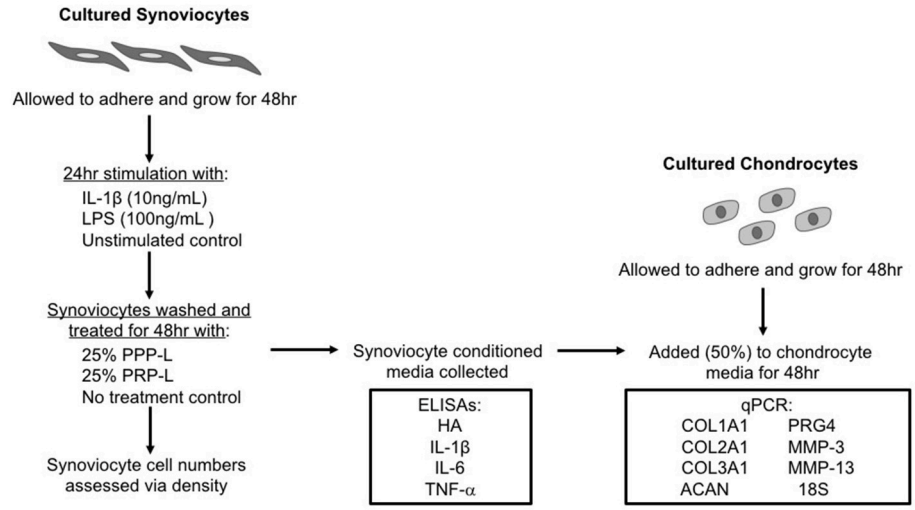

FIGURE 1 | Schematic of the study design.

resuspended in 12 mL of PPP to generate platelet-rich plasma (PRP) of approximately 10x the concentration of whole blood. Platelet numbers in PPP and PRP samples were determined by staining platelets with 1 μM Calcein-AM (Invitroge™ Molecular Probes™, ThermoFisher Scientific, Waltham, MA, USA), incubating for 20 min, and then counting the number of fluorescent cells using a Cellometer® Auto 2000 (Nexcelom Bioscience LLC, Lawrence, MA, USA). White blood cell (WBC) counts in PPP and PRP samples were determined using a Cellometer® Auto 2000 and ViaStain™ AOPI Staining Solution (Nexcelom Bioscience LLC, Lawrence, MA, USA). To generate PPP and PRP lysate, (PPP-L and PRP-L, respectively), the PPP and PRP then underwent five freeze/thaw cycles in liquid nitrogen. The majority of cell debris was removed from all PPP-L and PRP-L samples by centrifugation at 20,000 g for 20 min. PPP-L and PRP-L samples were then clarified by depth filtration using the ZetaPlus™ BC25 Capsule Filter, Medi 90ZB (3M Purification Inc., St Paul, MN, USA). The resultant PPP-L and PRP-L samples were then pooled, respectively, from all 6 horses and frozen at −80°C until use.

Synoviocyte Isolation

Synovium was harvested from the femoropatellar joints of 5 systemically healthy horses (ages 2–14 years) euthanized for reasons other than this study and free of femoropatellar joint disease. The isolated synovium was weighed and digested for 2 h at 37°C under constant rotation with synoviocyte media [high glucose (4.5 g/L) DMEM medium with 10% fetal bovine serum (FBS), 2 mM l-glutamine, 1 mM sodium pyruvate, 25 mM HEPES, penicillin (100 units/mL), and streptomycin (100 μg/ml)] added at 10 mL/g tissue and containing 1.5 mg/mL Gibco® collagenase type II (ThermoFisher Scientific, Waltham, MA, USA) (50, 51). The resulting digest was passed through a 100 μm filter and centrifuged at 800 g for 10 min. The cell pellet was then washed twice with fresh synoviocyte media and live synoviocyte count was determined using a Cellometer® Auto 2000 and ViaStain™ AOPI Staining Solution (Nexcelom Bioscience LLC, Lawrence, MA, USA). Synoviocytes were frozen in aliquots of 10×10^6 cells/mL in liquid nitrogen until use.

Chondrocyte Isolation

Cartilage was harvested from the femoral trochlear ridges of a 2-year-old Thoroughbred gelding free of orthopedic disease and euthanized for reasons other than this study. The isolated cartilage was weighed and digested overnight (16–18 h) at 37°C under constant rotation with chondrocyte media [Ham's F12 medium with 10% FBS, 25 mM HEPES, ascorbic acid (50 μg/mL), α-ketoglutarate (30 μg/mL), L-glutamine (300 μg/mL), penicillin (100 units/mL), and streptomycin (100 μg/ml)] containing 0.75 mg/mL of Gibco® collagenase type II (ThermoFisher Scientific, Waltham, MA, USA) (52, 53). The resulting digest was passed through a 100 μm filter and centrifuged at 800 g for 10 min. The cell pellet was then washed twice with fresh chondrocyte media. Cells were resuspended in chondrocyte media and live chondrocyte count was determined using a Cellometer® Auto 2000 and ViaStain™ AOPI Staining Solution. Chondrocytes were frozen in aliquots of 10×10^6 cells/mL in liquid nitrogen until use.

Synoviocyte Stimulation and Treatment

Cryopreserved synoviocytes were thawed, seeded onto a 12-well plate at 4×10^5 cells/well in synoviocyte media (50), and maintained at 5%CO_2, 90% humidity, and 37°C. Cells were brought to confluency over 48 h before media exchange and stimulated with either recombinant human IL-1β at 10 ng/mL (R&D Systems, Minneapolis, MN, USA) (54) or *E. Coli* O55:B5 LPS at 100 ng/mL (Sigma-Aldrich, St. Louis, MO, USA) (55, 56). Unstimulated control wells underwent media exchange only. After 24 h of stimulation, the stimulation media was removed and the cellular monolayer was washed twice with phosphate

buffered saline (PBS). Fresh media was then added to the cells either alone (no treatment controls) or with 25% PPP-L or 25% PRP-L. Synoviocytes were treated for 48 h and the resultant synoviocyte conditioned media was collected, centrifuged at 2,000 g for 15 min, and divided into aliquots for cytokine analyses and chondrocyte treatments. Aliquots for cytokine analyses were frozen at −80°C until use while aliquots for chondrocyte treatments were used fresh.

Synoviocyte Quantification

After removal of the synoviocyte conditioned media as described above, 1 mL of PBS was added to each synoviocyte well. Spectrophotometric quantification of synoviocyte cell numbers in each monolayer was then measured by density using a multiple detection plate reader set at an absorbance of 800 nm (Synergy™ 2, BioTek Instruments Inc., Winooski, VT, USA) (57). Fold change in cell density was determined as a change from the unstimulated, non-treated synoviocytes.

Synoviocyte Conditioned Media Analyses

Hyaluronic acid (hyaluronan; HA) concentrations were quantified in duplicate aliquots of all synoviocyte conditioned media samples using the commercially available Hyaluronan Quantikine ELISA kit (R&D Systems Minneapolis, MN, USA). Standards in each kit were used to generate standard curves and samples were analyzed for optical density on a multiple detection plate reader (Synergy™ 2, BioTek Instruments Inc., Winooski VT) at 450 nm with wavelength correction set at 540 nm.

Pro-inflammatory IL-1β and TNF-α and pro/anti-inflammatory IL-6 concentrations in synoviocyte conditioned media were determined using a truncated version of the commercially available equine multiplex assay (MILLIPLEX MAP Equine Cytokine/Chemokine Magnetic Bead Panel, EMD Millipore, Burlington, MA, USA) on a MAGPIX® System (Luminex Corp., Austin, TX, USA). All samples were analyzed in duplicate using a 96-well platform performed per manufacturer's instructions. A minimum bead count of 50 for each cytokine was acquired for data analysis. Data were analyzed using Milliplex Analyst 5.1 software (Luminex Corporation, Austin, TX, USA).

Chondrocyte Challenge With Synoviocyte Conditioned Media

Cryopreserved chondrocytes were thawed, seeded onto a 24-well plate at 2×10^5 cells/well in chondrocyte media (53) and maintained at 5%CO_2, 90% humidity, and 37°C. Cells were brought to confluency over 48 h before media exchange with an equal volume of synoviocyte conditioned media to chondrocyte media for each well. Challenge experiments were carried out for 48 h.

Chondrocyte RNA Extraction and qPCR

Total cellular RNA was extracted from chondrocytes using the RNeasy Mini Kit (Qiagen Inc., Germantown, MD, USA) according to the manufacturer's instructions. The RNA purity and quantity were evaluated using UV microspectrophotometry (NanoDrop 2000 Spectrophptometer, ThermoFisher Scientific, Waltham, MA, USA). RNA was stored at −80°C until cDNA construction by RT-PCR using the QuantiTect Reverse Transciption Kit (Qiagen Inc., Germantown, MD, USA) according to the manufacturer's instructions.

Previously published equine primers were used to amplify collagen types I (COL1A1), II (COL2A1), and III (COL3A1), aggrecan (ACAN), lubricin (PRG4), and matrix metallopeptidase 3 (MMP-3) and 13 (MMP-13) with 18S used as a housekeeping gene (**Table 1**). Quantitative real time RT-PCR (qPCR) was performed using the QuantiFast®SYBR®Green PCR Kit (Qiagen Inc., Germantown, MD, USA) according to the manufacturer's instructions with the QuantStudio®6 Flex System (applied biosystems®, ThermoFisher Scientific, Waltham, MA, USA). Relative gene expression, $2^{-\Delta\Delta Ct}$, was generated using Real-Time PCR Software v1.2 (applied biosystems®, ThermoFisher Scientific, Waltham, MA, USA). Chondrocytes cultured with the unstimulated, non-treated synoviocyte conditioned media were used as controls.

Statistical Analyses

All results were assessed for normality by means of Shapiro-Wilk test. Normally distributed data was analyzed by the analysis of covariance (ANCOVA) with horse as covariate, followed by the Tukey's test for multiple comparisons. Non-normally distributed data was analyzed by the non-parametric Wilcoxon rank sum test. Statistical analyses were performed within the non-treated group across stimulations to assess the effects of stimulation and then within each stimulation group to assess for treatment effects. Analyses were performed using JMP® Pro11 (SAS Institute Inc., Cary, NC, USA) and significance set at $p < 0.05$. All graphs were generated with GraphPad Prism 7 (GraphPad, La Jolla, CA, USA).

RESULTS

Verification of Platelet-Poor Plasma and Platelet-Rich Plasma Lysate Preparations

White blood cell (WBC) and platelet counts verified the generation of leukocyte-reduced PPP and PRP. The mean ± standard deviation ($n = 6$) WBC count in whole blood was 5.60 $\times 10^3/\mu L \pm 0.62 \times 10^3/\mu L$ compared to 0.05 $\times 10^3/\mu L \pm 0.02 \times 10^3/\mu L$ in PPP and 1.39 $\times 10^3/\mu L \pm 0.26 \times 10^3/\mu L$ in PRP. The mean ± standard deviation ($n = 6$) platelet count in whole blood was 134.50 $\times 10^3/\mu L \pm 35.67 \times 10^3/\mu L$ compared to 9.38 $\times 10^3/\mu L \pm 3.46 \times 10^3/\mu L$ in PPP and 1226.38 $\times 10^3/\mu L \pm 55.32 \times 10^3/\mu L$ in PRP. As such, the platelet concentration in each PRP sample was very close to our target of 10x the platelet concentration of whole blood.

Effects of Il-1β or LPS Stimulation on Synoviocytes and Chondrocytes Challenged With Synoviocyte Conditioned Media

Decreased synoviocyte growth compared to unstimulated controls was observed following 24 h of stimulation with either IL-1β ($p < 0.002$) or LPS ($p < 0.001$; **Figure 2A**). Synoviocytes stimulated with either IL-1β ($p < 0.03$) or LPS ($p < 0.03$) also

TABLE 1 | Equine primer sequences used for gene expression analyses.

Gene	Primer sequences
COL1A1, Collagen type I (58)	Forward, 5′-AAGGACAAGAGGCACGTCTG-3′ Reverse, 5′-GCAGGAAAGTCAGCTGGATG−3′
COL2A1, Collagen type II (53)	Forward, 5′-GCTACACTCAAGTCCCTCAAC-3′ Reverse, 5′-ATCCAGTAGTCTCCGCTCTT-3′
COL3A1, Collagen type III (58)	Forward, 5′-GGGTATAGCTGGTCCTCGTG-3′ Reverse, 5′-GCGCCTCTTTCTCCTTTAGC−3′
ACAN, Aggrecan (59)	Forward, 5′-CAACAACAATGCCCAAGACTAC-3′ Reverse, 5′-AGTTCTCAAATTGCAAGGAGTG−3′
PRG4, Proteoglycan 4 (Lubricin) (60)	Forward, 5′-TGCGGTGCTTCCCCATAC-3′ Reverse, 5′-AAACAGGAACCCATCAGAAAGTG−3′
MMP-3, Matrix metallopeptidase 3 (53)	Forward, 5′-ATGGACCTTCTTCAGGACTACC-3′ Reverse, 5′-GACCGACATCAGGAACTCCG-3′
MMP-13, Matrix metallopeptidase 13 (53)	Forward, 5′-ACAAGCAGTTCCAAAGGCTAC-3′ Reverse, 5′-CTCGAAGACTGGTGATGGCA-3′
18S, 18 small ribonucleic acid (53)	Forward, 5′-GCCGCTAGAGGTGAAATTCT-3′ Reverse, 5′-TCGGAACTACGACGGTATCT-3′

had increased production of IL-1β compared to unstimulated synoviocytes (**Figure 3A**). Increased production of TNF-α, however, was only observed following stimulation with LPS ($p < 0.02$; **Figure 3B**), and increased production of IL-6 was only observed following stimulation with IL-1β ($p < 0.03$; **Figure 3C**). Chondrocytes challenged with synoviocyte conditioned media stimulated with either IL-1β ($p < 0.03$) or LPS ($p < 0.03$) had increased gene expression of MMP-13 (**Figure 6B**), while only those challenged with synoviocyte conditioned media stimulated with LPS ($p < 0.03$) had increased gene expression of MMP-3 (**Figure 6A**) compared to control chondrocytes challenged with unstimulated synoviocyte conditioned media.

PRP-L Increases Growth and Hyaluronic Acid Production in Naïve and Inflamed Synoviocytes

Both PRP-L and PPP-L treatment increased the growth of unstimulated synoviocytes ($p < 0.0003$ and $p < 0.05$, respectively), but only PRP-L treatment was able to rescue the growth of synoviocytes stimulated with either IL-1β ($p < 0.0001$) or LPS ($p < 0.0001$; **Figure 2A**). Furthermore, only PRP-L treatment was able to increase total synoviocyte HA production in unstimulated synoviocytes ($p < 0.005$), synoviocytes stimulated with IL-1β ($p < 0.0001$) and synoviocytes stimulated with LPS ($p < 0.009$; **Figure 2B**). These results indicate that PRP-L treatment has powerful proliferative effects and is able to increase total synoviocyte HA production both under naïve conditions and in the face of inflammatory stimulation.

PRP-L Increases Anti-inflammatory IL-6 Production in Naïve and Inflamed Synoviocytes

PRP-L treatment increased the production of IL-1β from unstimulated synoviocytes ($p < 0.05$) but did not cause any further increase in IL-1β following stimulation either IL-1β or LPS compared to non-treated synoviocytes (**Figure 3A**). Similarly, both PRP-L and PPP-L treatments increased the production of TNF-α from unstimulated synoviocytes ($p < 0.05$) compared to non-treated synoviocytes (**Figure 3B**), but neither caused any further increase in TNF-α following stimulation with IL-1β and only PPP-L treatment caused a further increase in TNF-α following LPS stimulation ($p < 0.02$). Only PRP-L treatment was able to further increase production of IL-6 from synoviocytes under all conditions compared to non-treated synoviocytes ($p < 0.005$) and compared to PPP-L treated synoviocytes ($p < 0.05$; **Figure 3C**). These results indicate that PRP-L treatment does not reduce pro-inflammatory cytokine production from stimulated synoviocytes but does increase anti-inflammatory IL-6 production from both unstimulated and stimulated synoviocytes compared to both non-treated and PPP-L treated synoviocytes.

Conditioned Media From PRP-L Treated Synoviocytes Increases Anabolic Gene Expression in Cultured Chondrocytes

The anabolic effects of PPP-L treated, PRP-L treated, or non-treated synoviocytes either unstimulated or stimulated with IL-1β or LPS on cultured chondrocytes were assessed by measuring relative chondrocyte gene expression of collagen type I (**Figure 4A**), collagen type II (**Figure 4B**), collagen type III (**Figure 4C**), aggrecan (**Figure 5A**), and lubricin (**Figure 5B**). Chondrocytes challenged with conditioned media from PRP-L treated synoviocytes had increased collagen type II expression compared to chondrocytes challenged with conditioned media from non-treated synoviocytes when the synoviocytes where unstimulated ($p < 0.03$), IL-1β stimulated ($p < 0.003$), or LPS stimulated ($p < 0.001$) and compared to chondrocytes challenged with conditioned media from PPP-L treated synoviocytes when the synoviocytes where either IL-1β stimulated ($p < 0.01$) or LPS stimulated ($p < 0.01$; **Figure 4B**). No significant differences in chondrocyte gene expression of either collagen type I and collagen type III were found for any stimulation or treatment group of synoviocyte cultured media (**Figures 4A,C**). Chondrocytes challenged with conditioned media from PRP-L treated synoviocytes had increased aggrecan expression compared to chondrocytes challenged with conditioned media from either non-treated or PPP-L treated synoviocytes when the synoviocytes were unstimulated ($p < 0.02$), IL-1β stimulated ($p < 0.01$), or LPS stimulated ($p < 0.02$) (**Figure 5A**). Lubricin (proteoglycan 4) gene expression was largely unaffected apart from increased gene expression in chondrocytes challenged with PRP-L treated, IL-1β stimulated synoviocyte conditioned media ($p < 0.05$) compared to those challenged with non-treated, IL-1β stimulated synoviocyte conditioned media (**Figure 5B**). These

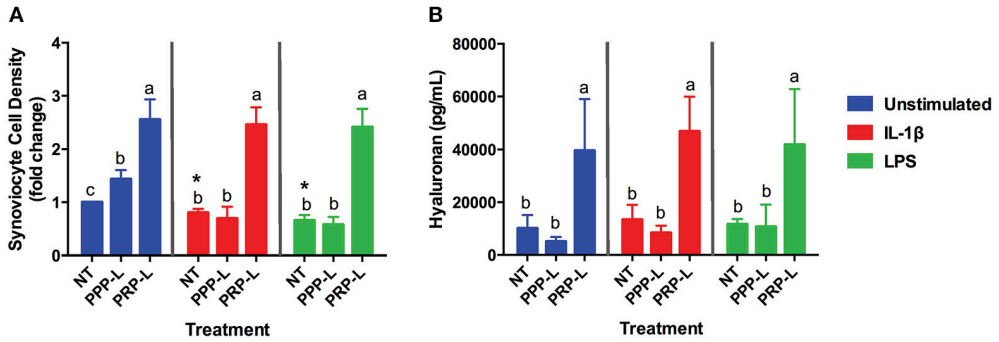

FIGURE 2 | PRP-L but not PPP-L treatment increases synoviocyte growth and production of hyaluronic acid regardless of synoviocyte stimulation. Equine synoviocytes were left unstimulated or stimulated with 10 ng/mL of IL-1β or 100 ng/mL of LPS for 24 h before being non-treated (NT) or treated with platelet-poor plasma lysate (PPP-L) or platelet-rich plasma lysate (PRP-L) for 48 h. **(A)** Synoviocyte growth was measured via optical density and displayed as a fold change from the unstimulated, non-treated group. **(B)** Production of hyaluronic acid (hyaluronan; HA) was measured in the media using a commercial ELISA kit. Data is shown as the mean ± standard deviation of $n = 5$. Differing letters indicate significant differences between groups ($p < 0.05$); statistical analysis was performed within each stimulation and not between stimulation groups. Asterisks (*) denote significant differences, when present, in stimulated NT groups from the unstimulated NT control. Blue bars = unstimulated synoviocytes, red bars = 10 ng/mL IL-1β stimulated synoviocytes, and green bars = 100 ng/mL LPS stimulated synoviocytes.

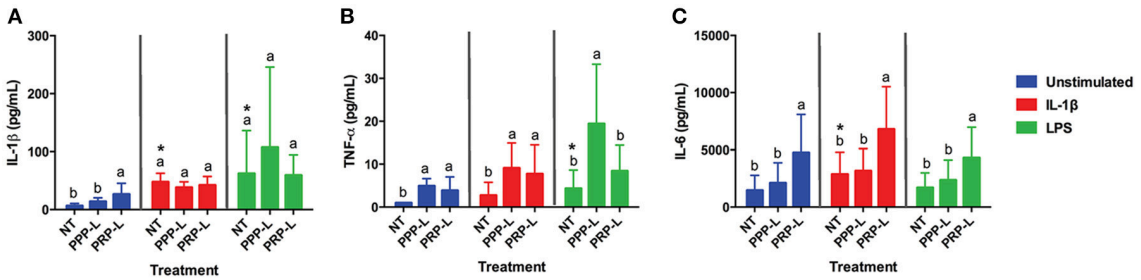

FIGURE 3 | PRP-L treated synoviocytes produce more anti-inflammatory IL-6 under all stimulations conditions and less pro-inflammatory TNF-α in response to LPS stimulation compared to PPP-L treated synoviocytes. Conditioned media from cultured equine synoviocytes was collected after stimulation with 10 ng/mL of IL-1β or 100 ng/mL of LPS for 24 h and subsequent non-treatment (NT) or treatment with platelet-poor plasma lysate (PPP-L) or platelet-rich plasma lysate (PRP-L) for 48 h. Media concentrations (pg/mL) of **(A)** IL-1β, **(B)** TNF-α, and **(C)** IL-6 were measured using a commercial equine multiplex assay. Data is shown as the mean ± standard deviation of $n = 5$. Differing letters indicate significant differences between groups ($p < 0.05$); statistical analysis was performed within each stimulation and not between stimulation groups. Asterisks (*) denote significant differences, when present, in stimulated NT groups from the unstimulated NT control. Blue bars = unstimulated synoviocytes, red bars = 10 ng/mL IL-1β stimulated synoviocytes, and green bars = 100 ng/mL LPS stimulated synoviocytes.

results indicate that PRP-L treatment increases the production of normal collagen type II found in mature articular cartilage rather than inferior collagen type I found in fibrocartilage or collagen type III found in cartilage undergoing repair. In addition, PRP-L treatment increases the production of aggrecan, the major structural proteoglycan of cartilage extracellular matrix.

Conditioned Media From Both PRP-L and PPP-L Treated Inflamed Synoviocytes Decreases Catabolic Gene Expression in Cultured Chondrocytes

The catabolic effects of PPP-L treated, PRP-L treated, or non-treated synoviocytes either unstimulated or stimulated with IL-1β or LPS on cultured chondrocytes were assessed by measuring relative chondrocyte gene expression of MMP-3 (**Figure 6A**) and MMP-13 (**Figure 6B**). Although no significant differences in chondrocyte gene expression of MMP-3 were found for conditioned media from PRP-L or PPP-L treated synoviocytes stimulated with either IL-1β or LPS compared to non-treated synoviocytes, there was a trend toward reduced MMP-3 gene expression under LPS stimulation conditions ($p = 0.09$) and in particular the PRP-L treated group decreased back down to the same level of MMP-3 gene expression observed in control chondrocytes challenged with conditioned media from unstimulated synoviocytes (**Figure 6A**). Chondrocytes challenged with conditioned media from both PRP-L and PPP-L treated synoviocytes had decreased MMP-13 gene expression compared to chondrocytes challenged with conditioned media from non-treated synoviocytes when the synoviocytes were unstimulated ($p < 0.03$) and also when the synoviocytes where stimulated with IL-1β ($p < 0.05$) or LPS ($p < 0.02$; **Figure 6B**). Similar to the trend observed for chondrocyte gene expression of MMP-3 under LPS stimulation conditions, there was a trend toward chondrocytes challenged with conditioned media from PRP-L treated synoviocytes that were stimulated with LPS to

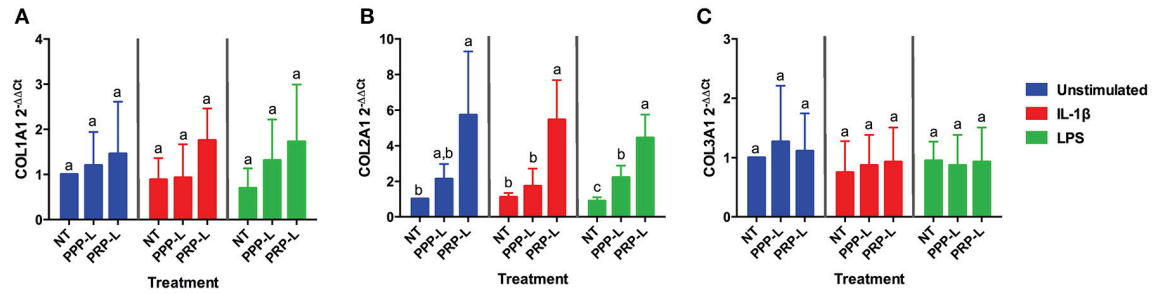

FIGURE 4 | Conditioned media from synoviocytes treated with PRP-L under all stimulation conditions increases collagen type II but not collagen type I or type I in cultured chondrocytes. Equine chondrocytes were challenged for 48 h with conditioned media from synoviocytes stimulated with IL-1β or LPS and either non-treated (NT) or treated with platelet-poor plasma lysate (PPP-L) or platelet-rich plasma lysate (PRP-L). Relative gene expression is represented as the $2^{-\Delta\Delta Ct}$ of **(A)** collagen type I (COL1A1), **(B)** collagen type II (COL2A1), and **(C)** collagen type III (COL3A1). Data is shown as the mean ± standard deviation of n=5. Differing letters indicate significant differences between groups ($p < 0.05$); statistical analysis was performed within each stimulation and not between stimulation groups. Asterisks (*) denote significant differences, when present, in stimulated NT groups from the unstimulated NT control. Blue bars = unstimulated synoviocytes, red bars = 10 ng/mL IL-1β stimulated synoviocytes, and green bars = 100 ng/mL LPS stimulated synoviocytes.

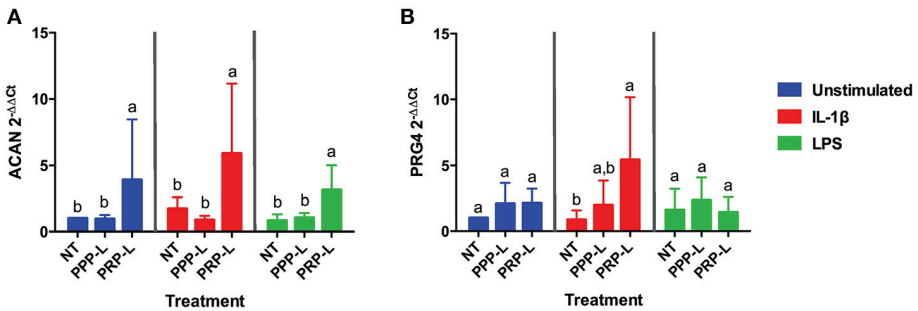

FIGURE 5 | Conditioned media from synoviocytes treated with PRP-L under all stimulation conditions increases aggrecan expression in cultured chondrocytes. Equine chondrocytes were challenged for 48 h with conditioned media from synoviocytes stimulated with IL-1β or LPS and either non-treated (NT) or treated with platelet-poor plasma lysate (PPP-L) or platelet-rich plasma lysate (PRP-L). Relative gene expression represented at the $2^{-\Delta\Delta Ct}$ of **(A)** aggrecan (ACAN), and **(B)** lubricin (PRG4) was measured in cDNA made from extracted chondrocyte RNA. Fold changes were generated from the chondrocytes cultured with unstimulated, non-treated synoviocyte conditioned media. Data is shown as the mean ± standard deviation of $n = 5$. Differing letters indicate significant differences between groups ($p < 0.05$); statistical analysis was performed within each stimulation and not between stimulation groups. Asterisks (*) denote significant differences, when present, in stimulated NT groups from the unstimulated NT control. Blue bars = unstimulated synoviocytes, red bars = 10 ng/mL IL-1β stimulated synoviocytes, and green bars = 100 ng/mL LPS stimulated synoviocytes.

have further reduced MMP-13 gene expression compared to chondrocytes challenged with conditioned media from PPP-L treated synoviocytes that were stimulated with LPS (**Figure 6B**). These results indicate that both PRP-L and PPP-L treatments are able to downregulate MMP-13 production in chondrocytes under LPS stimulation conditions, but with PRP-L treatment trending toward a closer return to unstimulated control levels of MMP-13.

DISCUSSION

The aim of this study was to examine the effects of a pooled allogeneic platelet-rich plasma lysate (PRP-L) preparation on equine synoviocytes and chondrocytes challenged with inflammatory mediators *in-vitro* to mimic the OA joint environment. The findings of this study support our overall hypothesis that PRP-L treatment of inflamed synoviocytes protects chondrocytes challenged with synoviocyte conditioned media. The protective effect of PRP-L, however, appears to be more through increase of synoviocyte anti-inflammatory cytokine production rather than through reduction of synoviocyte pro-inflammatory mediators. Such findings are consistent with those of previous studies on the anti-inflammatory effects of PL on other cell types (35, 41, 61) and are also consistent with previous studies on the anabolic and anti-catabolic effects of PRP preparations on chondrocytes (62–65). A particularly interesting, and unexpected finding of this study, however, was that treatment with PRP-L stimulated the growth of synoviocytes and the production of HA from synoviocytes even when challenged with IL-1β or LPS.

Numerous studies evaluating the efficacy of PL as a serum replacement for FBS in cell culture media have supported the proliferative effect of PL preparations on different cell types including bone marrow-derived stromal cells, adipose-derived stromal cells, synovial fluid stromal cells, and corneal endothelium cells (31, 38–40, 42, 45, 47). It is therefore

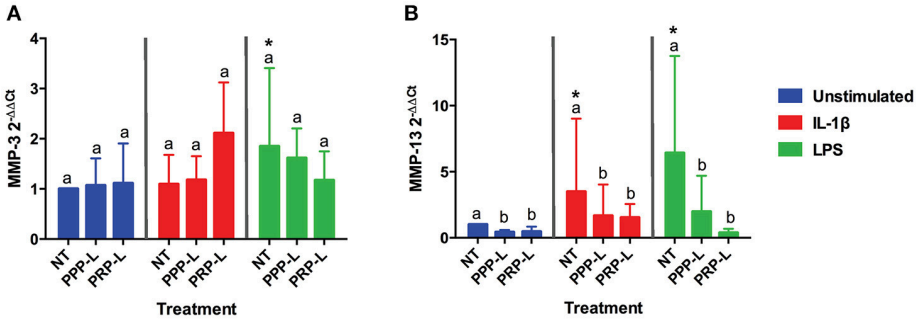

FIGURE 6 | Conditioned media from synoviocytes treated with PRP-L or PPP-L under all stimulation conditions decreases MMP-13 but not MMP-3 gene expression in cultured chondrocytes. Equine chondrocytes were challenged for 48 h with conditioned media from synoviocytes stimulated with IL-1β or LPS and either non-treated (NT) or treated with platelet-poor plasma lysate (PPP-L) or platelet-rich plasma lysate (PRP-L). Relative gene expression, $2^{-\Delta\Delta Ct}$, was generated using the unstimulated, non-treated conditioned media cultured chondrocytes as the control for **(A)** MMP-3 and **(B)** MMP-13. Data is shown as the mean ± standard deviation of $n = 5$. Differing letters indicate significant differences between groups ($p < 0.05$); statistical analysis was performed within each stimulation and not between stimulation groups. Asterisks (*) denote significant differences, when present, in stimulated NT groups from the unstimulated NT control. Blue bars = unstimulated synoviocytes, red bars = 10 ng/mL IL-1β stimulated synoviocytes, and green bars = 100 ng/mL LPS stimulated synoviocytes.

not surprising that the PRP-L used in this study would lead to enhanced cell growth of naïve synoviocytes under normal tissue culture conditions. It is surprising, though, that very similar fold changes in synoviocyte growth were observed for synoviocytes stimulated with either IL-1β or LPS and treated with PRP-L. The dramatic increases in synoviocyte growth observed under naïve and stimulated conditions when treated with PRP-L were consistent with the dramatic increases observed in HA production by synoviocytes under naïve and stimulated conditions when treated with PRP-L. PRP preparations have been previously reported to increase protein production of HA and to increase gene expression of Hyaluronan synthase-2 (HAS-2) in synoviocytes isolated from OA patients (63, 66). There is also a report of two different platelet gel supernatants isolated from a single horse that were able to increase HA production from synoviocytes under LPS stimulation compared to controls, but not to the same extent as observed in this current study (67). It is possible that the pooled nature of the PRP-L used in this study may have been in part responsible for the differences observed in HA production compared to previous studies evaluating a platelet preparation from a single donor. As discussed earlier, there is existing evidence in the literature to support increased efficacy of pooled PL preparations compared to individual donor preparations, as pooled preparations are able to capitalize on the natural cytokine variability that occurs in donors (31, 41). In this current study, we were unable to discern whether or not the dramatic increases in total HA concentration were due solely to the increases in synoviocyte cell numbers caused by PRP-L treatment or due in part to upregulation of HA production by synoviocytes treated with PRP-L. Future studies examining HAS-2 gene expression in synoviocytes following treatment with PRP-L are certainly warranted to determine all mechanisms involved.

The contribution of such a remarkable increase in synoviocyte HA production following PRP-L treatment on chondrocyte gene expression is unknown and also warrants further exploration. In our current study design, it is not possible to discern if the increases in collagen type II and aggrecan gene expression and the decrease in MMP-13 gene expression that were observed are due primarily to the high concentration of HA in the conditioned media of synoviocytes treated with PRP-L or due to other variables such as the high concentrations of platelet-derived growth factors found in PRP-L, the significant increase in synoviocyte IL-6 production caused by PRP-L treatment, or other factors that were not examined for. HA is known to bind to the cluster of differentiation 44 (CD44) receptors and thereby inhibit IL-1β expression resulting in decreased MMP production (68, 69). HA has also been shown to increase the proliferation of chondrocytes in tissue culture as well as to stimulate them to produce more collagen type II and aggrecan (70). Although IL-6 can be pro-inflammatory under certain conditions, it is known to induce the production of IL-1 and TNF antagonists by a variety of cell types including macrophages (71) and has been highlighted as a critical cytokine for the repair of other musculoskeletal tissues such as tendon (72, 73).

Another factor to consider that was not examined in this current study is the role of hypoxia-inducible factor (HIF) in PRP-L mediated chondroprotection and cartilage repair (37, 74–76). Several studies have previously demonstrated the critical role of HIF-1α in cartilage and in the nucleus pulposus for maintaining proper cellular function, including synthesis of extracellular matrix proteins, in a hypoxic environment (76–79). Both articular cartilage and the nucleus pulposus are highly avascular tissues with low intrinsic healing capacity and low oxygen tensions. This unique environment requires mechanisms adapted to support the survival of the tissue's resident cells, and HIF-1α is considered to be one of the main elements in such mechanisms. A recent study evaluating the effect of a pooled human PL preparation on growth-arrested progenitor cartilage cells found that PL induced the re-entry of such cells into the cell cycle (37). The cell activation and proliferation observed in this study was shown to correspond to induction of HIF-1 by PL (37). Consequently, we would speculate that PRP-L treatment of

inflamed or damaged cartilage may activate the HIF-1 pathway to increase cell proliferation and matrix synthesis, but this remains to be investigated.

In conclusion, treatment of inflamed synoviocytes with PRP-L *in-vitro* resulted in increased synoviocyte growth and increased total synoviocyte HA and IL-6 production. Challenge of chondrocytes with conditioned media from PRP-L treated synoviocytes then resulted in increased collagen type II and aggrecan gene expression as well as decreased MMP-13 gene expression. The results of this study support continued investigation into the use of pooled PRP-L for the treatment of osteoarthritis and warrant further *in-vitro* studies to discern the mechanisms of action of PRP-L.

AUTHOR CONTRIBUTIONS

We certify that all authors meet the qualifications for authorship as listed below: (1) substantial contributions to conception or design of the work or the acquisition, analysis, or interpretation of data for the work; (2) drafting the work or revising it critically for important intellectual content; (3) final approval of the version to be published; (4) agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Pharmacokinetics, Safety, and Clinical Efficacy of Cannabidiol Treatment in Osteoarthritic Dogs

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Objectives: The objectives of this study were to determine basic oral pharmacokinetics, and assess safety and analgesic efficacy of a cannabidiol (CBD) based oil in dogs with osteoarthritis (OA).

Methods: Single-dose pharmacokinetics was performed using two different doses of CBD enriched (2 and 8 mg/kg) oil. Thereafter, a randomized placebo-controlled, veterinarian, and owner blinded, cross-over study was conducted. Dogs received each of two treatments: CBD oil (2 mg/kg) or placebo oil every 12 h. Each treatment lasted for 4 weeks with a 2-week washout period. Baseline veterinary assessment and owner questionnaires were completed before initiating each treatment and at weeks 2 and 4. Hematology, serum chemistry and physical examinations were performed at each visit. A mixed model analysis, analyzing the change from enrollment baseline for all other time points was utilized for all variables of interest, with a $p \leq 0.05$ defined as significant.

Results: Pharmacokinetics revealed an elimination half-life of 4.2 h at both doses and no observable side effects. Clinically, canine brief pain inventory and Hudson activity scores showed a significant decrease in pain and increase in activity ($p < 0.01$) with CBD oil. Veterinary assessment showed decreased pain during CBD treatment ($p < 0.02$). No side effects were reported by owners, however, serum chemistry showed an increase in alkaline phosphatase during CBD treatment ($p < 0.01$).

Clinical significance: This pharmacokinetic and clinical study suggests that 2 mg/kg of CBD twice daily can help increase comfort and activity in dogs with OA.

Keywords: cannabidiol, CBD oil, hemp, canine, osteoarthritis, pharmacokinetic

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INTRODUCTION

Routine nonsteroidal anti-inflammatory drug (NSAID) treatments, though efficacious, may not provide adequate relief of pain due to osteoarthritis (OA) and might have potential side effects that preclude its use, particularly in geriatric patients with certain comorbidities, such as kidney or gastrointestinal pathologies (1–4). In a systematic review of 35 canine models of OA and 29 clinical trials in dogs, treatment with NSAIDs caused adverse effects in 35 of the 64 (55%)

studies, most commonly being gastro-intestinal signs (3). Although other pharmacological agents are advocated, such as gabapentin or amantadine, there is little evidence regarding their efficacy in dogs with chronic or neuropathic pain related to OA. Recent medical interest in alternative therapies and modalities for pain relief has led many pet owners to seek hemp related products rich in cannabinoids.

The endocannabinoid receptor system is known to play a role in pain modulation and attenuation of inflammation (5–7). Cannabinoid receptors (CB1 and CB2) are widely distributed throughout the central and peripheral nervous system (8–10) and are also present in the synovium (11). However, the psychotropic effects of certain cannabinoids prevent extensive research into their use as single agents for pain relief (5, 12). The cannabinoids are a group of as many as 60 different compounds that may or may not act at CB receptors. One class of cannabinoids, cannabidiol (CBD), may actually be an allosteric non-competitive antagonist of CB receptors (13). In lower vertebrates, CBD is also reported to have immunomodulatory (14), anti-hyperalgesic (15, 16), antinociceptive (17, 18), and anti-inflammatory actions (5, 19), making it an attractive therapeutic option in dogs with OA. Currently there are several companies distributing nutraceutical derivatives of industrial hemp, rich in cannabinoids for pets, yet little scientific evidence regarding safe and effective oral dosing exists.

The objectives of this study were to determine: (1) single-dose oral pharmacokinetics, (2) short-term safety, and (3) efficacy of this novel CBD-rich extract, as compared to placebo, in alleviating pain in dogs with OA. Our underlying hypotheses were that appropriate dosing of CBD-rich oil would safely diminish perceived pain and increase activity in dogs with OA.

MATERIALS AND METHODS

CBD Oil and Protocols Approval

The industrial hemp used in this study was a proprietary hemp strain utilizing ethanol and heat extraction with the final desiccated product reconstituted into an olive oil base containing ∼10 mg/mL of CBD as an equal mix of CBD and carboxylic acid of CBD (CBDa), 0.24 mg/mL tetrahydrocannabinol (THC), 0.27 mg/mL cannabichromene (CBC), and 0.11 mg/mL cannabigerol (CBG); all other cannabinoids were less than 0.01 mg/mL. Analysis of five different production runs using a commercial analytical laboratory (MCR Laboratories, Framingham, MA) show less than a 9% difference across batches for each of the detected cannabinoids listed above. The study was performed after the Cornell University institutional animal care and use committee (IACUC) approved the study following the guidelines for animal use according to the IACUC. Client owned dogs were enrolled after informed consent in accordance with the Declaration of Helsinki.

Abbreviations: CBD, cannabidiol; CB, cannabinoid; CBDa, carboxylic acid of CBD; THC, tetrahydrocannabinol; CBC, cannabichromene; CBG, cannabigerol; CBPI, Canine Brief Pain Inventory.

Pharmacokinetics

An initial investigation into single-dose oral pharmacokinetics was performed with 4 beagles (3.5–7 years, male castrated, 10.7–11.9 kg). Each dog received a 2 mg/kg and an 8 mg/kg oral dosage of CBD oil, with a 2-week washout period between each experiment. The dogs were fed 2 h after dosing. Physical examination was performed at 0, 4, 8, and 24 h after dosing. Attitude, behavior, proprioception, and gait were subjectively evaluated at each time point during free running/walking and navigation around standard traffic cones (weaving). Five milliliters of blood was collected at time 0, 0.5, 1, 2, 4, 8, 12, and 24 h after oil administration. Blood samples were obtained via jugular venipuncture and transferred to a coagulation tube for 20 min. Samples were centrifuged with a clinical centrifuge at 3,600 × g for 10 min; serum was removed and stored at −80°C until analysis using liquid chromatography-mass spectrometry (LC-MS) at Colorado State University Core Mass Spectrometry facility.

Extraction of CBD From Canine Serum and Mass Spectrometry Analysis

CBD was extracted from canine serum using a combination of protein precipitation and liquid-liquid extraction using n-hexane as previously described (20), with minor modifications for microflow ultra-high pressure liquid chromatography (UHPLC). Briefly, 0.05 mL of canine serum was subjected to protein precipitation in the presence of ice-cold acetonitrile (80% final concentration), spiked with deuterated CBD as the internal standard (0.06 mg/mL, CDB-d3 Cerilliant, Round Rock, TX, USA). 0.2 mL of water was added to each sample prior to the addition of 1.0 mL of hexane to enhance liquid-liquid phase separation. Hexane extract was removed and concentrated to dryness under laboratory nitrogen. Prior to LC-MS analysis, samples were resuspended in 0.06 mL of 100% acetonitrile. A standard curve using the CBD analytical standard was prepared in canine serum non-exposed to CBD and extracted as above. Cannabidiol concentration in serum was quantified using a chromatographically coupled triple-quadropole mass spectrometer (UHPLC-QQQ-MS) using similar methods as previously described (21).

CDB Serum Concentration Data Analysis

From the UHPLC-QQQ-MS data, peak areas were extracted for CBD detected in biological samples and normalized to the peak area of the internal standard CBD-d3, in each sample using Skyline (22) as well as an in-house R Script (www.r-project.org). CBD concentrations were calculated to nanograms per mL of serum as determined by the line of regression of the standard curve ($r^2 = 0.9994$, 0–1,000 ng/mL). For this assay, the limits of detection (LOD) and limits of quantification (LOQ) represent the lower limits of detection and quantification for each compound in the matrix of this study (23, 24). Pharmacokinetic variables were estimated by means of non-compartmental analysis, utilizing a pharmacokinetic software package (PK Solution, version 2.0, Montrose, CO, USA).

Inclusion and Exclusion Criteria for the Clinical Trial

The study population consisted of client-owned dogs presenting to Cornell University Hospital for Animals for evaluation and treatment of a lameness due to OA. Dogs were considered for inclusion in the study if they had radiographic evidence of OA, signs of pain according to assessment by their owners, detectable lameness on visual gait assessment and painful joint(s) on palpation. Each dog had an initial complete blood count ([CBC] Bayer Advia 120, Siemens Corp., New York, NY, USA) and serum chemistry analysis (Hitachi 911, Roche Diagnostics, Indianapolis, IN, USA) performed to rule out any underlying disease that might preclude enrolment. Elevations in alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were allowed if prior hepatic ultrasound was deemed within normal limits except for potential non-progressive nodules (possible hepatic nodular hyperplasia). All owners completed a brief questionnaire to define the affected limb(s), duration of lameness, and duration of analgesic or other medications taken. All dogs underwent radiographic examination of affected joints and a radiologist confirmed the presence or absence of OA, and excluded the presence of concomitant disease that might preclude them from enrolment (i.e., lytic lesions).

During the trial, dogs were only allowed to receive NSAIDs, fish oil, and/or glucosamine/chondroitin sulfate without any change in these medications for 4 weeks prior to or during the 10-week study period as standard of care for the disease process. Other analgesic medications used, such as gabapentin and tramadol, were discontinued at least 2 weeks prior to enrolment. Dogs were excluded if they had evidence of renal, uncontrolled endocrine, neurologic, or neoplastic disease, or were undergoing physical therapy. Every dog was fed its regular diet with no change allowed during the trial.

Clinical Trial

The study was a randomized, placebo-controlled, owner and veterinarian double-blind, cross-over trial. Dogs received each of two treatments in random order (Randomizer iPhone Application): CBD, 2 mg/kg every 12 h, or placebo (an equivalent volume of olive oil with 10 parts per thousands of anise oil and 5 parts per thousands of peppermint oil to provide a similar herbal smell) every 12 h. Each treatment was administered for 4 weeks with a 2-week washout period in between treatments. Blood was collected to repeat complete blood counts and chemistry analysis at weeks 2 and 4 for each treatment.

At each visit, each dog was evaluated by a veterinarian based on a scoring system previously reported (25) as well as by its owner (canine brief pain inventory [CBPI], Hudson activity scale) before treatment initiation and at weeks 2 and 4 thereafter (26–28).

Statistical Analysis

Initial power analysis was performed to assess number of dogs needed for this study as a cross over design with a power set 0.80 and alpha of 0.05 using prior data suggesting a baseline CBPI or Hudson score change of ∼15 points (two tailed) with a standard deviation of 20. When calculated it was assumed that 14 dogs would be necessary to find differences in outcomes of interest (29).

Statistical analysis was performed with a commercially available software package (JMP 12.0, Cary, NC, USA). All continuous data were assessed utilizing a Shapiro–Wilk test for normality. Considering a majority of our blood, serum and scoring data were normally distributed a mixed model analysis was used to analyze these outcomes, including the fixed effects of treatment, time, sequence of treatment assignment, gender, age, NSAID usage, treatment × time; as well as random effects of observation period, period nested within dog, time point nested within period nested within dog to account for the hierarchical nature of data in a cross-over design as well as repeated measurements for each dog. For ordinal veterinary scoring data a similar linear mixed model was used, but differences from baseline were first calculated to approximate a normal distribution to meet assumptions for a mixed model analysis. Residual diagnostics of all final models showed that residuals were normally distributed and fulfilled the assumption of homoscedasticity, and assumptions where therefore met. This statistical modeling approach allowed for adequate control of hierarchical data structure necessary in a cross-over design, as well as for the performance of easily interpretable time × treatment Tukey *post-hoc* comparisons that were our main interest, as compared to an ordinal logistical regression (30, 31). To control for baseline differences and therefore the possible difference in relative change in CBPI pain, and activity interference assessments and Hudson scoring across dogs, the initial CPBI or Hudson Scores were included for these analyses as a covariate. Pairwise comparisons between all-time points of both groups were corrected for multiple comparisons with Tukey's *post-hoc* tests to examine the interaction of time and treatment variables, and to assess differences between change from baseline at any time point as they related to treatment. A p-value of less than 0.05 was defined as the significance cut-off.

RESULTS

Pharmacokinetics

Pharmacokinetics demonstrated that CBD half-life of elimination median was 4.2 h (3.8–6.8 h) for the 2 mg/kg dose, and 4.2 h (3.8–4.8 h) for the 8 mg/kg dose (**Table 1**). Median maximal concentration of CBD oil was 102.3 ng/mL (60.7–132.0 ng/mL; 180 nM) and 590.8 ng/mL (389.5–904.5 ng/mL; 1.2 uM) and was reached after 1.5 and 2 h, respectively, for 2 and 8 mg/kg doses. No obvious psychoactive properties were observed on evaluation at any time point during the 2 and 8 mg/kg doses over 24 h. These results led to dosing during the clinical trial at 2 mg/kg body weight every 12 h, due the cost prohibitive nature of 8 mg/kg dosing for most larger patients, the impractical nature of more frequent dosing, the volume of oil necessary and anecdotal reports surrounding 0.5-2 mg/kg dosing recommended by other vendors.

TABLE 1 | Serum pharmacokinetic of single oral dosing (2 mg and 8 mg/kg) of CBD oil in dogs.

	Cmax (ng/mL)	Tmax (h)	T1/2 elim (h)	AUC 0-t (ng-hr/mL)	MRT (h)
DOSE (2 mg/kg)					
Dog 1	61	1	4.4	183	6.0
Dog 2	132	1	3.9	351	4.2
Dog 3	102	2	3.8	382	5.1
Dog 4	101	2	6.8	437	9.1
Median (Range)	102 (61–132.0)	1.5 (1.0–2.0)	4.2 (3.8–6.8)	367 (183–437)	5.6 (4.2–9.1)
DOSE (8 mg/kg)					
Dog 1	499	2	3.8	2,928	5.7
Dog 2	389	1	4.8	1,753	7.0
Dog 3	905	2	4.2	3,048	5.1
Dog 4	682	2	4.1	2,389	5.2
Median (Range)	591 (389–905)	2.0 (1.0–2.0)	4.2 (3.8–4.8)	2,658 (1,753–3,048)	5.6 (5.1–7.0)

Cmax, maximum concentration; Tmax, time of maximum concentration; T1/2 el, half-life of elimination; AUC 0-t, area under the curve (time 0–24 h); MRT, median residence time.

Dogs Included in the Clinical Trial

Twenty-two client-owned dogs with clinically and radiographically confirmed evidence of osteoarthritis were recruited. Sixteen of these dogs completed the trial and were included in the analyses; their breed, weight, age, sex, worse affected limb, radiographic findings, use of NSAIDs and sequence of treatments are summarized in **Table 2**. Dogs were removed due to osteosarcoma at the time of enrolment, gastric torsion (placebo oil), prior aggression issues (CBD oil), pyelonephritis/kidney insufficiency (CBD oil), recurrent pododermatitis (placebo oil), and diarrhea (placebo oil).

Clinical Trial

CBPI and Hudson change from baseline scores showed a significant decrease in pain and increase in activity ($p < 0.01$) at week 2 and 4 during CBD treatment when compared to baseline week 0, while no other statistical significances were observed across treatment in this cross-over design (**Table 3**). Lameness as assessed by veterinarians showed an increase from baseline in lameness with age ($p < 0.01$), whereas NSAID use ($p = 0.03$) reduced lameness scores. Veterinary pain scores showed a decrease from baseline in dogs on NSAIDs ($p < 0.01$). CBD oil resulted in a decrease in pain scores when compared to baseline on evaluation at both week 2 and week 4 ($p < 0.01$ and $p = 0.02$, respectively), and week 2 CBD oil treatment was lower than baseline placebo treatment ($p = 0.02$) and week 4 placebo treatment ($p = 0.02$). No other veterinary pain comparisons were statistically significant. No changes were observed in weight-bearing capacity when evaluated utilizing the veterinary lameness and pain scoring system (**Table 3**).

Chemistry analysis and CBC were performed at each visit. No significant change in the measured CBC values was noted in either the CBD oil or placebo treated dogs (data not shown). Serum chemistry values were not different between placebo compared to CBD oil (**Table 4**), except for alkaline phosphatase (ALP) which significantly increased over time from baseline by week 4 of CBD oil treatment ($p < 0.01$); with nine of the 16 dogs showing increases over time (**Figure 1**). Glucose was increased in dogs receiving the placebo oil at each time point ($p = 0.04$) and creatinine levels increased over time in both dogs receiving CBD oil and those receiving placebo oil ($p < 0.01$); though all values remained within reference ranges. Other notable significances in serum chemistry values were associated with primarily age or NSAID use. An increase in age was associated with significantly higher blood urea nitrogen (BUN; $p < 0.01$), calcium ($p = 0.01$), phosphorus ($p < 0.01$), alanine aminotransferase (ALT; $p = 0.03$), alkaline phosphatase (ALP; $p = 0.01$), gamma glutamyltransferase (GGT; $p = 0.02$), globulin ($p = 0.02$), and cholesterol ($p < 0.01$) values. NSAID use was associated with significantly higher BUN ($p = 0.003$), and creatinine ($p = 0.017$), and significant decreases in total protein ($p < 0.001$) and serum globulin ($p < 0.001$).

DISCUSSION

To date, an objective evaluation of the pharmacokinetics of a commercially available industrial hemp product after oral dosing in dogs is absent. This study showed that the terminal half-life of oral CBD, as the most abundant cannabinoid in this specific preparation when in an oil base, was between 4 and 5 h, suggesting it was bioavailable with a dosing schedule of 2 mg/kg at least twice daily. This half-life was shorter than a previous report after intravenous (1.88–2.81 and 3.75–5.63 mg/kg) and oral (7.5–11.25 mg/kg) administration (32). In the intravenous study, CBD distribution was rapid, followed by prolonged elimination with a terminal half-life of 9 h. When examining prior oral CBD bioavailability it was determined to be low and highly variable (0–19% of dose) with three dogs showing no absorption. This may be due to the first pass effect in the liver, and the product was not in an oil base, but a powder within a gelatin capsule being a different delivery vehicle (32). After initially seeing no neurological effects at the 2 mg/kg dose a 8 mg/kg dose was chosen to assess the potential neurological effects since mistaken overdosing can occur clinically, and a higher dose might have been necessary since the prior study showed poor absorption. Although our dogs were fasted the

TABLE 2 | Characteristics of dogs enrolled in a placebo-controlled study investigating the effects of CBD on osteoarthritis.

Breed	Weight (kg)	Age (years)	Sex	Radiographic findings and OA localization	NSAID
Rottweiler	35.3	10	FS	- Moderate, intracapsular swelling with moderate osteophytosis, left stifle	Carprofen (2.1 mg/kg BID)
Mix	30.6	13	MC	- Moderate-to-severe, right-shoulder osteoarthrosis; mild, left-shoulder osteoarthrosis - Moderate-to-severe, bilateral hip osteoarthrosis	None
Mix	27.2	9	FS	- Moderate medial coronoid remodeling (with fragmentation on the right) and bilateral elbow osteoarthrosis	None
Mix	30.5	14	MC	- Moderate enthesiopathies on right carpus; mild, left-antebrachiocarpal osteoarthrosis - Bilateral moderate coxofemoral osteoarthrosis	None
Mix	23.5	10	FS	- Moderate bilateral stifle osteoarthrosis and moderate intracapsular swelling	Carprofen (2.2 mg/kg)
Mix	28.1	10	FS	- Moderate bilateral elbow osteoarthrosis - Moderate left-stifle osteoarthrosis with intracapsular swelling	Metacam (0.1 mg/kg
English Bulldog	25.2	8	MC	- Severe osteoarthrosis, left elbow - Moderate intracapsular swelling and mild osteoarthrosis, right stifle	Carprofen (2 mg/kg BID)
German Shorthaired Pointer	21.5	14	FS	- Moderate bilateral elbow osteoarthrosis	Carprofen (2.4 mg/kg BID)
Labrador Retriever	26.1	13	FS	- Bilateral severe stifle osteoarthrosis due to cranial cruciate ligament disease	Meloxicam (0.1 mg/kg SID)
Mix	18.2	13	FS	- Bilateral moderate elbow osteoarthrosis and medial epicondylitis	Meloxicam (0.1 mg/kg SID)
Mix	22	9	FS	- Moderate, stifle osteoarthrosis with moderate intracapsular swelling	None
Bernese Mountain Dog	50	3	M	- Bilateral severe elbow osteoarthritis, medial coronoid disease, and medial epicondylitis	Carprofen (2 mg/kg SID)
Belgian Malinois	25.1	9	FS	- Severe bilateral elbow osteoarthrosis - Bilateral moderate hip osteoarthrosis	Carprofen (2 mg/kg BID)
Mix	28.6	13	FS	- Severe bilateral elbow osteoarthritis - Severe bilateral hip osteoarthritis	None
Border Collie	22	14	MC	- Severe thoracolumbosacral osteophytosis - Multifocal carpal enthesiophytes	None
Beagle	17.6	5	MC	- Mild left elbow osteoarthrosis, with possible medial coronoid disease - Moderate-to-severe bilateral stifle osteoarthrosis	None

FS, female spayed; MC, male castrated; Mix, mixed breed; SID, once daily; BID, twice daily.

delivery vehicle was olive oil which is a food item. The absorption may be greater and more consistent because of the oil-based vehicle which may be due to the lipophilic nature of CBD, hence delivery with food may be preferable (32, 33). As previously demonstrated, CBD biotransformation in dogs involves hydroxylation, carboxylation and conjugation, leading to relatively rapid elimination suggesting a more frequent dosing schedule (34). The dosing schedule of twice per day was chosen due to the practical nature of this dosing regimen even though the elimination is well within a three or four time a day dosing regimen. Our hope was that the lipophilic nature of CBD would allow for a steady state over time, and future studies examining 24 h pharmacokinetics with different dosing regimens with larger numbers of dogs, and steady state serum pharmacokinetics after extended treatment in a clinical population are sorely needed.

The main objective of this study was to perform an owner and veterinary double-blinded, placebo-controlled, cross-over study to determine the efficacy of CBD oil in dogs affected by OA. Despite our small sample size, short study duration and heterogeneity of OA signs, CBPI and Hudson scores showed that CBD oil increase comfort and activity in the home environment for dogs with OA. Additionally, veterinary assessments of pain were also favorable. Although a caregiver placebo effect should be considered with subjective evaluations by owners and veterinarians (35), the cross-over design limits confounding covariates since each dog serves as its own control. Our statistical model controlled for the possible effect of treatment sequence. The lack of a placebo effect in our study may be due to nine of the 16 owners being intimately involved in veterinary medical care, all of whom have an understanding of the placebo effect making them more cognizant of improvements when providing feedback. In addition, there was a noticeable decrease in Hudson scores and rise in CBPI scores during the initiation placebo treatment suggesting a potential carry over effect of CBD treatment indicating that a longer washout period might be indicated in future studies. This carry over effect may have resulted in some improved perceptions at the initiation of the placebo treatment which were eliminated by week 4 of placebo treatment, underscoring the importance of longer term steady state PK studies in dogs.

TABLE 3 | Canine Brief Pain Inventory (Pain and Activity questions) and Hudson Scale mean and standard deviation; lameness, weight-bearing and pain scores median and ranges at each time for cannabidiol (CBD) and placebo oils.

	CBD oil			Placebo oil		
	Week 0	**Week 2**	**Week 4**	**Week 0**	**Week 2**	**Week 4**
CBPI Pain (0–40)	21 ± 8	14 ± 6*	14 ± 8*	17 ± 7	19 ± 9	19 ± 9
CBPI activity interference (0–60)	35 ± 15	25 ± 15*	26 ± 14*	27 ± 15	29 ± 15	31 ± 16
Hudson (0–110)	54 ± 13	67 ± 15*	67 ± 10*	65 ± 14	64 ± 16	60 ± 19
Veterinary lameness§	3 (1–4)	3 (1–4)	3 (1–4)	3 (2–4)	3 (2–4)	3 (1–4)
Veterinary pain ∫	3 (3–4)	3 (2–4)*	3 (1–4)*	3 (2–4)**	3 (2–4)	3 (2–4)**
Veterinary weight-bearing =	2 (1–3)	2 (1–3)	2 (1–3)	2 (1–3)	2 (1–3)	2 (1–3)

**Represents significant difference ($p < 0.05$) from baseline week 0 of CBD treatment. **Represents significant differences ($p < 0.05$) from week 2 of CBD oil treatment. §Lameness was scored as follows: 1 = no lameness observed/walks normally, 2 = slightly lame when walking, 3 = moderately lame when walking, 4 = severely lame when walking, 5 = reluctant to rise and will not walk more than 5 paces. ∫Pain on palpation was scored as follows: 1 = none, 2 = mild signs, dog turns head in recognition, 3 = moderate signs, dog pulls limb away, 4= severe signs, dog vocalizes or becomes aggressive, 5 = dog will not allow palpation. =Weight-bearing was scored as follows: 1 = equal on all limbs standing and walking, 2 = normal standing, favors affected limb when walking, 3 = partial weight-bearing standing and walking, 4 = partial weight-bearing standing, non-weight-bearing walking, 5 = non-weight-bearing standing and walking.*

TABLE 4 | Serum chemistry values of dogs receiving CBD or placebo oils.

	Reference	CBD oil			Placebo oil		
		Week 0	**Week 2**	**Week 4**	**Week 0**	**Week 2**	**Week 4**
Sodium	145–153 mEq/L	149 ± 3	149 ± 2	149 ± 1	149 ± 1	149 ± 2	149 ± 2
Potassium	4.1–5.6 mEq/L	4.9 ± 0.3	4.9 ± 0.5	4.9 ± 0.3	4.8 ± 0.4	4.9 ± 0.4	4.9 ± 0.3
Chloride	105–116 mEq/L	110 ± 3	109 ± 3	109 ± 2	110 ± 2	110 ± 2	110 ± 2
SUN	10–32 mg/dL	20 ± 9	20 ± 7	20 ± 6	19 ± 6	21 ± 7	19 ± 6
Creatinine	0.6–1.4 mg/dL	1.0 ± 0.3	1.1 ± 0.3*	1.0 ± 0.3*	0.9 ± 0.3	1.0 ± 0.3*	1.0 ± 0.3*
Calcium	9.3–11.4 mg/dL	10.4 ± 0.5	10.4 ± 0.4	10.3 ± 0.4	10.4 ± 0.6	10.4 ± 0.4	10.4 ± 0.4
Phosphorus	2.9–5.2 mg/dL	3.8 ± 0.8	3.9 ± 0.8	3.9 ± 0.6	4.0 ± 0.7	3.9 ± 0.6	4.0 ± 0.5
Magnesium	1.4–2.2 mg/dL	1.8 ± 0.2	1.8 ± 0.2	1.8 ± 0.2	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1
Glucose	63–118 mg/dL	92 ± 9	89 ± 9	92 ± 9	97 ± 10*	93 ± 8	97 ± 10*
ALT	20–98 U/L	93 ± 86	93 ± 88	114 ± 119	90 ± 89	222 ± 606	166 ± 284
AST	14–51 U/L	31 ± 8	33 ± 13	34 ± 16	30 ± 8	56 ± 99	45 ± 34
ALP	17–111 U/L	160 ± 212	238 ± 268	323 ± 407*	204 ± 287	186 ± 287	175 ± 248
GGT	0–6 U/L	4 ± 3	3 ± 2	3 ± 2	3 ± 2	4 ± 6	5 ± 4
Bilirubin	0.0–0.2 mg/dL	0.1 ± 0.1	0.0 ± 0.1	0.1 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1
Total protein	5.3–7.0 g/dL	6.3 ± 0.4	6.4 ± 0.5	6.3 ± 0.4	6.3 ± 0.4	6.3 ± 0.4	6.3 ± 0.4
Albumin	3.1–4.2 g/dL	3.7 ± 0.2	3.7 ± 0.2	3.7 ± 0.2	3.7 ± 0.2	3.7 ± 0.2	3.7 ± 0.2
Globulin	1.9–3.6 g/dL	2.6 ± 0.3	2.6 ± 0.4	2.6 ± 0.4	2.6 ± 0.4	2.6 ± 0.4	2.6 ± 0.4
Cholesterol	138–332 mg/dL	291 ± 64	301 ± 62	302 ± 62	295 ± 71	300 ± 71	308 ± 83
CK	48–260 U/L	148 ± 81	147 ± 59	134 ± 61	139 ± 57	158 ± 80	168 ± 105

Data presented at mean + standard deviations. Asterisk (*) indicates significantly different ($p < 0.05$) serum concentration from baseline week 0 CBD treatment. SUN , serum urea nitrogen; ALT, alanine animotranferase; AST, aspartate animotransferase; ALP, alkaline phosphatase; GGT, gamma glutamyl transferase; CK, creatine kinase.

There was no significant difference in subjective veterinary lameness score and weight-bearing capacity throughout the study. Kinetic data was obtained from these dogs (data not shown), however 11 of the 16 dogs had significant bilateral disease (stifle, coxofemoral, or elbow) making evaluation of peak vertical force or symmetry tenuous at best. Unilateral disease in any of the aforementioned joints would be ideal to study the kinetic effects of this or similar extracts for pain relief leading to better objective outcomes. The population we used in our investigation was representative of dogs presenting in a clinical setting for management of OA and represents the typical OA patient.

Currently, NSAIDs are the primary treatment for OA and are associated with negative effects on the gastrointestinal tract and glomerular filtration (2). In the current study, no significant difference was noted in BUN, creatinine, or phosphorus between dogs treated with the CBD oil vs. the placebo oil, while NSAID treatment resulted in a higher creatinine concentration. A mild rise in creatinine from baseline was noted in both groups at weeks 2 and 4, the hydration status of the dogs was

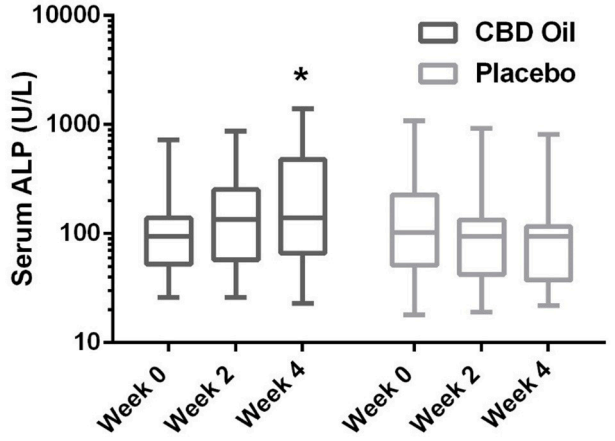

FIGURE 1 | Box-and-whisker plot of serum alkaline phosphatase (ALP) activity at each time for treatment and placebo oils. Box represents the mean and 25th and 75th percentile and the whiskers represent the 99th and 1st percentiles. *Indicates a significant difference ($p < 0.05$) from week 0 CBD treatment.

unknown; however changes in albumin sodium, and chloride were unchanged suggesting euhydration, and all creatinine values remained within the reference interval. Increased ALP activity is fairly sensitive for hepatobiliary changes in this age group, but not specific. Increased ALP activity noted in nine dogs in the CBD treatment group may be an effect of the hemp extract attributed to the induction of cytochrome p450 mediated oxidative metabolism of the liver (reported previously with prolonged exposure to cannabis) (36–38). Other causes of cholestasis, increased endogenous corticosteroid release from stress, or a progression of regenerative nodular hyperplasia of the liver cannot be ruled out. Without concurrent significant rise in ALT in the CBD treatment to support hepatocellular damage, or biopsy for further clarification, the significance is uncertain. As such, it may be prudent to monitor liver enzyme values (especially ALP) while dogs are receiving industrial hemp products until controlled long term safety studies are published.

A recent survey reported that pet owners endorse hemp based treats and products because of perceived improvement in numerous ailments, as hemp products were moderately to very helpful medicinally (39). Some of the conditions thought to be relieved by hemp consumption were: pain, inflammation, anxiety and phobia, digestive system issue, and pruritus (39). One immunohistochemical study suggested that cannabinoids could protect against the effects of immune-mediated and inflammatory allergic disorders in dogs (40) whereas another uncontrolled study suggested that CBD has anticonvulsant and anti-epileptic properties in dogs (41). The apparent analgesic effect of the industrial hemp based oil observed in the present study may be attributable to downregulation of cylooxygenase enzymes, glycine interneuron potentiation, transient receptor potential cation receptor subfamily V1 receptor agonism (peripheral nerves), and/or g-protein receptor 55 activation (immune cells), influencing nociceptive signaling and/or inflammation (14, 42, 43).

The industrial hemp product used in this study is a proprietary strain-specific extract of the cannabinoids outlined in the methods with relatively high concentrations of CBD and lesser quantities of other cannabinoids as well as small amounts of terpenes that may have synergistic effects often termed the "entourage effect." This brings to light that fact that different strains of cannabis produce differing amounts of CBD and other related cannabinoids making the results of this study specific to this industrial hemp extract that may not translate to other available products due to differing cannabinoid concentrations in this largely unregulated market.

In conclusion, this particular product was shown to be bioavailable across the small number of dogs examined in the PK portion of the study, and dogs with OA receiving this industrial hemp extract high in CBD (2 mg/kg of CBD) were perceived to be more comfortable and active. There appear to be no observed side effects of the treatment in either the dogs utilized in the PK study at 2 and 8 mg/kg, or dogs undergoing OA treatment for a month duration. There were some dogs with incidental rises in alkaline phosphatase that could be related to the treatment. Further long-term studies with larger populations are needed to identify long-term effects of CBD rich industrial hemp treatment, however short term effects appear to be positive.

AUTHOR CONTRIBUTIONS

L-JG was responsible for data analysis and interpretation, drafting of the manuscript and approval of the submitted manuscript. JB was responsible for the conception of the study and manuscript writing and revisions. CF was responsible for acquisition of data and manuscript revision. WS was responsible for pharmacokinetic evaluation and revision of the manuscript. SM was responsible for statistical analysis, data analysis and revision of the manuscript. LW was responsible for laboratory work including liquid chromatography-mass spectrometry. HB was responsible for interpretation of the blood work and manuscript revision. EB was responsible for acquisition of data, and data analysis. JW was responsible for the conception of study, supervised data collection, statistical analysis, and manuscript editing.

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REVIEW
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The Importance of Subchondral Bone in the Pathophysiology of Osteoarthritis

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Subchondral bone plays a critical role in the pathogenesis of osteochondral disease across veterinary species. The subchondral bone is highly adaptable, with the ability to model and remodel in response to loading stresses experienced by the joint. Repetitive stress injuries within the joint can result in primary or secondary pathologic lesions within the subchondral bone, which have been recognized to contribute to the development and progression of osteoarthritis. Recent advances in diagnostic imaging, particularly volumetric imaging modalities have facilitated earlier identification of subchondral bone disease. Despite these advancements, limitations in our knowledge about subchondral bone makes treatment and prevention of these conditions challenging. The purpose of this report is to review our current understanding of subchondral bone and its relationship to osteoarthritis across veterinary species, with a specific focus in the research that has been performed in horses. It can be concluded that our current understanding of subchondral bone is advancing, and future experimental, clinical and pathologic studies will provide additional insight about subchondral bone and its relationship to joint disease.

Keywords: subchondral bone, osteochondral unit, repetitive stress, chronic fatigue, exercise, horse, osteoarthritis

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INTRODUCTION

As our understanding of the underlying pathophysiology of osteoarthritis (OA) grows, we have begun to recognize that OA is a disease of not just articular cartilage, but of the osteochondral unit. The osteochondral unit is composed of articular cartilage, calcified cartilage, and subchondral and trabecular bone, which work synergistically to support functional loading of the joint (1, 2). Subchondral bone has received particular attention in recent years, as derangements in this essential tissue have been recognized for its contribution to the development and progression of OA. In this review, we will begin with a brief discussion of the anatomy, physiology, and biomechanical principles that guide subchondral bone function. We will then delve into the specific conditions of subchondral bone, specifically subchondral bone disease, repetitive stress injury, and chronic fatigue injury. We will conclude with some general principles of diagnosis and a brief discussion of treatment and prevention strategies.

ANATOMY AND PHYSIOLOGY OF SUBCHONDRAL BONE

The subchondral bone is located deep to the articular cartilage, but remains connected to it through a layer of calcified cartilage. The subchondral bone varies in architecture and physiology by region, from the more compact layer of bone adjacent to the calcified cartilage (subchondral bone plate),

to the trabecular bone closer to the medullary cavity. Normal subchondral bone plate is a thin layer of bone ranging from 10 μm to 3 mm in thickness, depending on the location (3). The character of subchondral bone also differs depending on the thickness: the thinner areas are predominantly appositional layers continuous with trabeculae and with a low number of haversian canals; while the thicker areas are composed predominantly of a network of osteons (4). The function of the subchondral bone is to attenuate forces generated through locomotion, with the compact subchondral bone plate providing firm support and the subchondral trabecular component providing elasticity for shock absorption during joint loading (3). Maintenance of this intrinsic joint elasticity is essential for the biomechanical principles of locomotion.

Subchondral bone is a biphasic material, which includes an inorganic component composed of hydroxyapatite crystals for rigidity, and an organic component composed of predominantly type I collagen, proteoglycan, glycosaminoglycans, and water affording elasticity and pliancy (3). The composition of subchondral bone is uniquely designed to disperse axial loads across the joint, sparing the overlying articular cartilage (5–8). Subchondral bone has the innate ability to display a range of responses, reflecting both acute stresses as well as more prolonged, chronic, adaptive responses within the joint. At one end of the spectrum, subchondral bone is responsible for dissipating forces generated by locomotion, considering it has been shown to be 10 times more deformable than the cortical shaft of long bones (9). Further along the spectrum, subchondral bone is able to physically adapt its morphology in response to stresses placed on the joint. The adaptive capabilities of subchondral bone follow Wolff's Law, which states that bone will adapt in response to the loading under which it is subjected (10). The adaptive response is facilitated through the formative and resorptive activities of osteoblasts and osteoclasts, respectively, and is macroscopically observed within the trabecular bone. The rich vascularization and innervation of subchondral bone facilitates a comprehensive and extensive local response to both physiologic and pathologic alterations within the bone.

BIOMECHANICS AND PATHOPHYSIOLOGY OF SUBCHONDRAL BONE

Unlike other tissues within the joint, subchondral bone is highly responsive to loading, with the ability to respond quickly to training and injury (11). The forces incurred by the articular cartilage are transmitted to the subchondral bone across the calcified cartilage layer, which is uniquely adapted to distribute forces and minimize shear stresses on the articular cartilage layer through an undulating association with subchondral bone (12). Deeper within the bone, compliance of the trabecular bone is essential for the joint to deform during loading and help to dissipate this energy across the layers of the joint. The different layers of the joint work in concert with one another to facilitate support and force distribution: the articular cartilage is supported by the calcified cartilage, which is supported by the subchondral bone plate, which is in turn supported by the subchondral trabecular bone and ultimately the cortical bone.

The complex balance of function between the different layers must compensate for the rate and coordinated loading of the joint, as these are the two most important factors in the bone's ability to respond to imposed stresses. Joint shape and ligamentous attachments confine joint motion and in doing so affect the pattern of response observed within the subchondral bone. The muscles or tendons which span the joint are the primary contributors of surface loads, which are generated to counteract the rotational forces secondary to the ground reaction force acting on the moment arm of the limb (2, 13) The generated forces are not equal however, as the moment arm of the tendon is typically shorter than that of the limb. Because of this, the force generated from the tendon is much larger than the ground reaction force, resulting in an amplification of contact forces within the joint and at the subchondral bone.

The joint is able to respond to repetitive loading through the adaptive processes of bone modeling and remodeling. Bone modeling is defined as bone formation and resorption at anatomically distinct sites to produce functionally and mechanically purposeful architecture (14). Bone modeling involves the geometric sculpting of bone by formation and/or resorption. Osteoclasts and osteoblasts work independently, and is typically characterized by a greater amount of bone formation than resorption. Modeling occurs at both the macroscopic and microscopic levels, with variation in the size and shape of the joints and microarchitecture observed over time. Modeling in subchondral bone typically manifests microscopically as changes in the microarchitecture, with trabecular infilling; and macroscopically as a thickening of the bone. These alterations change the mechanical properties in the bone, resulting in stiffer bone with decreased elasticity and reduced capacity for shock absorption. The bone may thicken in response to increased physical demands, as either a method to strengthen the bone or to reset the physiologic threshold, such as in cases of juvenile animals or those returning to exercise.

Bone remodeling is the coordinated activity of osteoclasts and osteoblasts to remove biomechanically inferior packets of bone and replace it with new bone (11). The processes of bone formation and resorption are asynchronously coupled, with small packets of abnormal or damaged bone resorbed by osteoclasts, followed by the recruitment of osteoblastic precursors which then differentiate and replace the removed bone (15). Importantly, there is a delay in the replacement of diseased bone, with osteoclastic processes occurring within weeks, while osteoblastic replacement of bone is slower and occurs over months. As this process occurs, the delay in new bone results in a relative osteoporosis at the site of bone replacement, as the bone is initially weakened after osteoclasts have removed the inferior bone, prior to the osteoblastic replacement with new bone. This transient osteoporosis is most

notable 60–120 days after the initial injury, and clinicians must be cognizant that affected bone is at an increased risk of fracturing during this period if compounded with a complete lack of physical activity (16). For this reason, controlled activity—such as hand walking or paddock turnout—is typically recommended to mitigate the potential for fracture during this time.

When the adaptive capabilities of the bone are exceeded—especially in cases of corresponding degradation of the articular cartilage layer—sclerosis, osteophytes and fibrocartilaginous repair tissues are visible within the osteochondral unit. Subchondral bone follows the innate properties of all tissues, as there is a strain threshold beyond which normal adaptive processes are unable to compensate and pathological events progress resulting in subchondral bone damage. Sclerosis may be observed with subchondral bone damage, which results in a decreased elasticity within the subchondral bone plate and trabecular bone. This thickening within the subchondral bone in turn affects the ability of the articular cartilage to withstand mechanical loading, by increasing transverse stresses at the base of the articular cartilage layer, resulting in horizontal clefts within the deep zone of cartilage. With continued loading, these clefts can progress to the articular surface of the cartilage, perpetuating the cycle of OA changes within the joint (**Figure 1**) (18–20). The point at which this adaptive process becomes pathological is influenced by a multitude of factors, and includes (but is not limited to) location (i.e., within and between joints), horse size, speed, discipline, and amount of training (2). Clinically these changes result in the perception of pain, as the rich nerve supply of subchondral bone is one of the main mechanisms of pain perception in the joint disease (3).

The complex balance of adaptive and maladaptive bone modeling is further complicated by the fact that microdamage is observed even in well-adapted bones. Osteoclasts remove damaged bone, which is then replaced with new bone by osteoblasts. Fatigue injuries, also sometimes referred to as "repetitive stress injuries" or "chronic fatigue injuries," have been observed most commonly in both Thoroughbred and Standardbred racehorses. These fatigue injuries occur when microdamage accumulates faster than remodeling can repair. Additionally, it is also accepted that remodeling activities are inhibited in a high load environment due to reduced recruitment of osteoclasts (16). Pathologically fatigued bone clinically manifests in one of two ways: either as overt, mechanical failure in the form of a fracture, or as biological or functional failure where subtle changes occur resulting in the inability of the bone to sustain the expected demands of activity. Biological or functional failure is typically the result of more insidious changes within the joint, resulting in abnormal or inefficient transmission of loads, incongruent articular surfaces, and may include compromised perfusion or inadequate physical support from the adjacent articular cartilage (2). Fractures of fatigued bone can be further characterized as to whether they are the result of supra-physiological loads or the result of cumulative microdamage resulting in excessive fatigue within the bone. Taken together, the more contemporary research has demonstrated strong evidence that subchondral bone changes are not simply a secondary sign of OA, but rather can be an initiating factor of degeneration of the health of the joint (21, 22).

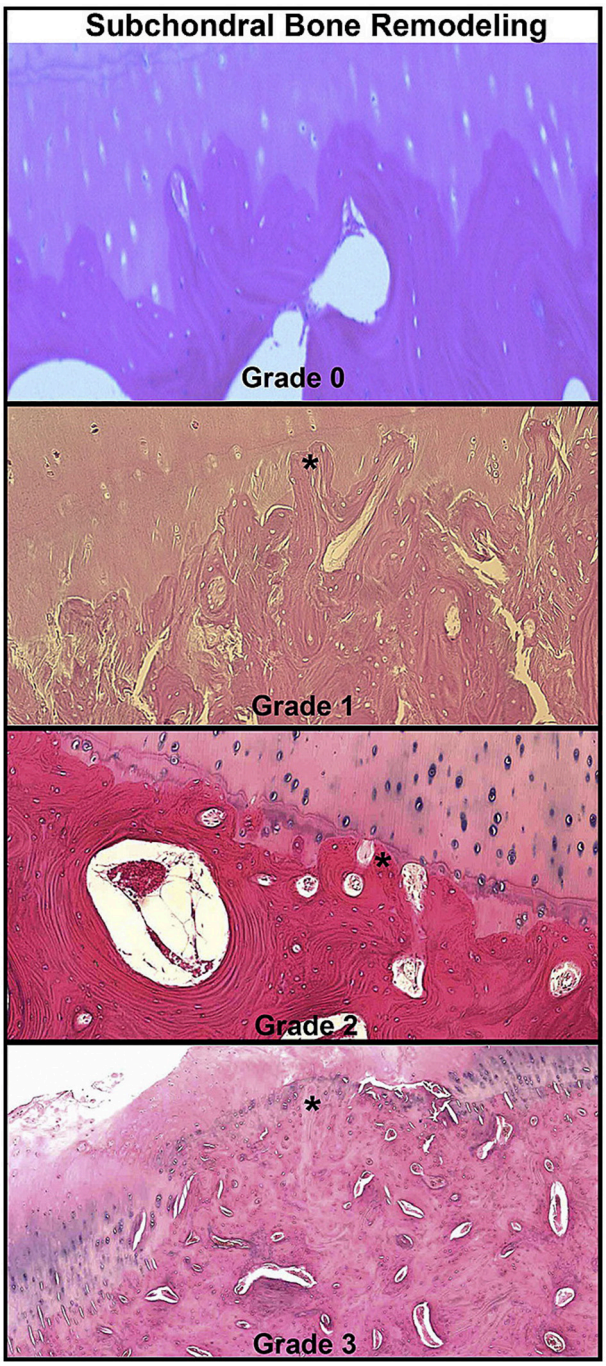

FIGURE 1 | Histopathologic grading examples of osteochondral lesions in spontaneous osteoarthritis cases in equine metacarpophalangeal joints. Microscopic images showing advancement of subchondral bone remodeling through calcified cartilage layer (star). Reprinted from McIlwraith et al. (17), with permission from Elsevier.

SUBCHONDRAL BONE DISEASE AND CHRONIC FATIGUE INJURY

Historically, traumatic arthritis has been held as a condition of synovial-mediated degradation of articular cartilage and direct mechanical damage to the joint surface. More recently, the joint has been re-conceptualized as an organ system, within which multiple tissues can be damaged and contribute to overall injury and dysfunction. A general approach to understanding the fundamental mechanisms for OA have been noted as either abnormal loading on normal cartilage or normal loading on abnormal cartilage (23). Damage of the articular cartilage can occur from a variety of different circumstances, including damage to the subchondral bone, synovial membrane, fibrous joint capsule, peri-ligamentous support structures, or direct trauma to the articular cartilage itself (24). Damage to the subchondral bone has received increasing interest for its role in joint injury, as changes in the composition or mechanical properties of the subchondral bone appear to mediate some of the changes observed in OA. For example, sclerosis reduces the shock-absorbing capability of the subchondral bone, and increases the risk of shear-induced tensile failure of the articular cartilage cross-links (12); and subchondral bone has also been demonstrated as a potential source of inflammatory mediators that have been associated with degradation of deeper layers of articular cartilage (25, 26). Changes are also observed on the microarchitectural level, with the coalescing of microcracks or microfractures within the bone. Rapid, excessive bone formation may result in the development of bone sclerosis, which may be of reduced mineral quality and integrity (27). Furthermore, expansion of the trabecular bone observed in subchondral bone undergoing excessive new bone formation, reduces the size of the bone marrow spaces, potentially resulting in an ischemic state, or at the very least a change in tissue nutrition (28). Integration between the old bone and the newly added bone also takes time, and if there is a disparity between mineral properties, this may further predispose this area of bone to failure (27).

Although many terms are used by clinicians and researchers alike, the term "subchondral bone disease" most accurately represents the different phases and spectrum of pathologic changes observed within the subchondral bone. At the 2015 Dorothy Russell Havemeyer Foundation workshop in Newmarket, UK focused on subchondral bone, a consensus definition for subchondral bone disease included, "a repetitive stress injury of subchondral bone" (2). The delay between bone resorption and remodeling, where bone is resorbed at a faster rate than it is replaced, leaves the equine athlete susceptible to injury, especially when it is subjected to excessive training and stress. This type of subchondral bone injury is frequently referred to as "maladaptive," however this term is somewhat misleading, as the bone does not truly display a maladaptive response, but rather the subchondral bone does not have the opportunity—typically due to time or ongoing physical stresses—to appropriately complete the repair processes. Compensation for these stresses is only possible up to a point, after which time the bone enters a spectrum of pathologic changes, which may be further complicated by the concurrent presence of clinical disease. The degree of the adaptive response tolerated by a given horse before showing clinical signs indicative of greater pathology appears somewhat individualized, which makes understanding the line between "normal" and "pathologic" responses difficult to distinguish.

The athletic horse is a well-studied model for repetitive stress injury, with multiple locations and manifestations of the disease. The metacarpal and metatarsal condyles are the most recognized locations of repetitive stress response and injury, with macroscopic abnormalities including increased radiopharmaceutical uptake on nuclear scintigraphy, radiolucency within the condyles on radiography, increased sclerosis within the bone on computed tomography and longitudinal fractures within the bone. Slab fractures of the carpal and tarsal bones, parasagittal fractures of the proximal phalanx, mid-body fractures of the proximal sesamoid bone, dorsoproximal fractures of the third metacarpal and metatarsal bones, wing fractures of the distal phalanx, palmar/plantar osteochondral disease, and intra-articular fragmentation are some of the other well-recognized examples of this condition. In some cases, it may be difficult to distinguish whether these injuries are primary diseases of subchondral bone, or whether they reflect more complex traumatic injuries to the bone, cartilage, and supporting soft tissues of the joint. An acute injury to a bony or ligamentous structure around or within a joint may alter weight-bearing during exercise and stimulate alterations within the subchondral bone that may result in the development of disease. Recent work in Thoroughbred racehorses has recognized a significant association between catastrophic biaxial proximal sesamoid bone fractures and disease of the subchondral bone of the third metacarpal bone of the contralateral limb (29). Subchondral bone disease is most frequently recognized in racehorses, but can affect horses in a variety of disciplines, including steeplechasers, jumpers, and three-day event horses.

Investigation of injury in subchondral bone has been reported in both clinical cases and experimental models. Microdamage within the subchondral bone has been experimentally investigated using an equine model with controlled treadmill exercise, showing that it can develop early under exercising conditions (18). The metacarpal condyles of these horses displayed changes indicative of a milder version of what has been observed in clinical cases. Investigation of subchondral bone changes in clinical cases of racehorses euthanized for other catastrophic injuries has revealed changes on both a macroscopic and microscopic level. On gross examination, the metacarpo/tarsophalangeal joints from racehorses without catastrophic fractures displayed a spectrum of disease, ranging from fibrillation of the articular cartilage to focal cartilage erosions and cavitation within the subchondral bone (20). Lesions in the subchondral bone varied from thickening of the subchondral and trabecular bone to advancing sclerosis with increasing amounts of osteocyte necrosis, the presence of vascular channels filled with matrix debris and osteoclastic remodeling. Changes in the subchondral bone were not limited to architectural microdamage alone, as osteocyte death was also identified. Changes in the subchondral bone—such as necrosis

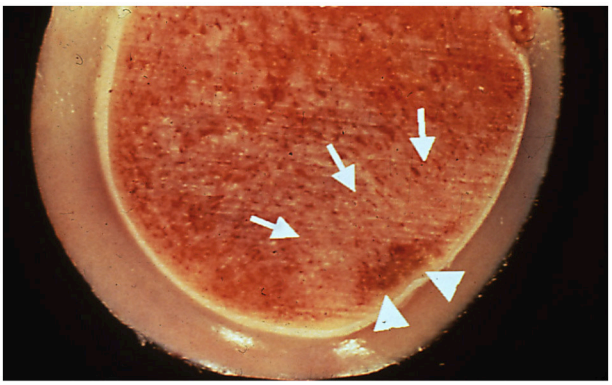

FIGURE 2 | Postmortem sample of a distal metacarpus from the leg opposite to that suffering a catastrophic injury in a racehorse. Although there is intact articular cartilage, subchondral bone necrosis (arrowheads) and sclerosis (arrows) can be seen. Reprinted from McIlwraith et al. (3), with permission from Elsevier.

and sclerosis—could also be present in the face of intact articular cartilage (as has been observed in the palmar/plantar aspect of the distal third metacarpal/tarsal bones) (20). In many of these cases, on gross examination the articular cartilage appeared largely viable, with limited erosion and degeneration within the superficial layers (**Figure 2**). Focal disruption of the calcified cartilage layer appeared to result in cartilage in-folding. The disparity between these experimental and clinical findings is not necessarily unexpected, as it is unrealistic to believe clinical conditions can be perfectly modeled in a laboratory setting. Taken together however, these findings would suggest that microdamage within the subchondral bone not only results in the loss of mechanical support to the articular cartilage, but local factors (i.e., cytokines) released from the bone can influence—potentially permanently—the state and health of the articular cartilage (11, 18, 20). Furthermore, progressive injury within the subchondral bone can result in complete failure, with pathologic changes within the third metacarpal/tarsal bones culminating in condylar fracture (11, 30–32). More recently, the term "impact fracture" has been used to describe these types of pathologic fractures that correspond to areas of radiographic lucency within the bone (33).

Chronic fatigue injury has also been used to describe injury to the subchondral bone. Chronic fatigue injury is a broader and more encompassing term than "subchondral bone disease," as the subchondral bone is typically not the only tissue affected. Chronic fatigue injuries result from cyclic loading of the tissue below the biomechanical threshold of tissue failure, and occurs commonly in the subchondral bone of the equine athlete (3, 11). Currently, it seems that chronic fatigue injuries and repetitive stress injuries are terms used interchangeably throughout the literature and amongst clinicians, suggesting these terms are frequently used to describe the same underlying disease processes of subchondral bone. As more is understood about the biological behavior of subchondral bone under abnormal physiologic conditions, further clarification or consensus about these terms will likely be important. Chronic fatigue injury in the subchondral bone was initially described in clinical cases of injured racehorses (34); and validated through numerous clinical studies and experimental models (18–20, 35–37). There are three mechanisms by which damage can occur: (1) microdamage formation within the tissues; (2) an area of weakness within tissues secondary to biomechanical and tissue responses to cyclic loading, predisposing this tissue to damage; or (3) adaptive tissue responses that chronically fatigue the tissue, resulting in a change in material properties and ultimately to injury (3). One of the best clinical examples of chronic fatigue injury is in the palmar aspect of the third metacarpal condyles. In young Thoroughbred racehorses in training, sclerosis in the palmar aspect of the condyles is common, but the difficulty lies in discerning when these changes are indicative of a pathologic response. It is rare to observe clinical abnormalities in this region until these processes have resulted in subchondral bone pain or gross damage within the joint (38).

Advances in diagnostic imaging have improved identification of these pathologic changes and is an attractive avenue for further research into understanding subchondral bone disease. Although the significance of osteochondral injury in joint disease has been well-discussed (39), our understanding of the complex relationship between the subchondral bone and articular cartilage in repetitive stress injury and chronic fatigue injury continues to evolve, as great progress in understanding have and will likely continue to be made over the next decade through advancements in imaging and experimental disease models.

PRINCIPLES OF DIAGNOSIS AND DIAGNOSTIC IMAGING

The tenets of diagnosis for subchondral bone disease remain the same as for many other musculoskeletal conditions in the horse—a thorough clinical examination (including static and dynamic, and subjective and objective evaluations) to localize the source of lameness, diagnostic analgesia, and diagnostic imaging examination. Subchondral bone injury may be identified on standard radiographic projections, but also may be missed depending on the location and time-course of the disease. Diseased bone may radiographically have the appearance of areas of decreased opacity surrounded by areas of increased opacity, or may be visible as a distinct fracture. A lack of radiographically observable abnormalities does not rule out the presence of subchondral bone disease and repeat imaging (in 10–14 days) or use of a different, more advanced imaging modality should be considered. Depending on the severity and chronicity of the injury, nuclear scintigraphy, magnetic resonance imaging (MRI), and computed tomography (CT) can be considered for further evaluation of changes within the subchondral bone. In severe cases where overlying articular cartilage damage is also present, diagnostic arthroscopy may be considered for further evaluation of the subchondral bone. Volumetric imaging techniques, such as MRI and CT that provide information in three-dimensions have arguably revolutionized our clinical ability to assess subchondral bone and the health of the joint as a whole.

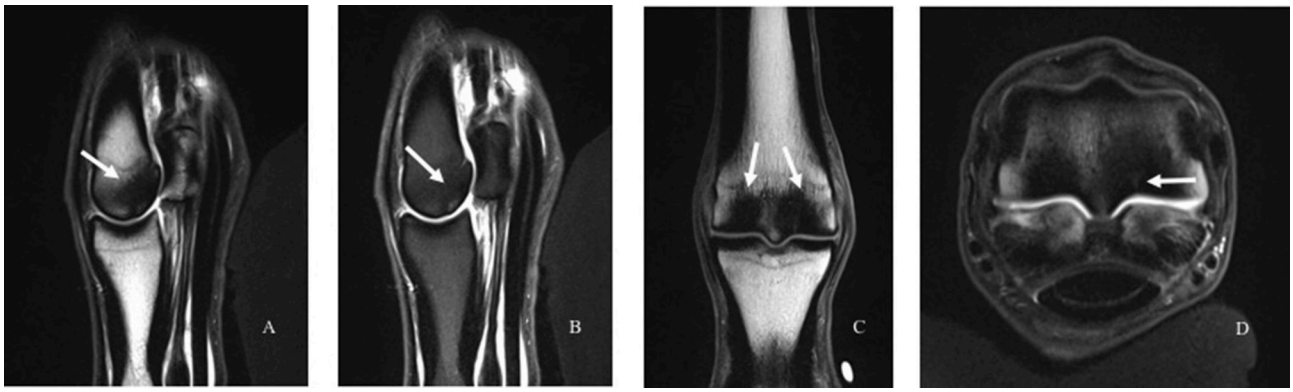

FIGURE 3 | Images of subchondral bone injury as detected using magnetic resonance imaging (MRI). An intermediate-weighted T1 TSE sequence with **(A)** and without **(B)** fat suppression in the sagittal **(A, B)**, dorsal **(C)**, and transverse **(D)** planes. There is marked subchondral and trabecular bone sclerosis in the palmar aspect of the third metacarpal condyle (white arrows), with the lateral condyle slightly more affected than the medial condyle. There is a fissure visible within the bone on the palmar-axial aspect of the lateral condyle that also exhibits increased signal (**D**, arrow).

Magnetic resonance imaging has facilitated the identification of diseased subchondral bone. Terms such as "bone marrow edema," "bone bruise," or "bone contusion" have all been used to describe what are now referred to as bone marrow lesions (40). These terms all describe lesions within the subchondral bone with high signal intensity on fluid-sensitive sequences (**Figure 3**). Bone marrow lesions have a characteristic decreased signal intensity on T1-weighted images and increased signal intensity of T2-weighted images (41), Multiple theories and etiologies have been proposed for the formation of bone marrow lesions, and histologic examination of these lesions have identified a wide spectrum of abnormalities (42). More recently, compelling reports have been published citing bone marrow lesions as early indicators of structural deterioration of the joint and may serve as a marker for maladaptive changes within the subchondral bone and articular cartilage (43–46). Further research is essential and forthcoming in this field with the continued development of high-field MRI systems that can provide increased detail.

In addition to identification of bone marrow lesions, increased bone mineral density or sclerosis is frequently identified on MRI in areas of signal changes. These changes are identified as areas of low signal intensity, but this depends on the sequences used for evaluation. Furthermore, diseased subchondral bone is not always sclerotic and although increased bone density may be present, signal changes on MRI are non-specific and may represent an increased volume of lower density bone. Increased signal intensity on MRI should be interpreted with caution, as this may not truly represent the trabecular thickening inherent to sclerosis (2).

Magnetic resonance imaging remains as the only modality capable of being used to identify fluid (e.g., hemorrhage, edema, fibrosis, necrosis) within bone; however CT has superior resolution for structural imaging of bone. Fine bone detail can be very challenging to identify using MRI, as the appearance of bone and soft tissue can overlap. In cases of confluent tissue—such as in a joint with osseous proliferation with adjacent soft tissue thickening—the signal intensity of MRI will be the same and it can be challenging or impossible to distinguish between the structures. The volumetric information from reconstructed CT images can illustrate subtle to extensive internal and external osseous remodeling (**Figure 4**). In specific sites, remodeling changes observed on CT have been validated to indicate pathologic change and impending fractures (47). The tissue density observed on CT can be translated into numerical values known as Hounsfield units (HU). Information about the specific densities of the bone may provide valuable insight into unique patterns of bone change, and furthermore provides an objective metric for comparison if serial examinations are performed.

The information afforded through volumetric imaging modalities such as CT and MRI, have increased our knowledge in the behavior of subchondral bone and its response to training and injury. Historically, CT and MRI evaluation in the equine patient has been limited due to gantry size or requirements for general anesthesia. Recent developments have yielded MR systems and rapid-scan CT units dedicated for equine use with an enlarged gantry size (Pegaso, Epica Medical Innovations, San Clemente, CA USA) or separate generator-receiver robotically-controlled arms (4DDI Equimagine, Equine Imaging LLC, Milwaukee, WI, USA), all of which can be used in the standing horse. MRI and CT should be considered as complimentary to one another when evaluating the subchondral bone, as each provides unique and valuable information. An additional notable volumetric imaging modality that is receiving increasing attention as a developing technology for assessment of subchondral bone is positron emission computed tomography (PET). Recent work has demonstrated that PET is able to identify lesions that were not visible using other imaging modalities, and furthermore to distinguish between active and inactive lesions (48). As PET gains further justification, the potential applications for evaluation of subchondral bone injury will likely warrant further discussion. With increasing accessibility to these volumetric imaging modalities and evolving understanding of the role of subchondral bone in joint disease, it is likely that

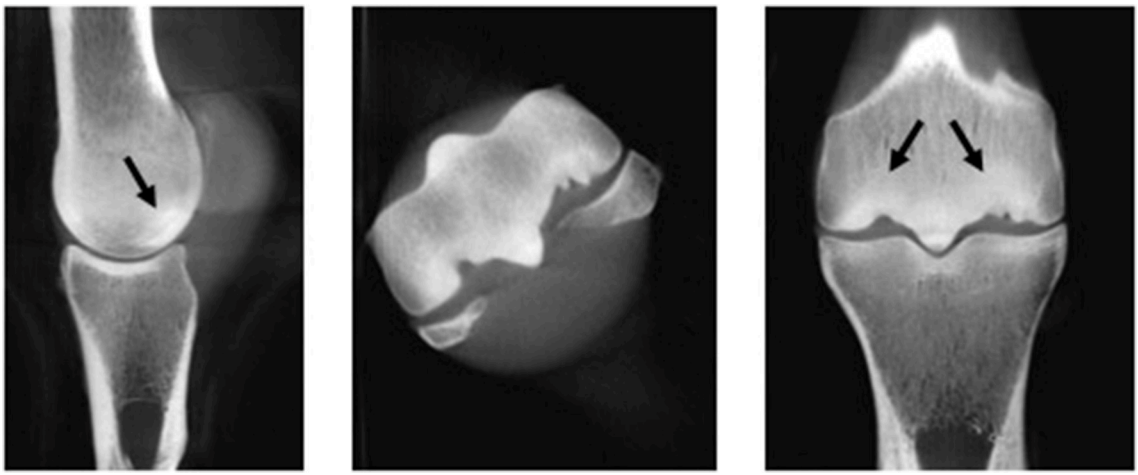

FIGURE 4 | Image of the distal metacarpus of a 10-year-old Thoroughbred racehorse obtained post-mortem with marked palmar osteochondral disease obtained using computed tomography (CT) in the sagittal, transverse and dorsal planes. Subchondral bone sclerosis and lysis (black arrows) and extensive articular cartilage loss is visible on these images and was present on gross evaluation.

assessment of subchondral bone will become an integral part of diagnostic imaging for veterinary patients affected by orthopedic conditions.

TREATMENT AND PREVENTION

The causes of pain associated with diseased subchondral bone, including bone resorption, still remain poorly understood. Proposed contributors to pain may include instability, increased intraosseous pressure, hypertension, and impingement of sensitive structures such as the periosteum, ligaments or joint capsule. The two consensus goals for treatment of subchondral bone disease include: (1) restoring function and (2) preventing progression of disease to failure through pain relief and restoration of normal bone architecture (2). It is challenging to create a specific treatment regimen since subchondral bone disease encompasses a large spectrum of abnormalities. The plethora of methods proposed to treat subchondral bone disease reflects the variety of challenges associated with managing this condition. If pain or lameness are recognized early in the maladaptive response process, exercise modifications including altering the intensity, duration or type (e.g., reduced work, turn out, swimming, underwater treadmilling) for a short period may be all that is necessary to resolve clinical signs. A notable critique of these alternative training methods is that they utilize instability, or alterations in the distribution of weightbearing forces across the articular surfaces (49). Instability has been shown to prevent vessel ingrowth, and despite the fact that subchondral bone disease is not necessarily a primary vascular condition, avascular areas are commonly present within diseased subchondral bone (2). Bisphosphonates have also been used commonly to treat subchondral bone disease, but their potential effectiveness has yet to be fully elucidated. Proponents of bisphosphonate therapy argue that inhibition of osteoclastic activity benefits those cases undergoing active degradation of the subchondral bone, and additional analgesic and anti-inflammatory effects have also been suggested (2, 50, 51). Despite these potential benefits and positive reports in the human medical literature, there has been no definitive consensus in the treatment of subchondral bone disease using bisphosphonates in veterinary species. Bisphosphonates are also not licensed for use in juvenile horses, which likely represent the largest population of cases of subchondral bone injury through the racehorse industry.

The frequency and intensity that subchondral bone disease is observed would justify this as an economic issue, however there is a paucity of data on the incidence and specific demographics of this disease. The inherent difficulties in identifying subchondral bone disease prior to the occurrence of more severe sequelae makes epidemiologic studies very challenging. At the current time, the best prevention of subchondral bone disease is focused on reducing risk. Despite inconsistent agreement in a specific definition for clinical signs of subchondral bone disease, it is well-agreed that cumulative exercise is associated with an increased risk of subchondral bone disease (2). Exercise and training regimes should be tailored to each specific horse, with specific attention to the clinical condition and response of the horse. Published work has recognized that exposure to exercise at the end of growth, but before skeletal maturity is beneficial for subchondral bone development, and this must be balanced with the adaptive response of each animal. Further work is essential in order to understand those cases that may be at an increased risk for development of subchondral bone disease through the use of multi-modality imaging or potentially biomarker panels. Despite the voids in the current knowledge about subchondral bone disease, exercise modulation will likely remain a central tenet of disease management.

CONCLUSION

Substantial insight has been gained about the biomechanical influences of the joint on the subchondral bone, with the relationship between subchondral bone injury and articular cartilage loss and the development of degenerative joint disease only beginning to be elucidated. Continued investigation of the adaptive and maladaptive changes within the subchondral bone by researchers and clinicians alike will continue to yield valuable information about the behavior of this unique component of the joint. Further discovery of the delicate balance of factors that maintain the integrity of the subchondral bone and homeostasis within the joint will surely enhance and direct our understanding of subchondral bone disease in across both veterinary and human patients.

AUTHOR CONTRIBUTIONS

Both HS and CK contributed equally to the preparation, developing, writing and editing of the review article submitted.

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Galectins-1 and-3 Increase in Equine Post-traumatic Osteoarthritis

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Galectins are potent regulators of cell adhesion, growth and apoptosis in diverse cell types, including chondrocytes and synovial fibroblasts. Elevations in synovial fluid galectin-3 have been observed in rheumatoid arthritis, juvenile idiopathic arthritis and experimental inflammatory arthritis in animal models, whereas galectin-1 is thought to be protective. Less is known about galectins-1 and-3 in osteoarthritis (OA). Therefore, the purpose of this study was: (1) to determine whether galectin-1 and-3 synovial fluid concentrations and synovial membrane and cartilage histochemical staining were altered following osteochondral injury in an experimental equine osteoarthritis (OA) model and (2) to measure galectin-1 and-3 mRNA expression and synovial fluid concentrations in naturally occurring equine carpal OA. Synovial fluid galectin-1 and-3 concentrations were quantified using custom ELISAs in two research horse cohorts undergoing experimental OA induction ($n = 5$ and 4) and in a cohort of horses with naturally occurring carpal OA ($n = 57$). Galectin mRNA expression in synovial membrane and cartilage tissue obtained from carpal joints of horses with naturally occurring OA was measured using RT-qPCR, and galectin immunostaining was assessed in synovial membrane and osteochondral tissues in the experimental model ($n = 5$). Synovial fluid galectin-1 and-3 concentrations increased following experimental carpal osteochondral fragmentation. Cartilage galectin-1 mRNA expression increased with OA severity in naturally occurring disease. The superficial zone of healthy articular cartilage stained intensely for galectin-3 in sham-operated joints, whereas galectin-1 staining was nearly absent. Chondrocyte galectin-1 and-3 immunoreactivity increased following cartilage injury, particularly in galectin-1 positive chondrones. Galectins-1 and-3 are present in healthy equine synovial fluid and increase following post-traumatic OA. Healthy superficial zone chondrocytes express galectin-3, whereas galectin-1 chondrocyte staining is limited predominantly to chondrones and injured cartilage. Further work is needed to clarify the functions of galectins-1 and-3 in healthy and OA joints.

Keywords: inflammatory arthritis, cartilage, synovium, chondrocyte, synoviocyte, horse, rheumatoid arthritis

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INTRODUCTION

Galectins are potent regulators of cell adhesion, growth, and apoptosis in diverse tissues and organs, including synovial joints. Galectin-1 and galectin-3 are expressed in synovial fibroblasts, articular chondrocytes, and hypertrophic growth plate chondrocytes (1–3). Synovial fibroblasts express higher levels of galectin-1 and-3 as compared to articular chondrocytes (4), and intracellular galectin-3 promotes chondrocyte survival in both articular and hypertrophic chondrocytes (5, 6).

Associations between increased galectin-3 in synovial fluid, synovial tissues, and sera of human patients with inflammatory arthritis have been observed in several studies (7–9), and strong expression of galectin-3 at sites of joint destruction has led authors to suggest that galectin-3 plays a role in rheumatoid arthritis (RA) pathogenesis (10). Some authors have even proposed that galectin-3 may be a potential therapeutic target for RA (11, 12). Conversely, decreased galectin-1 concentrations and increased anti-Gal-1 antibodies have been detected in RA patients (13). Galectin-1 expression is downregulated and galectin-3 expression is upregulated in synovial tissue from patients with juvenile idiopathic arthritis (8). Most rodent experimental models of inflammatory arthritis suggest that galectin-1 is protective (14–17), whereas galectin-3 promotes joint inflammation (16, 18). For example, galectin-1 knockout (KO) mice develop earlier onset and more severe collagen-induced arthritis (14), and galectin-3 KO mice have reduced inflammation and bone erosion in response to antigen-induced arthritis as compared to wild-type mice (18). In addition, while both recombinant protein and genetic delivery of galectin-1 are protective in rodent models of collagen-induced arthritis (15–17), administration of galectin-3 shRNA protects rodents from collagen-induced arthritis (16). Classification of galectin-3 as a driver or inhibitor of inflammatory arthritis is likely affected by its intracellular or extracellular localization (6, 19), with rodent knockout models emphasizing intracellular galectin signaling.

The role of galectins in osteoarthritis (OA) and post-traumatic osteoarthritis (PTOA) is not well understood. What little is known about galectin-1 and-3 synovial fluid levels or synovial membrane localization in human OA is derived from studies where OA patients were used as a comparison group to RA patients (7). Galectin-1 and-3 levels have not been evaluated in synovial fluid from healthy human patients, with the exception of a "healthy control" group in two studies in which synovial fluid was obtained pre-operatively from patients with knee trauma or meniscal tears (7, 20). Lectin/galectin staining in human OA cartilage has revealed increased galectin-1 and galectin-3 chondrocyte immunostaining at sites of cartilage damage (21–23), with increasing galectin-1 positivity correlated with cartilage Mankin scores (21, 23). Galectin-1 and-3 mRNA was expressed in human OA chondrocytes at higher levels than galectins-2, 4, 7, 8, and 9; however, levels of galectin expression in healthy chondrocytes were not studied (23). Investigation of *in vitro* signaling pathways in human OA chondrocytes revealed that both galectins-1 and-3 promote an inflammatory gene signature, at least in part through their role as upstream NF-κB signaling effectors (21, 24). On the other hand, galectin-3 KO mice demonstrate increased cartilage damage in a mono-iodoacetate injection model of OA (6), and galectin-3 KO mice also demonstrate increased bone resorption and accelerated trabecular bone loss as compared to wild-type mice (25), suggesting a protective role for galectin-3 in OA.

To the authors' knowledge, galectins have only been evaluated in animal models of inflammatory arthritis and not in PTOA models. Therefore, critical gaps in knowledge include understanding: (1) how synovial fluid galectin-1 and-3 concentrations change over time following joint injury, (2) how galectin-1 and-3 mRNA expression and synovial fluid levels differ in healthy as compared to OA joints, and (3) whether galectin-1 and-3 immunostaining differs between healthy and OA cartilage in PTOA. Horses are athletic animals that commonly develop PTOA in the course of their performance careers (26). Synovitis, cartilage impact injury, osteochondral fragmentation and subchondral bone injury are common in the high-motion carpal joints (27). Because PTOA in horses can better recapitulate certain aspects of human PTOA pathogenesis than chemically induced models in rodents (28), we chose to evaluate galectins-1 and-3 in the equine model. Synovial fluid and articular tissues were obtained from horses with naturally occurring OA, and the carpal osteochondral fragment high-speed treadmill exercise model of OA was used to evaluate serial changes in synovial fluid galectins and cartilage immunohistochemistry. Biochemical, histologic, and inflammatory changes are well-characterized in the equine carpal osteochondral fragment model of OA (27, 29, 30), and this model is commonly used to test the therapeutic effects of intra-articular or systemic OA therapies (27, 31).

Therefore, the objectives of this study were to: (1) compare galectin-1 and-3 mRNA expression and synovial fluid concentrations in healthy and OA joint tissues from horses with naturally occurring OA, and (2) to determine whether galectin-1 and-3 serial synovial fluid concentrations and galectin immunostaining were altered following osteochondral injury in an experimental equine OA model.

MATERIALS AND METHODS

Ethics Statement

All experimental protocols were approved by the university Institutional Animal Care and Use Committee (protocol numbers: 2011-0027 and 2012-0097). All sample collection was performed following humane euthanasia of horses using sodium pentobarbital or obtained from discarded tissues following arthroscopic surgery of horses with informed consent from owners.

Equine Carpal Osteochondral Fragment Model

Synovial fluid samples collected from two distinct equine experimental cohorts ($n = 5$ and $n = 4$) undergoing carpal fragmentation were used to measure serial galectin-1 and-3 concentrations. Synovial membrane biopsies and osteochondral tissues were collected from the first cohort ($n = 5$) for

immunohistochemistry following euthanasia on day 70 post-fragmentation. Analysis of synovial fluid lubricin concentrations and lubricin immunostaining has previously been reported in the first cohort (32). Horses in both cohorts were subjected to carpal osteochondral fragmentation in one randomly assigned joint, while the opposite joint served as a sham-operated control. Two weeks post-operatively, horses commenced a high-speed treadmill exercise program 5 times weekly, continuing throughout the study duration of either 70 or 75 days. Five Thoroughbred horses ($n = 3$ females and 2 castrated males), aged 2–6 years old, were enrolled in the first cohort, and four Thoroughbred horses ($n = 2$ females and 2 castrated males), aged 2–6 years old, were enrolled in the second cohort. Experimental protocols were roughly similar between the two groups; however, the timing of synovial fluid collection and study duration differed slightly. All horses were housed in 3.65 × 3.65 m box stalls and engaged in similar treadmill exercise programs, consisting of walking (5 km/h) for 5 min, followed by trotting (16–18 km/h) for 2 min, galloping (28–32 km/h) for 2 min, and ending with 2 min of trotting (16–19 km/h) exercise performed in the morning. Synovial fluid aspirates were processed similarly, and synovial fluid supernatants were stored in aliquots at −80°C following centrifugation at 3,000x g for 5 min to pellet any cellular debris. Synovial fluid samples were collected and banked from the first cohort approximately 2 years prior to the second cohort, and all samples were frozen at −80°C for long-term storage. Synovial fluid samples from the first cohort were subjected to up to 3 freeze-thaw cycles prior to ELISA measurements, whereas samples from the second cohort were only subjected to 1 freeze-thaw cycle.

Naturally Occurring Equine OA

Synovial fluid and discarded tissues, including synovial membrane and osteochondral tissues, were harvested where available from the antebrachialcarpal (ACJ) and middle carpal joints (MCJ) of horses undergoing arthroscopic surgery at the Cornell University Equine Hospital, with informed owner consent. Each joint was assessed as healthy (grade 0) or assigned an osteoarthritis (OA) severity score of mild (1), moderate (2) or severe (3) on the basis of radiographic evidence of osteophytes, enthesiophytes, osteoproliferation, joint space narrowing or chronic fracture lines as previously described (33). Synovial fluid and tissues were also collected from horses donated for research purposes, and joint scores were assessed on the basis of radiographic and/or gross dissection findings. Thoroughbred, Standardbred or Quarter Horse females ($n = 34$), intact males ($n = 6$), or castrated males ($n = 17$) ranging in age from 2 to 13 years were included. A total of 54 and 52 synovial fluid samples were quantified using galectin-1 and-3 ELISAs, and 57 synovial membrane and 34 cartilage tissue samples were analyzed via RT-qPCR.

Galectin-1 and-3 Synovial Fluid ELISA

Equine galectin-1 (GenBank ID: KY264050.1) and galectin-3 (GenBank ID: KY264051.1) were cloned, recombinantly expressed in *E. coli,* and purified using lactosyl sepharose chromatography and FPLC gel filtration as previously reported (34). Galectin ELISA antibody reactivity to recombinant equine galectin standards has previously been described (4). Both the goat anti-mouse Gal-1 antibody (AF1245; R&D Systems, Minneapolis, MN) and the goat anti-human Gal-3 antibody (sc-19280; Santa Cruz Biotechnology, Dallas, TX) were biotinylated using the Mix-n-Stain biotin antibody labeling kit (Biotium Inc., Fremont, CA), and biotinylated goat anti-mouse Gal-3 antibody (BAF1197, R&D Systems, Minneapolis, MN) was obtained from the manufacturer. Synovial fluid samples were resolved on 12% SDS-PAGE gels and probed with the biotinylated primary antibodies to confirm antibody reactivity to equine synovial fluid galectins. All 3 biotinylated primary antibodies were used at a concentration of 0.15 μg/mL in blocking buffer (3% BSA in 0.1% PBS-Tween for anti-mouse Gal-1 and anti-mouse Gal-3 antibodies; 1% Gelatin in 0.1% PBS-Tween for anti-human Gal-3). Streptavidin-HRP was applied at 4 ng/mL in 0.1% PBS-Tween for all blots.

Custom galectin-1 competitive inhibition ELISAs (4) were performed on banked equine synovial fluid samples from two cohorts of horses subjected to carpal osteochondral fragmentation. Briefly, 96-well high-binding plates (Corning Inc., Corning, NY) were coated with 1 μg/mL of goat anti-mouse Gal-1 capture antibody in sodium carbonate buffer, pH 9.6 at 4°C. After 3 rinses in 0.1% PBS-Tween, protein free blocking buffer (Thermo Fisher Scientific, Rockford, IL) was added for 1 h. Unlabeled recombinant equine galectin-1 standards (2 μg/mL to 15.6 ng/mL) were diluted in 200 ng/mL biotinylated recombinant equine galectin-1 in 0.1% PBS-Tween. Synovial fluid samples pre-diluted 1:50 in PBS were further diluted 1:1, for a final dilution of 1:100, in 200 ng/mL of biotinylated recombinant equine galectin-1. Blocking buffer was aspirated, and 100 μL of recombinant equine galectin-1 standards or synovial fluid samples were added to the plate in duplicate and incubated for 1 h at RT. Plates were rinsed in 0.1% PBS-Tween, incubated with 100 μL of streptavidin HRP for 30 min. TMB reagent was added for 10 min prior to halting the reaction with 1N H_2SO_4. Absorbance was measured at 450 nm with 540 nm background subtraction.

For the custom galectin-3 sandwich ELISA, 96-well plates were coated with 2 μg/mL of goat anti-human Gal-3 capture antibody (sc-19280) using similar methodology as for Gal-1. After rinsing in 0.1% PBS-Tween, protein-free blocking buffer was added for 1 h, followed by serial dilutions of recombinant equine galectin-3 standards (400 ng/mL to 1.6 ng/mL) in duplicate. Synovial fluid samples pre-diluted 1:50 in PBS were further diluted 1:1 in 1% BSA in PBS, for a final dilution of 1:100, and added in duplicate to the plate. Following 1 h incubation at RT, the plate was rinsed in 0.1% PBS-Tween, and biotinylated goat anti-mouse Gal-3 pAb (BAF1197) was added at 200 ng/mL for 1 h. Following rinsing, 100 μL of streptavidin HRP was added for 30 min prior to adding 1N H_2SO_4. Absorbance was measured at 450 nm with 540 nm background subtraction.

Galectin-1 and-3 RT-qPCR

Synovial membrane and cartilage tissues were snap-frozen in liquid nitrogen and stored at −80°C for up to 3 years. The frozen tissues were crushed and ground into fine powder in

liquid nitrogen with a mortar and pestle prior to isolation of RNA. Total RNA was extracted using the E.Z.N.A Tissue RNA Kit (Omega BioTek, Inc., Norcross, GA) for synovial membrane or the RNeasy Lipid Tissue Mini Kit (QIAGEN Sciences Inc., Germantown, MD) for cartilage. DNase I was added during RNA extraction to remove genomic DNA. RNA purity and concentration were assessed with a multimode plate reader (Tecan Spark® 10M, Tecan, Austria) with a NanoQuant Plate™. Expression of galectin-1 and galectin-3 mRNA was quantified using the Taqman RNA-to-CT one-step kit (Applied Biosystems, Foster City, CA) and the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). As previously reported, Primer Express Software Version 2.0 (Applied Biosystems, Foster City, CA) was used to design forward and reverse primers and probes for equine galectins-1 and-3 (4). Primer and probe sequences are listed in **Table 1**. Equal amounts (10 ng per reaction) of total RNA were added in a 20 µl reaction volume for all samples. The RT-qPCR reactions were all performed in duplicate. The levels of gene expression were calculated using the standard curves generated with serial dilutions of *E. coli*-expressed equine *Gal-1* and *Gal-3* as standards. All data were normalized with the housekeeping gene equine 18S rRNA. A qPCR checklist is provided to document the technical aspects of qPCR Protocols (**Supplementary Material**).

Galectin-1 and-3 Immunohistochemistry in Experimental Equine OA

Synovial membrane and osteochondral tissue sections from the radial carpal bone and opposing third carpal bone were stained for galectin-1 and galectin-3 using previously reported techniques (34). Briefly, osteochondral sections were fixed in 4% paraformaldehyde, de-calcified in 10% EDTA for 3 weeks and embedded in paraffin, while synovial membrane sections were embedded in paraffin immediately after fixation. Following deparaffinization, sections were treated with 1% hyaluronidase (Sigma-Aldrich, St. Louis, MO) in 20 mM sodium acetate for 30 min at 37°C, followed by 3% hydrogen peroxide for an additional 30 min. Blocking in normal rabbit serum was performed, followed by incubation with a goat anti-mouse galectin-1 antibody (AF1245; R&D) or goat anti-human galectin-3 antibody (sc-19280; Santa Cruz) at 1:100 dilution for 1 h at room temperature. After washing, sections were incubated with a biotinylated rabbit anti-goat IgG (Vectastain, Vector Labs) and immunodetected with the Vectastain ABC Kit and ImmPACT DAB reagent (Vector Labs). Negative controls were performed by omission of primary antibody. Sections were rinsed in PBS, counterstained with Harris hematoxylin, coverslipped and imaged with a 20x objective using a ScanScope (ScanScope CS0, Aperio). Images were saved as.tif files in Aperio's Image Scope software, cropped in Adobe Photoshop CC and formatted in Adobe Illustrator CC.

Synovial membrane tissue sections were imaged but not quantified due to the presence of strong galectin-1 and galectin-3 immunostaining in all sections. Osteochondral sections from the radial carpal bone and third carpal bone were scored independently by two observers. Although observers were blinded to individual animal identity, blinding to treatment group (sham-operated or OA) was not possible due to the presence of obviously injured articular cartilage within some osteochondral sections. Where possible, separate scores were assessed for injured cartilage regions vs. healthy cartilage regions within the same section. Chondrocytes within each cartilage zone (superficial, middle and deep) were assigned an immunostaining intensity score for both galectin-1 and galectin-3 where: 0–none, 1–weak, 2–moderate, and 3–strong. In addition, the percentage of galectin-1-positive chondrocytes was calculated for the entire articular cartilage section, including superficial, middle, and deep zones combined.

Statistical Analysis

To assess the effect of treatment (sham vs. OA) and time (day) on galectin concentrations in synovial fluid, galectin ELISA data were first tested for normality using a Shapiro-Wilk W test and were found to be right skewed. Log transformation was performed to achieve normality. In order to account for the hierarchical nature of the data in the experimental models, a mixed linear model was employed because each horse was repeatedly measured on each limb and each limb repeatedly over days. The fixed effects in the model included treatment (sham vs. OA), day and a treatment*day interaction term, and random effects included horse and individual limb nested within horse to account for the non-independence of the observations. Predefined *post hoc* comparisons of specific contrasts for each time point were performed to assess differences between sham and OA joints, with a Bonferroni correction applied based on the number of multiple comparisons to correct for the false discovery rate. Model diagnostics were performed and showed normality and homoscedasticity of residuals. Spearman correlation analysis

TABLE 1 | Genes, primers, and probes for TaqMan RT-qPCR.

Genes	Accession number	Primer sequences	Probe sequences	Amplicon size (bp)
EqGal-1	KY264050.1	For: CAAGGCAGACCTGACCATCA Rev: TGACGGCCTCCAGGTTGA	6-FAM/CTGCCGGAT/ZEN/GGCTACTCGT TCAAGTTC/IABkFQ	77
EqGal-3	KY264051.1	For: TAAATTTCAACAGAGGGCATGATG Rev: CAATGACTCTCCTGTTGTTCTCGTT	6-FAM/TGCCTTCCA/ZEN/CTTTAACCCG CGCTT/IABkFQ	75
Eq 18S rRNA	NR_046271.1	For: GGCGTCCCCCAACTTCTT Rev: AGGGCATCACAGACCTGTTATTG	6-Fam/TCGAACGTCTGCCCTATCAACT TTCGAT/IABkFQ	77

was performed to determine associations between synovial fluid galectin-1 and galectin-3 levels and previously published lubricin ELISA data for the first experimental cohort and for the naturally occurring OA cases (32). Raw RT-qPCR and ELISA data from naturally occurring samples were log-transformed to achieve normality of the data, and data were analyzed using a one-way ANOVA with Dunnett's *post hoc* tests for multiple comparison correction, designating healthy carpal joints (OA severity = 0) as the control group. Significance was set at $\alpha = 0.05$.

Immunohistochemistry scores (0 to 3 scale) were treated as ordinal categorical outcomes, and weighted kappa statistics were calculated for inter-observer agreement. Immunostaining results were assessed using Wilcoxon matched-pairs signed rank tests due to the small sample size and non-normal distribution of data. For the percentage of galectin-1 positive chondrocytes throughout the entire cartilage section, scores were treated as continuous outcomes and analyzed using Wilcoxon matched-pairs signed rank tests. All modeling and parametric analyses were performed using JMP Pro 13 software (SAS; Cary, NC), and

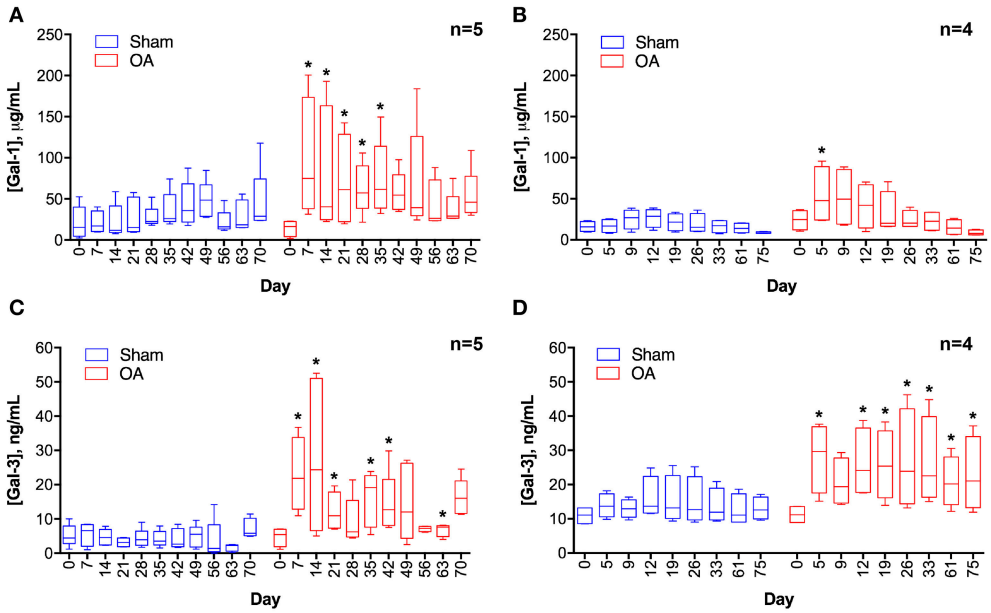

FIGURE 1 | Galectin-1 **(A,B)** and galectin-3 **(C,D)** concentrations in equine synovial fluid prior to (day 0) and after arthroscopically-induced osteochondral fragmentation (OA) or sham operation (Sham). Data are displayed as box-and-whisker plots representing the first and third quartiles, median, and spread of concentrations for each serial sampling time point. ELISA data in **(A,C)** were obtained from the same cohort of horses ($n = 5$), and ELISA data in **(B,D)** were obtained from a second cohort of horses ($n = 4$). Mixed linear model derived P-values for the fixed effect of treatment and day were <0.001 for models A-D, except for D where day $P = 0.32$. An interaction between treatment and day was identified for model A (treatment * day $P = 0.0007$) and C (treatment * day $P = 0.03$). Note that there is variation in the date of synovial fluid sampling between the two cohorts. Asterisks denote days where OA galectin concentrations were significantly increased as compared to Sham, * = $P < 0.05$ after Bonferroni correction.

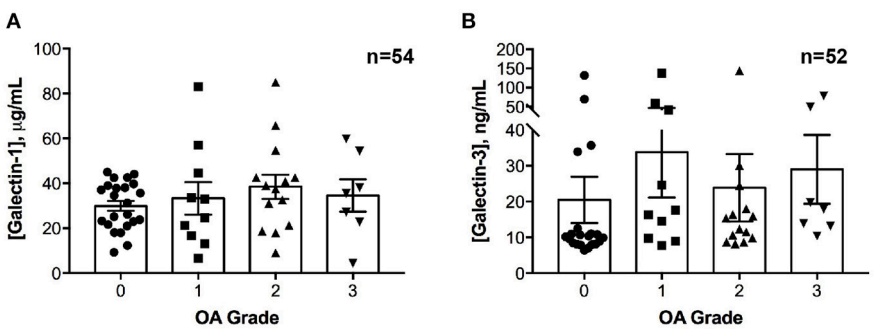

FIGURE 2 | Galectin-1 and-3 concentrations in equine synovial fluid obtained from carpal joints of horses with naturally occurring osteoarthritis (OA), classified by severity as mild (1), moderate (2) or severe (3) and from healthy carpal joints (0). Individual data are shown as scatterplots, in addition to means ± S.E.M. There were no significant differences in synovial fluid galectin-1 **(A)** or galectin-3 **(B)** concentrations between healthy and OA joints.

non-parametric test statistics, kappa statistics and graphs were generated using Prism 7 (GraphPad; La Jolla, CA).

RESULTS

Synovial Fluid Galectin Protein Concentrations

Experimental OA

Synovial fluid galectin-1 and galectin-3 concentrations increased following carpal osteochondral fragmentation in both experimental cohorts (**Figure 1**). In the carpal osteochondral fragment model, galectin-1 synovial fluid concentrations increased by up to 4-fold (median: 74.8 μg/mL vs. 16.9 μg/mL on day 7), and galectin-3 synovial fluid concentrations increased by as much as 5-fold in the OA joint as compared to the sham-operated joint (median: 24.3 ng/mL vs. 4.6 ng/mL on day 14). Galectin-1 synovial fluid concentrations were most elevated acutely after injury, whereas elevations in galectin-3 were sustained up to the end of the study duration (day 75) in one cohort of horses (**Figure 1D**). Galectin-3 was moderately correlated with synovial fluid lubricin ($\rho = 0.47$, $P < 0.0001$), whereas galectin-1 was weakly correlated with lubricin ($\rho = 0.27$, $P = 0.007$) (32). Galectin-1 and-3 synovial fluid levels were weakly correlated ($\rho = 0.31$, $P = 0.002$).

Naturally Occurring OA

No differences in galectin-1 or galectin-3 concentrations were detected in healthy joints as compared to joints with naturally occurring OA (**Figure 2**). Galectin-1 and galectin-3 synovial fluid concentrations were weakly correlated ($\rho = 0.28$, $P = 0.04$).

mRNA Expression in Naturally Occurring OA

There were no differences in galectin-1 or galectin-3 mRNA expression in synovial membrane from healthy and OA joints (**Figures 3A,B**). In contrast, galectin-1 mRNA expression was significantly upregulated in moderate and severe OA cartilage, with an approximately 12- and 75-fold increase as compared to healthy cartilage (**Figure 3C**). Galectin-1 mRNA expression was minimal in healthy (grade 0) cartilage. Galectin-3 mRNA levels were greater in severe OA as compared to mild OA (**Figures 3C,D**).

Immunolocalization in Experimental OA

Synovial membrane tissue sections stained intensely for both galectin-1 and galectin-3 (**Figure 4**), with the most prominent immunoreaction observed in perivascular and intimal regions. Consistent with RT-qPCR data, no differences were noted in galectin synovial membrane immunostaining between sham-operated and OA joints on day 70 post-fragmentation. Weighted kappa statistics revealed moderate inter-observer agreement for both galectin-1 and-3 chondrocyte immunostaining (0.55 and 0.43, respectively). Safranin O staining was decreased in areas of partial-thickness cartilage fibrillation in the osteochondral fragment joint as compared to the sham-operated joint (**Figures 5A–D**). Superficial zone

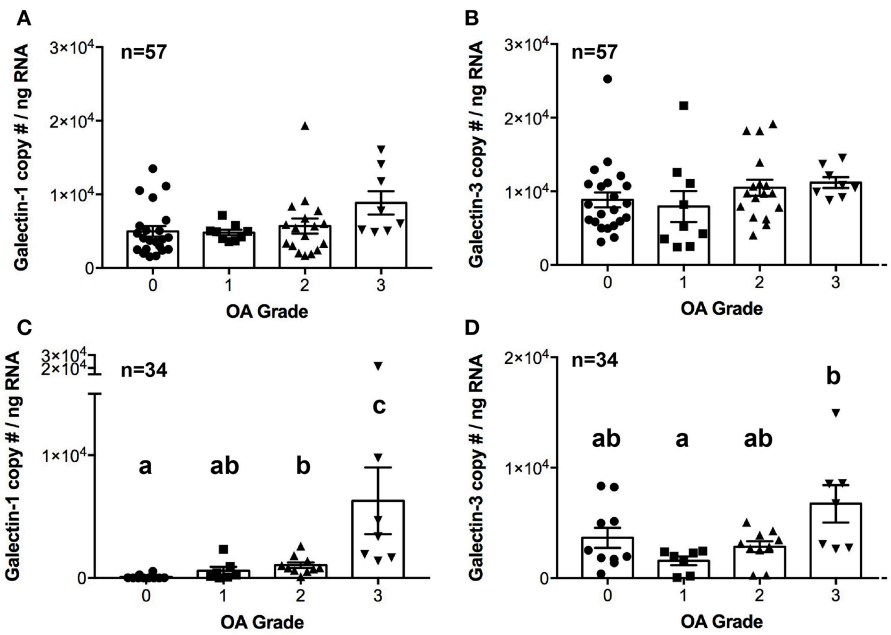

FIGURE 3 | Galectin-1 and-3 copy # per ng of RNA from synovial membrane **(A,B)** or cartilage **(C,D)** tissue obtained from healthy carpal joints (0) or carpal joints with naturally occurring osteoarthritis (1–mild, 2–moderate, 3–severe OA). There were no differences in galectin-1 or-3 mRNA expression in synovial membrane tissue **(A,B)**. Cartilage galectin-1 mRNA expression increased with OA severity score (**C**, $P = 0.0002$), whereas galectin-3 mRNA expression was increased in severe OA cartilage as compared to mild OA cartilage (**D**, $P = 0.03$). Scatterplots are displayed, with bars representing mean ± S.E.M. Differing letters note statistically significant differences, $P < 0.05$.

chondrocytes from healthy articular cartilage stained intensely for galectin-3 (**Figures 5I,J**), whereas galectin-1 staining was nearly absent (**Figures 5E,F**), consistent with gene expression data and prior immunostaining results in healthy equine cartilage (34). Galectin-1 immunostaining was increased in superficial and middle zone chondrocytes from injured cartilage (**Figures 5G,H**, $P = 0.02$ and 0.03, respectively) and was minimal to absent in deep zone chondrocytes in all cartilage sections. The most intense galectin-1 staining was localized to dividing chondrocytes and chondrocyte clusters (chondrones) within the superficial zone (**Figure 5H**, arrow). Chondrocyte galectin-1 immunoreactivity was significantly increased in cartilage from the osteochondral fragment joint as compared to the sham-operated joint (**Figure 6**).

There were no differences in galectin-3 chondrocyte immunostaining between healthy and injured cartilage. Superficial zone chondrocytes demonstrated intense galectin-3 expression in both healthy and injured cartilage (**Figures 5I–L**).

DISCUSSION

Galectins-1 and-3 are present in healthy equine synovial fluid and synovial tissue but increase in response to osteochondral injury. In prior work, we have demonstrated greater galectin-1 and-3 mRNA expression in healthy equine synovial membrane as compared to healthy articular cartilage (4), suggesting that the synovium may be the predominant source of synovial fluid galectins. Synovial fluid galectins-1 and-3 were elevated in experimental OA, with a transient increase in galectin-1 and a sustained increase in galectin-3. Cartilage galectin-1 mRNA expression increased with increasing OA severity, and galectin-1 immunostaining was increased in superficial and middle zone chondrocytes in injured cartilage. Whereas, galectin-3 was constitutively produced by superficial zone chondrocytes, galectin-1 immunostaining was nearly absent in healthy articular cartilage. Thus, although both galectin-1 and-3 synovial fluid concentrations are increased in joint injury, galectin-1 upregulation in injured chondrocytes appears to be a specific response to cartilage injury in horses.

Synovial Fluid Galectin Protein Concentrations

Galectin-1 and-3 synovial fluid concentrations were increased in injured as compared to sham-operated joints. To our knowledge, this is the first study to profile serial galectin synovial fluid measurements prior to and after joint injury, revealing a transient elevation in galectin-1 after injury and a

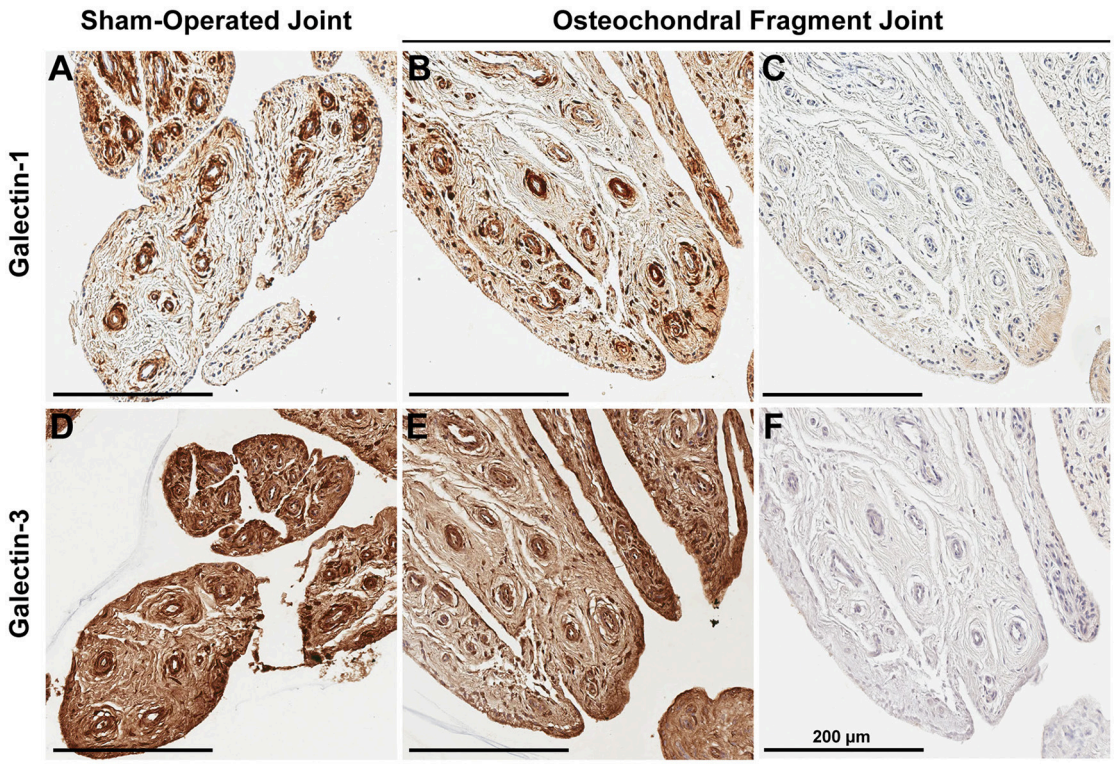

FIGURE 4 | Synovial membrane galectin-1 and galectin-3 immunostaining reveals constitutive galectin expression in both sham-operated **(A,D)** and osteochondral fragment joints **(B,E)** from a representative horse 70 days post-injury. Galectin-1 and-3 staining is most prominent in perivascular regions. No differences were observed in galectin-1 or-3 staining between sham-operated and osteochondral fragment joints. Primary antibodies were omitted in **(C,F)**, revealing absence of antigen-independent staining. Scale bar: 200 µm.

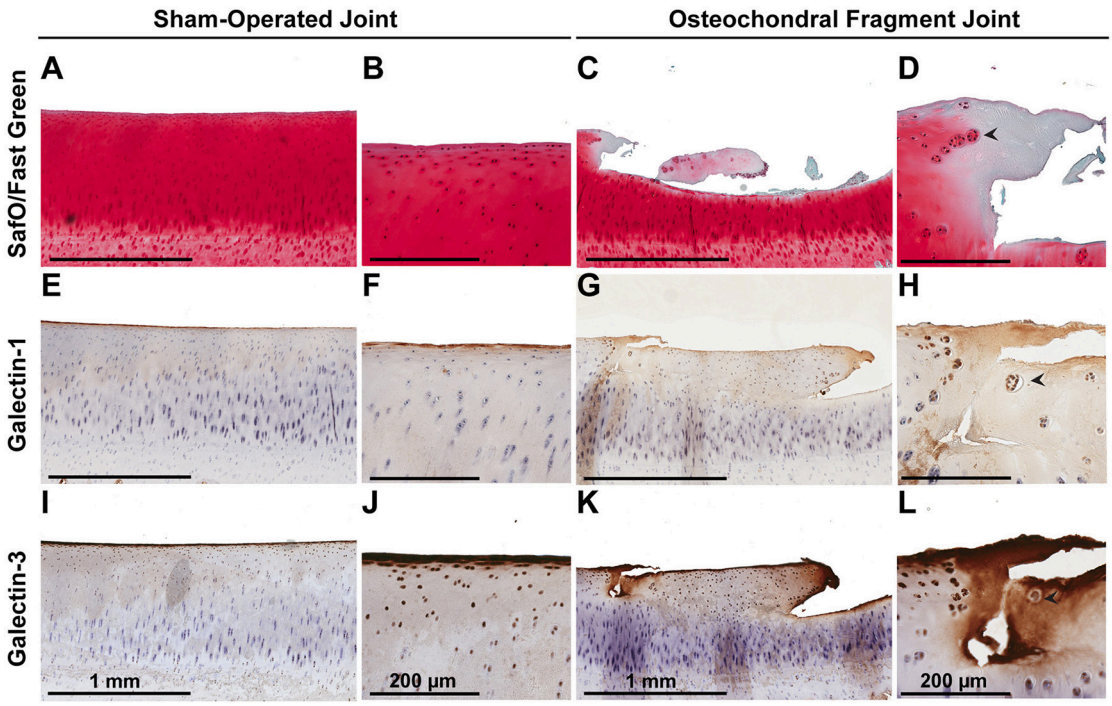

FIGURE 5 | Safranin O/Fast Green **(A–D)**, galectin-1 **(E–H),** and galectin-3 **(I–L)** immunostaining of third carpal bone cartilage from sham-operated and osteochondral fragment joints from a representative horse 70 days post-injury. Superficial zone chondrocytes and some middle zone chondrocytes stain positively for galectin-3 **(I,J)** but not galectin-1 **(E,F)** in sham-operated joints. Cartilage fibrillation, proteoglycan loss and chondrone (arrow) formation is observed in cartilage from the osteochondral fragment joint **(C,D)**. Chondrones stain positively for galectin-1 **(G,H**-arrow) and galectin-3 **(K,L**-arrow).

more sustained elevation in galectin-3. This longitudinal data suggests that galectins are increased in response to traumatic joint injury. Although these observations were supported by two distinct equine experimental cohorts, differences in synovial fluid galectin levels were not observed in horses with naturally occurring carpal OA. Because the OA severity grading scale for naturally occurring carpal injury involves assessment of radiographic changes, which lag behind inflammatory changes, and because the OA severity score doesn't account for the duration of injury prior to presentation, we may be missing more acute, transient elevations in galectins in horses at the time that they present to the hospital for surgical treatment.

mRNA Expression

We observed increased galectin-1 mRNA expression and immunostaining in injured equine cartilage. Interestingly, galectin-1 cartilage mRNA expression increased proportionally with the severity of arthritis. Our findings that galectin-1 mRNA expression is increased in OA cartilage and that galectin-1 immunostaining is increased in regions of focal cartilage damage coincide with prior studies in human OA cartilage (21–23). Differences in galectin-1 immunostaining were not observed in deep zone chondrocytes in the current study; however, cartilage pathology was primarily restricted to the superficial and middle zones in this equine OA model and could explain why galectin-1 immunostaining was not observed in deep zones.

Galectin-1 stimulates a network of NF-kB downstream signaling in human OA chondrocytes as demonstrated by microarray and RT-qPCR (21). Synergistic effects of galectins-1,-3, and−8 have been demonstrated in human OA chondrocytes, providing evidence for cooperativity within this galectin signaling network (24, 35). On the other hand, galectin-1 is suggested to mitigate inflammatory arthritis in rodent models by altering immune cell function, inducing apoptosis of CD4+ T cells and decreasing pro-inflammatory cytokine expression (14–17). Notably, inflammatory arthritis and post-traumatic osteoarthritis are distinct entities despite sharing some similarities, such as synovial inflammation and synovitis (36, 37).

Immunolocalization

In addition to gene expression data demonstrating that synovial membrane is the predominant source of galectins in synovial joints, galectin immunostaining revealed prominent galectin-1 and-3 immunolocalization in synovial membrane tissue from both sham-operated and OA joints. Whereas, superficial zone chondrocytes consistently stained positively for galectin-3 in healthy cartilage, middle and deep zone chondrocytes were negative. Similar patterns of strong superficial zone chondrocyte galectin-3 staining have been previously observed

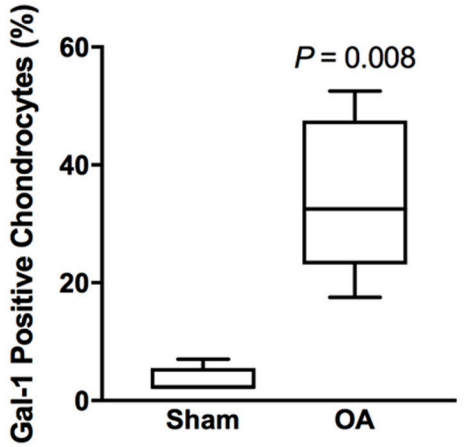

FIGURE 6 | Chondrocyte galectin-1 positive immunoreactivity over the entire thickness of articular cartilage in the equine carpal fragmentation experimental model 70 days post-injury ($n = 5$). Data are presented as box-and-whiskers plots, where boxes represent the first and third quartiles, the lines within the boxes represent the median, and the lines outside the boxes represent the spread of galectin-1 scores. The *P*-value indicates the difference between injured (OA) and healthy (Sham) cartilage, produced by Wilcoxon matched-pairs signed rank test.

in healthy equine and human cartilage (2, 34). Chondrocytes in healthy cartilage were immune negative for galectin-1. Galectin-1 positive chondrocytes were only detected in injured cartilage, especially in dividing chondrocytes or chondrones in the superficial and middle zones of injured, fibrillated cartilage.

The identification of increased galectin-3 in RA (7, 38) and juvenile idiopathic arthritis (8) has led authors to hypothesize that galectin-3 precipitates inflammatory arthritis. Elevations in serum galectin-3 concentrations have been detected in patients with early RA and correlated with MRI bone lesions 1 year later (39). However, the link between galectin-3 and OA is less clear and, to our knowledge, no human studies have documented elevations in synovial fluid galectin-3 levels preceding the development of arthritis. Data on the role of galectin-3 in experimental rodent models is conflicting. Gal-3 KO mice are predisposed to OA (6), potentially due to the protective role that galectin-3 plays in chondrocyte survival (5, 6). Galectin-3 KO mice also demonstrate decreased bone formation, increased bone resorption, accelerated trabecular bone loss and reduced bone strength as compared to wild-type mice, suggesting an important role for galectin-3 in bone remodeling and biomechanics (25). On the other hand, exogenous intra-articular galectin-3 administration promoted the development of arthritis in mice (19), and inhibition of galectin-3 through lentiviral-mediated delivery of galectin-3 shRNA ameliorated collagen-induced arthritis in rats (16). The conflicting data with respect to galectin-3 and OA may be due to the distinct functions of intracellular vs. extracellular galectin-3 and differences between inflammatory and PTOA models of arthritis. Intracellular gal-3 promotes chondrocyte survival both *in vitro* (6) and *in vivo* (6); whereas administration of exogenous, extracellular galectin-3 exacerbates inflammation (16, 18). Notably, most rodent studies investigating galectins and arthritis have focused on inflammatory models which more closely mimic RA. Horses and other large animal models are more commonly used to study PTOA and better represent the clinical scenario for translation to PTOA in humans (28, 40). Therefore, future work is needed to determine whether increased synovial fluid galectin-3 concentrations in equine PTOA are functioning to protect articular chondrocytes, promote synovial inflammation or both.

Here, we demonstrate constitutive expression of both galectin-1 and-3 in healthy synovial membrane tissue and synovial fluid, while also elucidating the time course of galectin-1 and-3 upregulation following induction of post-traumatic OA. Toegel and Weinmann et al. have suggested that both galectin-1 (21) and galectin-3 (24) promote OA through upstream regulation of NF-kB signaling in chondrocytes. Accordant with these findings, we show that galectin-1 mRNA expression and immunostaining is increased in equine OA cartilage as compared to healthy cartilage. Overall, our data suggests that galectin-1 mRNA expression and protein production is increased in injured equine articular chondrocytes, similar to injured human cartilage (21, 22). Galectin-1 appears to be more specific to articular cartilage injury in horses than galectin-3. In addition, our data suggests that synovial membrane and cartilage galectin expression patterns differ, with constitutive galectin-1 and-3 synovial membrane expression present in all sham-operated, healthy joints.

This study provides evidence for the constitutive expression and production of galectins-1 and-3 in healthy synovial joints. Whereas, galectin-3 is constitutively produced in superficial zone chondrocytes in healthy articular cartilage, both galectin-1 and-3 are expressed in healthy synovial membrane tissue. In addition, we demonstrate that synovial fluid galectins are elevated in response to PTOA and that cartilage galectin-1 expression strongly correlates with OA progression. Galectins may be potential upstream therapeutic targets in OA; however, further work is needed to clarify the mechanistic roles of galectins-1 and-3 in synovial membrane tissue, cartilage and synovial fluid in PTOA. Several small-molecule galectin antagonists and anti-galectin monoclonal antibodies are currently undergoing preclinical testing for fibrosis and cancer therapy and may have applications in other chronic inflammatory diseases, such as OA and RA (41). However, therapeutic targeting of galectins also poses significant challenges due to the context-dependent multifunctionality of galectin signaling (42) and the ability of other galectin family members to compensate for the loss of an individual galectin (35). In addition, because galectins are constitutively expressed in several tissues, off-target effects are of potential concern (43, 44). Constitutive expression of galectins in synovial fluid also suggests that there may be functional roles for galectins in healthy synovial joints, including beneficial roles in cartilage lubrication (34). Additional research is needed to clarify the functions of galectins-1 and-3 in healthy joints and in PTOA in both experimental animal models and human patients. Translational animal models will be critical for pre-clinical testing of galectin-targeted therapies for human OA.

DATA AVAILABILITY STATEMENT

Datasets for equine galectin genomic sequences can be found in the GENBANK repository [https://www.ncbi.nlm.nih.gov/genbank/]. The raw gene expression and ELISA data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

HR conceived and designed the project; obtained funding for the project; acquired, analyzed and interpreted data; drafted the article; and approved the final submitted version of the article. AN obtained funding for the project, and both AN and AW contributed to the acquisition, analysis and interpretation of data; critically revised the article for important intellectual content; and approved the final submitted version of the manuscript. JS, SL, RS, and RP contributed to the acquisition, analysis and interpretation of the data; and revised and approved the final submitted version of the manuscript. SM provided statistical expertise for the analysis and interpretation of the data; critically revised the article for important intellectual content; and approved the final submitted version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2018.00288/full#supplementary-material

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frontiers in Veterinary Science

ORIGINAL RESEARCH
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A Pilot Study on the Efficacy of a Single Intra-Articular Administration of Triamcinolone Acetonide, Hyaluronan, and a Combination of Both for Clinical Management of Osteoarthritis in Police Working Dogs

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Objectives: To describe and compare the use and effectiveness of a single intra-articular injection (IA) of triamcinolone acetonide (TA), hyaluronan (HA), and a combination of both (TA+HA) in police working dogs with natural occurring hip osteoarthritis (OA).

Study Design: Prospective, randomized, single-blinded study.

Sample Population: Thirty animals with naturally occurring hip OA.

Methods: Animals were randomly divided in three groups: GT, treated with 20 mg of TA per hip joint; GH, treated with treated 20 mg of HA per hip joint; and GTH, treated with a combination of 20 mg of TA and 20 mg of HA per hip joint. Response to treatment, measured by the Canine Brief Pain Inventory (divided in Pain Interference Score—PIS and Pain Severity Score—PSS) and the Hudson Visual Analog Scale (HVAS), was evaluated in seven different time points: T0 (before treatment), T1 (after 15 days), T2, T3, T4, T5, and T6 (after 1, 2, 3, 4, and 5 months, respectively). Results were compared using a Kruskal-Wallis test or a Wilcoxon signed ranks test, and $p < 0.05$ was set.

Results: Comparing results of the different time points considered with T0, significant differences were registered in GH at T1 for HVAS ($p = 0.03$) and PIS ($p = 0.04$); and in GTH at T1 ($p = 0.05$ for HVAS and $p < 0.05$ for PIS), T2 ($p < 0.04$ for PIS), T3 ($p < 0.03$ for HVAS and $p = 0.05$ for PIS), T4 ($p < 0.03$ for HVAS and $p < 0.05$), and T5 ($p < 0.05$ for HVAS). No significant differences were found

between groups when comparing scores in each time point. Individual treatment is considered successful with a reduction of ≥1 for PSS or ≥2 for PIS. In GTH, treatment was successful in four animals between T1 and T5 (40%, $n = 10$) and three at T6–T7 (30%, $n = 10$) for PSS and three animals of GTH at T1 (30%), two at T2 (20%), three between T3 and T4 (30%), and two between T5 and T7 (20%).

Conclusions and Clinical Relevance: This study provides direct information on the use of these treatment modalities in patients with hip OA. Intra-articular injection with TA and HA may be a treatment option for dogs with naturally occurring OA, particularly when simultaneously used, as they provide significant improvements of PIS and HVAS scores. Individual scores improved in some animals with PIS, PSS, and HVAS.

Keywords: animal model, osteoarthritis, pain, triamcinolone, hyaluronan

INTRODUCTION

Osteoarthritis (OA) is a complex joint disease with a high negative impact on patient's quality of life and a high financial burden. Characterized by its inflammatory character and degradation of cartilage layers, it is a source of chronic pain, which affects all mammals, including humans and dogs (1–3). In adult active dogs, OA presents a prevalence around 20% (4–6). This value is expected to rise, due to a simultaneous increase in life expectancy and obesity. Both surgical and natural occurring canine models have been widely studied, and since pathologic process, clinical presentation and response to treatment are very similar in both species—humans and dog, this animal model is the closest to a gold standard (7–10). The changes that occur in slowly progressive spontaneous dog OA closely match those of human OA, in contrast with those seen in rapidly advancing experimental surgical induced OA (11). The grades of canine hip OA are similar to those in the classification of human OA (mild/minimal, moderate, and severe) (12, 13). In addition, companion animals share the same environment and suffer similar co-morbidities as humans, with OA usually being present for prolonged periods. Therefore, these naturally occurring painful disease models may better reflect the complex genetic, environmental, temporal, and physiological influences present in humans (12). Exploring spontaneous dog OA under the One Medicine concept can promote new insight on the disease, improving therefore the health and well-being of both species, humans, and dogs (12, 13).

Intra-articular (IA) corticosteroids (CS) have been used for several decades in humans and horses to successfully palliate pain and control inflammation associated with OA and surrounding tissues (14, 15). Different guidelines for the management of human OA provide varying strength of recommendation for the use of intra-articular CS, from weak to strong recommendation (16–20). On the other hand, other guidelines state an inability to recommend for or against the use of intra-articular corticosteroids, in this case specifically for patients with symptomatic knee OA (21). Corticosteroids reduce the number of inflammatory cells such as lymphocytes, macrophages and mast cells, and also slow down the synthesis of inflammatory mediators such as interleukin 1β, Tumor necrosis factor α, and Cycloxygenase 2 in the synovial fluid (22–25). The pain relief they provide is attributed to the inhibition of prostaglandin synthesis (24). Triamcinolone is recommended over other CS due to an extended duration of action (26, 27). Hyaluronan (HA), the high molecular glycosaminoglycan, occurs naturally in synovial fluid, and provides joint lubrication, helps limit inflammation, pain and cartilage degradation while acting as a shock absorber, allowing the joint to move in a smooth manner (16, 17). Its mechanism of action is not completely understood, but anti-inflammatory, anti-nociceptive, and chondroprotective properties have been suggested, through the enhancement of cartilage synthesis, blunting response to interleukin 1, protection from the damage of oxygen free radicals, and protection of chondrocytes from apoptosis (28–31). Guidelines for the management of OA provide a weak recommendation for the use of IA HA (20), a conditional recommendation against (19) or that they should not be offered as an option (18). The choice to use IA HA or a CS, or which CS to use, is often determined by individual preference of the clinician (18, 19). A popular approach is their combined administration, thus providing rapid onset of action (obtained from the CS), with prolonged effect and decreasing the potential side effects of intra-articular CS therapy (obtained from HA) (20, 21). Being an incurable chronic disease, treatment success in OA is often defined as the ability to manage its symptoms, mainly pain. The similarities in neurophysiology across mammals strongly suggest that the type of pain experienced by humans and animals is analogous (22). The Canine Brief Pain Inventory (CBPI) was developed to assess the impact of chronic pain in the patient's life. It is divided in two sections, a pain severity score (PSS) that assesses the magnitude of the animal's pain, and a pain interference score (PIS) that assesses the degree in which pain affects daily activities (23). The Hudson Visual Analog Scale (HVAS) has been validated for the assessment of mild to moderate lameness in dogs, using force plate analysis as a criterion-reference standard (24).

With this study, we aimed to determine (1) if the intra-articular administration of triamcinolone acetonide (TA) or HA can reduce pain scores in a naturally occurring canine osteoarthritis model and if (2) the combined administration of both substances provides better results for longer periods of time.

METHODS

The study used a sample of 30 working dogs ($N = 30$) from the *Guarda Nacional Republicana* (Portuguese Gendarmerie Canine Unit) of both genders (6 females and 24 males), with a mean age of 6 ± 2.4 years old and body weight of 33.3 ± 6.65 kg. Breeds included German Shepherd Dogs ($n = 20$), Belgian Malinois Shepherd Dogs ($n = 5$), Labrador Retriever ($n = 4$), and Dutch Shepherd Dog ($n = 1$). All had bilateral naturally occurring mild and moderate hip OA, classified according to the Orthopedic Foundation for Animals scoring, This method was chosen due to the unavailability of other evaluation methods, such as PennHip. Radiographic evaluation was performed by one of the authors (JCA), not a board radiologist but with extensive training and experience in radiographic examination.

Patients were included based on trainer complaints, physical examination, and standard pelvis radiographic evaluation consistent with bilateral hip OA. Animals with other diseases were ruled out through physical examination, complete blood count, and basic serum chemistry profile (BUN, Creat, ALT, AST, Gluc) were not included in the study. Patients under any treatment, therapy or supplement were also excluded. Written, informed consent was obtained from the Institution responsible for the animals.

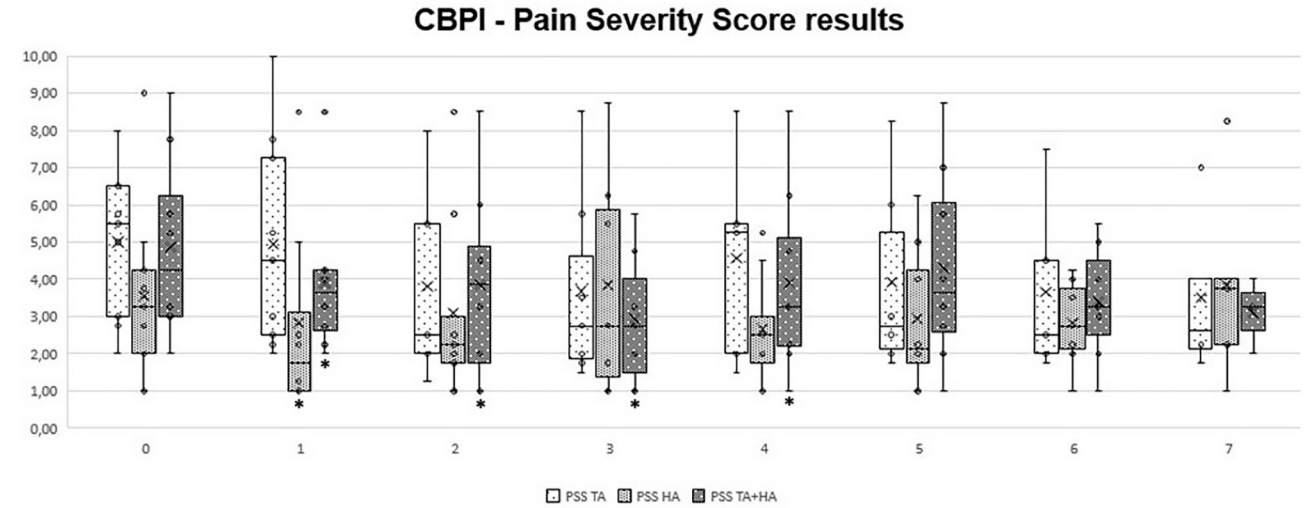

FIGURE 1 | Overall Pain Severity Scores, by section and instant for triamcinolone acetonide (TA), hyaluronan (HA), and TA+HA. Box plots represent median, 25th and 75th percentiles, and whiskers represent 10th and 90th percentiles. *indicates significant differences within the group, when compared with T0.

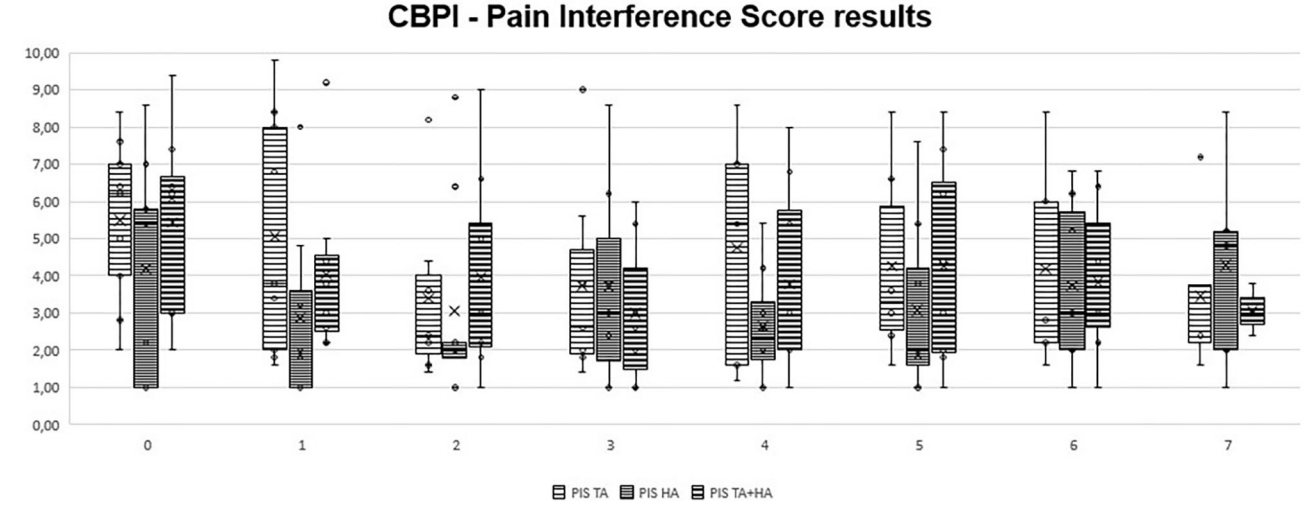

FIGURE 2 | Overall Pain Interference Scores, by section and instant for triamcinolone acetonide (TA), hyaluronan (HA), and TA+HA. Box plots represent median, 25th and 75th percentiles, and whiskers represent 10th and 90th percentiles.

Dogs were randomly divided in three groups according to the type of drug used for hip joint IA administration, namely: GT (treated with 20 mg of TA per hip joint—Trigon depot, Bristol-Myers Squibb®, Spain), GH (treated with 20 mg of hyaluronan per hip joint—Hyalart, Grunenthal®, Portugal), and GTH (treated with the combination of both substances per hip joint). Breeds were similarly distributed amongst groups: GT had 7 German Shepherd Dogs, 2 Belgian Malinois Shepherd Dog and 1 Labrador Retriever; GH had 6 German Shepherd Dogs, 2 Belgian Malinois Shepherd Dogs, 1 Labrador Retriever and 1 Dutch Shepherd Dog; and GTH had 7 German Shepherd Dogs, 1 Belgian Malinois Shepherd Dogs and 2 Labrador Retrievers.

The IA administration was always made by the same clinician and conducted with dogs under light sedation using medetomidine (0.01 mg/kg) and buthorphanol (0.1 mg/kg), both administered intravenously, and with intravenous fluids of NaCl 0.9% in the dose of 2 ml/Kg/h. Animals were placed in lateral recumbency, and a small window of 4 cm × 4 cm area surrounding the greater trochanter was clipped and aseptically prepared, using a chlorhexidine solution 0.2% followed by 70% alcohol application, using sterile gloves and 10 cm × 10 cm gauzes. With the limb parallel to the table surface and in a neutral position, the operator inserted a 22-gauge with 75 mm length spinal needle, closely dorsal to the greater trochanter and perpendicular to the long axis of the limb (32). Confirmation of correct needle placement was obtained through the collection of synovial fluid. Both hips were treated with the same treatment in all animals. After treatment, animals were rested for 3 consecutive days. Signs of exacerbated pain during daily activities or physical examination (pain during joint mobilization, stiffness, and reduced range of motion), persistent stiffness of gait and changes in posture exhibited by the dogs, were evaluated by the veterinarian on the days 1 and 3 after the IA procedure. With IA treatments, some side effects were documented, and include local pain and inflammation, swelling and infection. These are usually self-limiting, and take 2–10 days to resolve (33, 34). If no complaints were registered, the animal could resume its normal activity over a period of 5 days (35, 36).

To evaluate the response to treatment and comparing it with the initial clinical condition, two validated tools for dog pain assessment were used: the CBPI (**Appendix A**) and the HVAS (**Appendix B**). These were completed by the trainers, who were unaware of which treatment the animal received. Evaluations were conducted at T0 (before IA treatment), T1 (15 days after IA treatment), T2, T3, T4, T5, T6, and T7 (1, 2, 3, 4, 5, and 6 months after IA treatment, respectively).

From all the sampled individuals, three dogs from the GT were excluded—two after T2 due to having developed unrelated medical conditions (one developed gastric dilatation volvulus and the other suffered a third phalanx avulsion), and one after T3 due to an inability to maintain medical follow-up. Data collected from these animals was considered up to the point of their exclusion. Data was analyzed with IBM SPSS Statistics version 20, and a significance level of $p < 0.05$ was set. Normality was accessed with a Shapiro-Wilk test and results of all groups in each time point considered were compared using a Kruskal-Wallis test. When comparing each time point with T0 within each

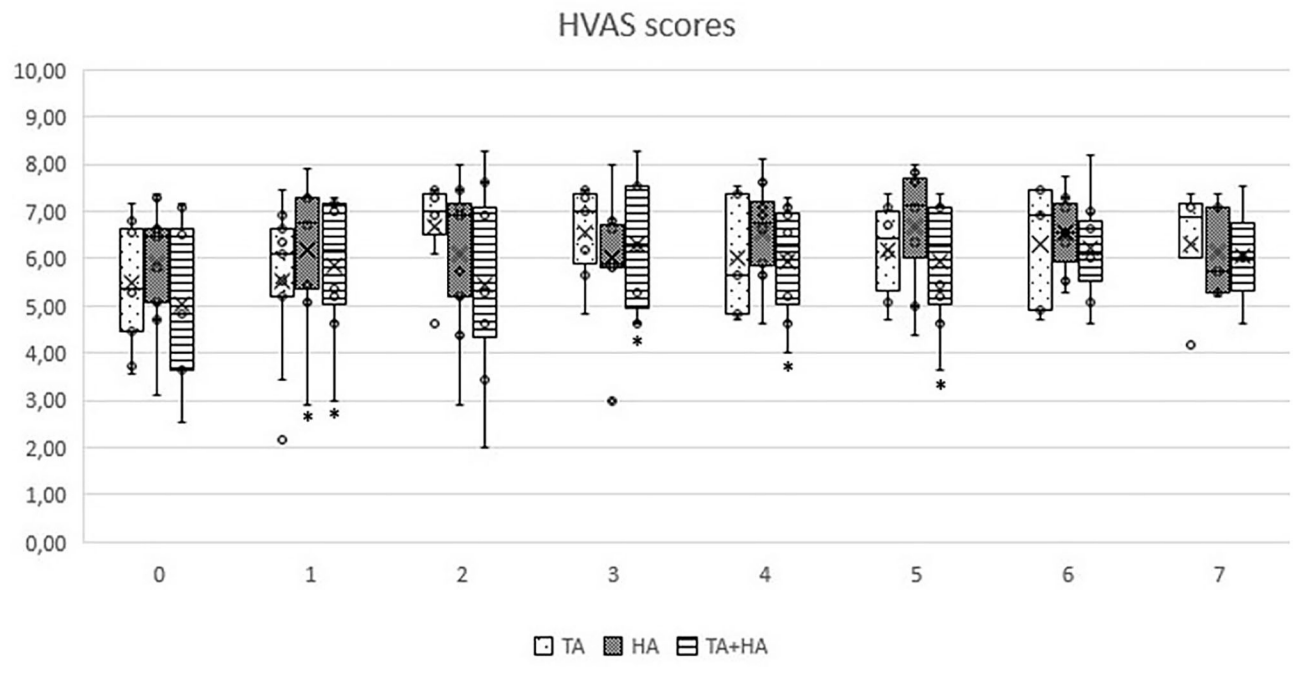

FIGURE 3 | Overall Hudson Visual Analog scores, by instant for triamcinolone acetonide (TA), hyaluronan (HA), and TA+HA. Box plots represent median, 25th and 75th percentiles, and whiskers represent 10th and 90th percentiles. *indicates significant differences within the group, when compared with T0.

group, a Kruskal-Wallis test or a Wilcoxon signed ranks test was used.

RESULTS

In GT, when comparing clinical results from T1 to T7 with patient initial condition (T0), no significant differences were recorded. In GH, significant differences were observed only at T1 ($p = 0.03$ for HVAS and $p = 0.04$ for PSS). In GTH, significant differences were observed at T1 ($p < 0.05$ for HVAS and $p < 0.05$ for PSS), T2 ($p < 0.04$ for PSS), T3 ($p < 0.03$ for HVAS and $p < 0.05$ for PSS), T4 ($p < 0.03$ for HVAS and $p < 0.05$ PSS), and T5 ($p < 0.05$ for HVAS). Comparing results of the three groups in each evaluation moment, no significant differences were found. Evolution of PSS, PIS, and HVAS scores can be observed in **Figures 1**–**3**, respectively. Evolution of mean PSS, PIS, and HVAS scores (±standard deviation), *p* values and percentage variations in each group, are presented in **Table 1**.

During the study, no side effects were detected in any of the animals. All patients were able to resume normal activity after treatment.

DISCUSSION

OA is a chronic disease with no cure, but with the possibility to be effectively managed in a largely palliative approach, aiming to relieve symptoms and especially pain (17, 29). Non-steroidal anti-inflammatory drugs (NSAIDs) are often considered as the first line of OA treatment. For active patients, or with more advanced OA stages, the control they provide over signs and symptoms may be insufficient (30, 31, 37). Since OA is symptomatic only in the affected joint, while lacking obvious extra-articular manifestations, it is well-suited to have a local therapy administered by intra-articular injection, reducing the total amount required to produce an effect, compared with a systemic administration (38–40). Still, this approach presents some disadvantages, as the need of a precise diagnosis, the learning curve inherent to the execution of the procedure (particularly when considering hips), better conducted with the assistance of fluoroscopy or ultrasound, and the need of placing the patient under sedation or general anesthesia. Results showed that both TA and HA, when administered through intra-articular injection, are able to reduce pain levels to some degree and up to certain time points, in a naturally occurring canine osteoarthritis model. The combination of both substances, in particular, can be an effective therapeutic option, with a majority of treated patients showing improved results in their clinical condition, lasting for several months.

Limitations of this study are associated with sample size and the lack of a control group. Additionally, even though both the CBPI and HVAS have been validated as tools for the assessment of pain, lameness, and response to treatment in dogs, further studies should include another evaluation method such as Force Plait Gait or Stance Analysis.

The CBPI is often the analysis of choice in the veterinary literature, recommended for comparisons of pain scores between

TABLE 1 | Evolution of mean pain severity score (PSS) and pain interference score (PIS) of the Canine Brief Pain Inventory (CBPI), and improvements in HVAS scores (±standard deviation), by group and moment.

Survey	Group	T0		T1				T2				T3				T4				T5				T6				T7			
		Score	SD	Score	SD	*p*	%	Score	SD	*p*	%	Score	SD	*p*	%	Score	SD	*p*	%	Score	SD	*p*	%	Score	SD	*p*	%	Score	SD	*p*	%
PIS	GT	5.00	1.90	4.94	2.33	1.00	1.1	3.82	2.15	0.42	23.6	3.68	1.97	0.49	26.4	4.55	2.24	0.46	9.0	3.92	2.14	0.42	21.7	3.65	2.15	0.19	27.0	3.50	1.75	0.27	30.0
	GH	3.56	2.32	2.81	1.97	0.17	20.9	3.08	1.80	0.23	13.3	3.86	2.55	0.75	−8.5	2.66	1.11	0.87	25.3	2.94	1.61	0.79	17.4	2.82	1.08	0.68	20.6	3.85	1.82	0.36	−8.3
	GTH	4.88	2.35	3.91	1.34	0.18	19.9	3.84	2.03	0.21	21.2	2.93	1.42	0.06	39.9	3.91	1.95	0.16	19.9	4.31	2.14	0.53	11.5	3.39	1.48	0.27	30.4	3.08	0.72	1.00	36.8
PSS	GT	5.49	2.07	5.07	2.83	0.86	7.7	3.40	1.71	0.09	38.1	3.74	2.05	0.11	31.8	4.76	2.69	0.69	13.3	4.27	2.16	0.21	22.3	4.20	2.59	0.22	23.5	3.45	1.88	0.27	37.1
	GH	4.20	2.76	2.85	1.86	0.04	32.1	3.04	2.02	0.35	27.5	3.71	2.13	0.35	11.6	2.65	1.16	0.35	36.9	3.08	1.89	0.46	26.8	3.74	2.13	0.35	10.9	4.28	2.22	0.47	−1.9
	GTH	5.43	2.37	4.05	1.61	0.04	25.3	3.95	2.19	0.03	27.2	3.00	1.54	0.04	44.7	3.78	2.22	0.04	30.4	4.28	2.33	1.04	21.2	3.83	1.99	0.23	29.4	3.07	0.49	0.66	43.5
HVAS	GT	5.51	1.29	5.54	1.29	0.52	0.6	6.69	0.76	0.24	21.5	6.55	0.86	0.18	18.9	6.02	1.15	0.89	9.3	6.18	0.88	0.92	12.3	6.29	1.22	0.35	14.3	6.32	1.07	0.36	14.8
	GH	5.89	1.30	6.19	1.28	0.03	5.2	6.09	1.37	0.51	3.4	6.00	0.99	0.31	1.9	6.57	0.88	0.39	11.5	6.68	1.08	0.26	13.5	6.55	0.83	0.74	11.1	6.13	0.88	0.23	4.0
	GTH	5.03	1.63	5.85	1.31	0.04	16.4	5.44	1.62	0.16	8.2	6.31	1.27	0.03	25.5	5.95	1.01	0.03	18.4	5.94	1.22	0.04	18.2	6.23	1.10	0.11	24.0	6.06	0.99	0.11	20.5

groups (41, 42). In this study, significant variations were observed in GH only at the first follow up, and for PSS. In GTH, improvements were also observed in PSS scores, but for a longer period. It was in GTH that the biggest improvement was observed, with a 44.7% improvement at T3. Individual treatment success has been set as a decrease in PSS $\geq$ 1 and in PIS $\geq$ 2 (28, 43). Both IA treatments with HA and TA were able to significantly reduce individual scores in naturally occurring canine OA model, particularly PSS, while improving the results for the majority of patients. In some cases of GT and in one patient of GH, beneficial results spread up to the last evaluation point, while most improvements in both groups declined around T4-T5. Results for PIS showed no significant variations, considering group results. Individual results registered some improvements, but less marked than PSS results. This may be because, for some patients, PIS scores were low to begin with, making it difficult to reach a significant reduction. In addition, these are dogs with very high prey drive and work motivation, which may lead to a good performance and low perception of pain interference during daily activities. Individual HVAS results improved in almost all animals. However, when considering group results, significant improvements were observed only in GH (at T1) and GTH, in this case up to T5. In GTH, similarly to PSS scores, improvements reached a highest of 25.5% at T3, and declined from this point, reaching an 18.2% improvement at T5. The IA use of HA for the treatment of OA is still somewhat controversial due to its mode of action being unclear and clinical trials have provided contradictory results (38). The results of our study showed improvements in some animals at T1, with the maximal number of significant improvements obtained at T2, and maintained for a couple of months. Additional administrations, compared to a single injection, may be required in order to obtain sustained results. IA CS have been used for several decades in humans to successfully palliate pain and control inflammation associated with OA and surrounding tissues (14, 15). A systematic review has deemed triamcinolone more effective in relieving pain and improving function than betamethasone and methylprednisolone acetate (44). The choice to use IA HA or CS, or which CS to use, is often determined by individual preference of the clinician (18, 19). There are reports presenting deleterious effects of IA CS, as they may induce the production of a low quantity and high viscosity synovial fluid. These results are often based on multiple injections, particularly of methylprednisolone, while a single dose does not seem to cause long-term detrimental effects (45, 46). In a canine model of OA, animals treated with IA triamcinolone showed a significant reduction of osteophyte size compared with a control group. At the histological level, it significantly reduced the severity of OA structural changes of cartilage and had no deleterious effects on normal cartilage (47). Our results show that a single IA TA is effective in reducing pain scores in some animals, but not the majority of them. For those that it was, benefits were detected for several months, in some cases up to the last evaluation point. We did not observed any clinical side-effects in the animals treated with TA, and this seems to be a safe therapeutic options in patients with hip OA. As we did not performed a follow-up radiographic evaluation of the joints, we cannot comment on the evolution of radiographic signs in the three considered groups, but it should be addressed in future studies.

Results observed in GTH support the hypothesis that combined administration of HA and TA is superior in positive effects when comparing to the individual use of each one. PSS scores show significant improvements until T3, raising from a 25.3% at T1 to a 44.7% improvement at T3. Individual PSS scores improved in several patients up to the six-month evaluation point. HVAS in GTH also improved significantly from T1 to T5, in contrast to what was observed in GT and GH. This result is in accordance with what was observed with PIS, reflecting increased mobility, presumably due to decreased pain.

According to the author's knowledge, this is the first study that presents the description of the clinical effect of IA CS, HA and the combined use of both products in a naturally occurring canine model. The study was able to establish that all therapeutic approaches are safe, since no side effects were observed after the IA procedure in all animals of the three groups considered. All treatments can be effective for the treatment of OA, particularly the combined use of both products (TA + HA). Future studies should enroll a larger sample and considered the effect of different doses and administration frequency.

CONCLUSIONS AND CLINICAL RELEVANCE

Intra-articular administration of TA and HA may be a treatment option for natural occurring OA, particularly when used simultaneously. This study provides information on the use of these treatment modalities in patients with hip OA. Further studies are required, involving a larger number of patients and the use of a more objective evaluation method.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

This study is a part of a project approved by the ethical review committee of the University of Évora (Órgão Responsável pelo Bem-estar dos Animais da Universidade de Évora, approval no. GD/32055/2018/P1, September 25, 2018). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

JA designed the protocol, conducted treatments, and prepared the manuscript. PJ and AS selected patients and conducted treatments. CL and LC revised the protocol and prepared the

manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.512523/full#supplementary-material

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Macrophage Activation in the Synovium of Healthy and Osteoarthritic Equine Joints

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Synovitis is a major component of osteoarthritis and is driven primarily by macrophages. Synovial macrophages are crucial for joint homeostasis (M2-like phenotype), but induce inflammation (M1-like) when regulatory functions become overwhelmed. Macrophage phenotypes in synovium from osteoarthritic and healthy joints are poorly characterized; however, comparative knowledge of their phenotypes during health and disease is paramount for developing targeted treatments. This study compared patterns of macrophage activation in healthy and osteoarthritic equine synovium and correlated histology with cytokine/chemokine profiles in synovial fluid. Synovial histology and immunohistochemistry for M1-like (CD86), M2-like (CD206, IL-10), and pan macrophage (CD14) markers were performed on biopsies from 29 healthy and 26 osteoarthritic equine joints. Synovial fluid cytokines (MCP-1, IL-10, PGE_2, IL-1β, IL-6, TNF-α, IL-1ra) and growth factors (GM-CSF, SDF-1α + β, IGF-1, and FGF-2) were quantified. Macrophage phenotypes were not as clearly defined *in vivo* as they are *in vitro*. All macrophage markers were expressed with minimal differences between OA and normal joints. Expression for all markers increased proportionate to synovial inflammation, especially CD86. Synovial fluid MCP-1 was higher in osteoarthritic joints while SDF-1 and IL-10 were lower, and PGE_2 concentrations did not differ between groups. Increased CD14/CD86/CD206/IL-10 expression was associated with synovial hyperplasia, consistent with macrophage recruitment and activation in response to injury. Lower synovial fluid IL-10 could suggest that homeostatic mechanisms from synovial macrophages became overwhelmed preventing inflammation resolution, resulting in chronic inflammation and OA. Further investigations into mechanisms of arthritis resolution are warranted. Developing pro-resolving therapies may provide superior results in the treatment of OA.

Keywords: joint homeostasis, osteoarthiritis, synovitis, inflammatioin, activation, polarization

INTRODUCTION

Osteoarthritis (OA) is a leading cause of lameness and morbidity and presents significant treatment challenges in horses and people (1, 2). The pathophysiology of OA is incompletely understood; however, there is increasing evidence that macrophages play a central role in the synovial inflammation leading to OA (3–6). Macrophage depletion in studies of rheumatoid (7) and experimental arthritis (8, 9) have established that macrophages drive synovial inflammation (7, 8, 10) by showing dramatically decreasing expression of OA biomarkers in the absence of macrophages (6, 11). Although other cells, such as chondrocytes, can further amplify the inflammatory reaction, they cannot induce it in the absence of macrophages (5, 6, 12–14). More recent studies have shown that activation of macrophages in osteoarthritic synovium is directly related to disease activity, severity, and pain (15, 16). Conversely, macrophages are also key regulators of joint homeostasis and chondrogenesis (4, 17). In healthy conditions, macrophages promote synovial integrity through phagocytic activity (i.e., clearance of foreign material, tissue debris, and efferocytosis), and secretion of synovial fluid, cytokines, chemokines, and growth factors (4, 17). When these homeostatic functions become overwhelmed, synovial macrophages upregulate inflammation, recruiting other immune cells to respond to the increased demands for repair and recovery of homeostasis (6, 18–20).

Upon defined stimulation *in vitro*, macrophages activate into a spectrum of phenotypes, with the extremes represented by cells displaying classical pro-inflammatory (M1) or pro-resolving/healing (M2) responses (21). *In vivo*, macrophages respond to oscillating environmental stimuli, displaying marked phenotype plasticity, and play such a fundamental role in resolving inflammation and promoting tissue repair, that macrophage exhaustion or depletion results in severely compromised wound healing or chronic inflammation (22–25). *Ex vivo* chondrogenesis of synovial progenitor cells is impeded by classically activated (M1-like) macrophages from the osteoarthritic synovium, while alternatively activated (M2-like) macrophages are required for efficient chondrogenesis (17, 26). Inflammation in arthritic joints is dampened by M2-like macrophages, improving clinical and histological signs of joint disease (19, 27, 28). Collectively, these findings suggest that enhancing the M2-like response in diseased joints may provide a mechanism for resolving joint inflammation and restoring a healthy synovial environment with improved capacity for tissue repair.

Specific information regarding macrophage phenotypes in joint disease is limited to *in vitro* studies, experimental animal models, or end stage OA in people (6, 10, 17, 26, 29). Comparisons of macrophage responses between diseased and healthy joints include extrapolations from other types of arthritides, such as rheumatoid arthritis. Additional reports are limited to low numbers of macrophages in the synovial fluid, shedding from the synovium following mechanical detachment or hyperactivation. Reports evaluating synovial fluid macrophages may not represent the response of the synovial membrane tissue itself (30, 31). Defining patterns of macrophage activation in the synovium of healthy and naturally developing osteoarthritic joints will enhance the understanding of the roles of macrophages *in vivo,* which is paramount for optimizing therapeutic strategies targeting macrophage-driven joint homeostasis (28).

The objective of this study was to compare the expression of macrophage markers in the synovium of healthy equine carpal and metacarpophalangeal (MCP) joints and those with naturally occurring OA. The well-defined equine model for the study of OA (1) used in this study will enable translation of information to the treatment of both equine and human patients suffering from OA. We hypothesized that synovial macrophages in osteoarthritic joints would exhibit increased ratios of M1:M2(-like) marker expression compared to healthy joints and that differences in gross pathology, histology, and concentrations of pro- and anti-inflammatory cytokines in synovial fluid would be associated with differences in M1:M2(-like) macrophage ratios in synovium. Antibodies targeting markers of M1 (CD86), M2 (CD 206 and IL-10), and all mature macrophages (CD14) were used to identify macrophage phenotypes in the synovium and a multiplex bead-based assay was used to determine concentrations of synovial fluid cytokines, chemokines, and growth factors.

MATERIALS AND METHODS

Experimental Design

Synovial fluid and synovial membrane biopsies were collected from 26 osteoarthritic joints (16 MCP joints and 10 radiocarpal/middle carpal joints) of horses undergoing arthroscopy or following euthanasia at the Hagyard Equine Medical Institute (Lexington, KY) or the Virginia-Maryland College of Veterinary Medicine (Blacksburg, VA). Written informed consent was received from owners prior to inclusion of horses in the study. Control samples from healthy joints (15 MCP and 14 carpal joints) were collected at the same hospitals from horses without history and evidence of lameness referable to the harvested joints and with grossly healthy articular surfaces at euthanasia. All procedures were performed under IACUC approval. Both healthy and OA samples were harvested from 13 horses, OA samples only from 8, and healthy samples only from 7 horses. The mean age of control horses used to harvest healthy samples (7.4 years) was similar to those with OA (6 years), and comparable to horses used for both purposes (8 years). Synovial inflammation was assessed by gross pathology, synovial membrane histology, and synovial fluid cytology and immunoassay quantification of concentrations of pro- and anti-inflammatory cytokines and growth factors. Synovial macrophage phenotype activation *in situ* was defined by immunohistochemistry.

Abbreviations: MCP, metacarpophalangeal joints; M1, classically activated/pro-inflammatory; M2, suppressive/healing; MCP-1, macrophage chemoattractant protein 1; OA, osteoarthritis/osteoarthritic; PGE_2, prostaglandin E_2; SDF-1, stromal cell–derived factor 1; TNCC, total nucleated cell count; TP, total protein; CD14, LPS co-receptor along with Toll-like receptor 4— proposed mature macrophage marker; CD86, /T cell costimulatory receptor (B7.2)—proposed M1 marker; CD206, Mannose receptor 1 (MRC1)—proposed M2 marker.

Inclusion Criteria

A total of 29 horses (11 females and 18 castrated males), 3–15 years old (skeletally mature, but not aged) were recruited and lameness exams performed, including response to joint manipulation, joint flexion, gait analysis at the trot, and radiography. OA joints were from horses exhibiting Grade 1-3 (out of 5) lameness (32), localized to the selected joint. Diagnostic analgesia was performed at the discretion of the referring veterinarian, and therefore not in all horses. Inclusion was based on arthroscopic or post mortem findings of cartilage abnormalities according to the OARSI scale (0-3, for metacarpophalangeal joints; 0-4 for carpal joints) (33). Only moderate OA joints (OARSI grade 2) were included, as representative of those most commonly treated clinically and when synovium cellularity is highest (34). As per the OARSI guidelines, carpal joints were selected according to degree of macroscopic cartilage erosion (grade 2= partial thickness), and MCP joints were included if presenting a score of 2 for one of the three macroscopic diagnostic parameters: wear lines (3–5 partial-thickness or 1–2 full-thickness wear lines), erosion (partial-thickness erosion, >5 mm in diameter), or palmar arthroses (partial-thickness erosion, purple discoloration, >5 mm in diameter). Horses with a history of septic arthritis, non-steroidal anti-inflammatory therapy, or intra-articular diagnostic anesthesia within 2 weeks, intra-articular corticosteroids within 2 months prior to sample collection, or evidence of osteochondrosis were excluded from the study. Only healthy horses with a body condition score between 4 and 6 (out of 9) were included.

Sample Collection

Synovial fluid (2 mL) was aseptically collected and aliquoted (EDTA and Protein LoBind microfuge tubes, Eppendorf®, Westbury, CT). Anticoagulant-free synovial fluid was immediately centrifuged at 12,000 × g for 10 min at 4°C and the supernatant stored at −20°C for cytokine and growth factor quantification. Two synovial membrane biopsies were obtained from each OA joint adjacent to the major cartilage alterations (35), using 6 mm dermal biopsy punches. Two control samples were harvested at sites where each joint is traditionally most commonly affected (33). Samples were fixed (AZF Fixative®; Newcomer Supply, WI) at room temperature for 24 h, rinsed, and stored in PBS at 4°C until processing.

Synovial Fluid Analysis

Synovial fluid cytology was processed for total nucleated cell count (TNCC) by hemocytometer and total protein (TP) by refractometer. Differential cell counts were performed following Romanowski stain (Microscopy Hemacolor®, Merck, Germany). Concentrations of pro- (IL-1β, IL-6, GM-CSF, TNF-α) and anti-inflammatory cytokines (IL-10, IL-1ra), chemokines (MCP-1, SDF-1), growth factors (IGF-1, FGF-2), and PGE_2 in synovial fluid were quantified. Thawed samples (200 μL) were hyaluronidase-digested (10 μL of 100 IU hyaluronidase/mL acetate buffer; Worthington Biochemical Corporation, Lakewood, NJ) for 30 min at 37°C, centrifuged at 12,000 × g for 10 min at 4°C, and the supernatant recovered. Based on previous experience and interfering factors in cytokine detection in synovial fluid (36, 37), spike-and-recovery assays were performed for the PGE_2 ELISA and 4 representative serially-diluted targets in the multiplex assay (IL-1β, IL-6, IL-10, TNF-α). Based on the results, a dilution of 1:2 was selected for PGE_2 quantification and no dilution was deemed necessary for the multiplex assay.

PGE_2 was quantified by ELISA (R&D Systems, Minneapolis, MN). Hyaluronidase-digested samples were solid-phase extracted (500 μL synovial fluid in 490 μL 100% ethanol and 10 μL glacial acetic acid incubated at 23°C for 5 min), centrifuged at 2,500 × g for 8 min at room temperature, and the supernatant collected. Remaining analytes were quantified by bead-based multiplex assay (MILLIPLEX MAP Equine Cytokine/Chemokine Multiplex Assay with manufacturer modification to include IGF-1, SDF-1, and IL-1ra; Luminex 200 plate reader Millipore Sigma, Burlington, MA).

Synovial Membrane Histology and Immunohistochemistry

Fixed synovial membrane biopsies were paraffin-embedded, sectioned at 5 μm, and H&E-stained. Synovitis was scored based on the OARSI histopathology guide and included cell infiltration, vascularity, hyperplasia, edema, and fibrosis (33). Immunostaining for macrophage markers was assessed using a previously described semi-quantitative approach considering cell staining intensity, cell compartment distribution of the staining, distribution of the staining pattern over the synovial villi, and tissue compartment distribution of the staining (28, 38). Cell staining intensity was scored as: absent (0); mild (1); moderate (2); or intense (3). Staining distribution across synovial villi was scored as restricted to the base of the synovial villus (1); reaching portions of the synovial villus tip (2); or throughout the entire synovial villus (3). Cell compartment distribution was scored as cytoplasm (1), nucleus (2), or both (3). Tissue compartment distribution was scored as restricted to the cell (1), more evident in the matrix (2), or evident on both cell and matrix. Staining patterns were scored on 3 different tissue sections and averaged. Composite scores for each marker were compared between normal and OA samples for both histology and immunohistochemistry. For immunohistochemistry, all tissue sections were baked at 66°C overnight, deparaffinized, and incubated in antigen recovery solution (Antigen Retrieval Citra Plus, BioGenex, Fremont, CA) at 95°C for 10 min. Slides were stained (Super Sensitive™ Polymer-HRP IHC Detection System, BioGenex) using antibodies targeting the following markers: pan macrophage (equine CD14, Wagner Lab, Cornell University); M1 (mouse anti-human CD86 [clone 2331(FUN-1), BD Biosciences, San Jose, CA]); M2 (mouse anti-human CD 206 [clone ab64693, Abcam, Cambridge, UK]); and IL-10 (mouse anti-equine IL-10, Wagner Lab). Markers were selected based on extensive literature review [CD14 (39–43), CD 86 (44–47), CD206 (48–53), and IL-10 (54–57)] and specificity or validated cross-reactivity to equine samples (58–61). Positive controls using tissues know to express each marker, and negative controls using tissues known to be negative for each marker and those stained without primary

antibody were included. All samples were blindly scored (BCM), and scores corroborated by a experienced investigators and a board certified pathologist.

Statistical Analysis

Data analysis was performed using SAS version 9.4 (SAS Institute, Inc, Cary, NC). Effects of different joints sampled (carpi vs. MCP) and effects of disease (healthy vs. OA) on outcomes were assessed using linear General Estimating Equations (GEE) in an incomplete block design. Each of the linear models specified joint, disease, and the interaction between joint and disease as fixed effects. Correlation between observations within horse (the blocking factor) were modeled by specifying a compound symmetry covariance matrix. The interaction between joint and disease was further analyzed (sliced) to extract comparisons between disease conditions within joint. Scatterplots and analysis of covariance models were used to determine associations between synovial fluid cytology, synovial membrane histology, and synovial membrane immunohistochemistry parameters with joint condition (healthy vs. OA). For the analysis of covariance models, immunohistochemistry parameters (macrophage markers) were specified as covariates (one parameter at a time) while disease was the design effect. Statistical significance was set to $p < 0.05$.

RESULTS

Synovial Fluid Cytology

To determine whether synovial fluid macrophage counts differed between healthy and OA joints, standard synovial fluid cytology analysis was performed (**Table 1**). Overall, TP was overall significantly higher in OA compared to normal joints ($P = 0.0331$). TNCC was also higher in OA compared to normal joints, but failed to reach significance ($p = 0.0532$). No overall differences were detected between normal and OA joints for differential cell counts (relative counts of synovial fluid macrophages, lymphocytes and neutrophils), and macrophages were the predominant cell type in all groups.

Cytokine/Chemokine and Growth Factor Quantification

To assess the secretory response of synovial lining macrophages, concentrations of pro- (IL-1β, IL-6, GM-CSF, TNF-α, PGE_2) and anti-inflammatory cytokines (IL-10, IL-1ra), chemokines (MCP-1, SDF-1) and growth factors (IGF-1, FGF-2) were quantified in synovial fluid (**Table 2**). GM-CSF was below detectable limits (3.7 pg/mL) for all samples. Detection of MCP-1, SDF-1α + β, IL-10, and PGE_2, was possible in the majority of samples. The remaining analytes (IL-1β, IL-6, TNF-α, IL-1ra, IGF-1, and FGF-2) were detected in only a minority of samples, precluding statistical analysis. Concentrations of PGE_2 did not vary overall or when comparing OA to healthy MCP or carpal joints. Concentrations of synovial fluid IL-10 were overall lower in OA than healthy joints; however, only significantly lower in OA compared to healthy MCP joints ($p = 0.0462$). The concentrations of key chemokines for recruitment of circulating monocytes (MCP-1) and homing of myeloid progenitors (SDF-1) were altered in the osteoarthritic synovial fluid. Overall MCP-1 concentrations were significantly higher in OA than healthy joints ($p = 0.0443$). In contrast, SDF-1 concentrations were significantly lower in the overall comparison of OA to healthy joints ($p = 0.0243$) and within healthy and OA MCP joints ($p = 0.0378$).

Synovial Membrane Histology

Overall scores for histological assessment of the synovium for intimal hyperplasia ($p = 0.0076$) were significantly higher in OA compared to normal joints (**Table 3**, **Figure 1**). Overall scores for subintimal edema ($p = 0.0514$), cell infiltration ($p = 0.0818$), vascularity ($p = 0.1398$), and fibrosis ($p = 0.3053$) were higher for OA joints, but were not significant (**Table 3**). The composite of these individual scores was significantly higher overall in OA compared to normal joints ($p = 0.0122$). Within MCP joints, only subintimal edema was significantly higher in OA joints

TABLE 1 | Synovial fluid cytology from healthy and OA equine metacarpophalangeal and carpal joints (median, 95% Confidence Interval).

Synovial fluid cytology						
		Total protein g/dL	**TNCC cells/μL**	**Macrophages%**	**Lymphocytes%**	**Neutrophils%**
Metacarpophalangeal joints	**Control**	2.1 (1.5–2.4)	91 (24–256)	65 (55–73)	28 (24–43)	0 (0–3)
	OA	2.7 (1.1–3.9)	68 (21–607)	68 (49–79)	27 (4–44)	0 (0–3)
	P–value	*P = 0.1402*	*P = 0.6253*	*P = 0.6278*	*P = 0.5702*	*P = 0.5805*
Carpal joints	**Control**	2.4 (1.6–2.8)	24 (19–221)	58 (50–67)	33 (28–48)	2 (0–3)
	OA	3.1 (1.7–3.8)	124 (14–204)	61 (46–77)	31 (16–39)	2 (0–19)
	P–value	*P = 0.0595*	*P = 0.3370*	*P = 0.8715*	*P = 0.2167*	*P = 0.2251*
Overall	**Control**	2.1 (1.9–2.4)	91 (24–156)	64 (55–71)	30 (25–43)	1 (0–3)
	OA	2.7 (1.8–3.6)	110 (36–173)	65 (54–73)	29 (18–39)	0 (0–3)
	P–value	***P = 0.0331***	*P = 0.0532*	*P = 0.8780*	*P = 0.2699*	*P = 0.1995*

No significant differences were detected between samples from control and OA joints.
TNCC, Total Nucleated Cell Count. P-values <0.05 highlighted in bold.

TABLE 2 | Cytokine, chemokine, and growth factor concentrations in synovial fluid of healthy and OA equine joints (median, 95% Confidence Interval).

Analytes											
		FGF−2	**IGF-1**	**IL-1β**	**IL-6**	**IL1-ra**	**MCP-1**	**SDF-1**	**IL-10**	**PGE_2**	**TNF-α**
Min. D.C.		11.5 pg/mL	0.3 pg/mL	15.5 pg/mL	2.3 pg/mL	0.02 pg/mL	9 pg/mL	20.5 pg/mL	23.2 pg/mL	39 pg/mL	1.5 pg/mL
Metacarpo-phalangeal joints	**Control** *N* = 15	*N* = 4 44* (23–137)	U	*N* = 4 335* (55–617)	*N* = 5 6* (3–19)	U	*N* = 10 799 (128–1,508)	*N* = 14 241 (129–292)	*N* = 15 86 (55–97)	*N* = 14 69 (53–73)	*N* = 5 3.5* (2–10)
	OA *N* = 16	*N*= 3 20* (13–53)	*N* = 1 506*	*N* = 5 4 (28–4014)	*N* = 5 25 (3–65)	*N* = 1 3*	*N* = 13 773 (128–1,463)	*N* = 13 137 (89–208)	*N* = 11 68 (40–96)	*N* = 13 71 (53–75)	*N* = 3 5* (3–24)
	P–value	–	–	–	–	–	*P = 0.0803*	***P = 0.0378***	***P = 0.0462***	*P = 0.7206*	–
Carpal joints	**Control** *N* = 14	*N* = 2 78* (16–141)	*N* = 2 1917* (196–3639)	*N* = 7 199 (21–5501)	*N* = 6 13 (3–89)	*N* = 4 6* (1–219)	*N* = 14 786 (230–1,867)	*N* = 14 334 (152–467)	*N* = 14 64 (41–98)	*N* = 14 67 (53–73)	*N* = 5 9* (6–35)
	OA *N* = 10	*N* = 3 18* (16–42)	*N* = 4 270* (83–863)	*N* = 6 169 (18–695)	*N* = 5 11 (3–172)	*N* = 1 15*	*N* = 10 933 (260–2,526)	*N* = 10 267 (73–498)	*N* = 10 64 (57–108)	*N* = 10 73 (62–83)	*N* = 5 5 (3–41)
	P–value	–	–	–	–	–	*P = 0.1360*	*P = 0.1943*	*P = 0.7362*	*P = 0.3740*	–
Overall	**Control** *N* = 29	*N* = 6 44 (16–141)	*N* = 2 1,917 (196–3,639)	*N* = 11 283 (42–1,014)	*N* = 11 8 (3–22)	*N* = 4 6* (1–219)	*N* = 24 799 (240–1,508)	*N* = 28 276 (188–320)	*N* = 29 80 (55–92)	*N* = 28 68 (58–72)	*N* = 10 6 (3–11)
	OA *N* = 26	*N* = 6 19 (12–53)	*N* = 5 407 (83–863)	*N* = 11 64 (18–4014)	*N* = 10 18 (4–40)	*N* = 2 9* (3–14)	*N* = 23 880 (442–1,096)	*N* = 23 150 (109–278)	*N* = 21 66 (57–92)	*N* = 23 71 (64–75)	*N* = 8 5 (3–25)
	P–value	–	–	–	–	–	***P = 0.0443***	***P = 0.0243***	*P = 0.2052*	*P = 0.5159*	–

*Min. D.C., Minimum detectable concentration; U, Undetectcted; –, p–values could not be determined due to small number of samples in which the analyte was detected; *, the actual confidence level is < 95%. N in analyte columns, number of samples in which the analyte was detected. P-values <0.05 highlighted in bold.*

TABLE 3 | Individual and composite histological parameters for H&E-stained equine synovial membrane (median, 95% Confidence Interval).

Synovial membrane histology							
		Cell infiltration	**Vascularity**	**Intimal hyperplasia**	**Subintimal edema**	**Fibrosis**	**Composite scores**
Metacarpo-phalangeal joints	**Control**	2 (1–2)	2 (1–3)	1 (0–1)	1 (0–2)	2 (2–3)	7 (4–11)
	OA	2 (1–3)	3 (1–4)	1 (0–3)	1.5 (1–3)	2 (1–3)	9.5 (6–14)
	P–value	*P = 0.3084*	*P = 0.1099*	*P = 0.1747*	***P = 0.0158***	*P = 0.8501*	*P = 0.0711*
Carpal joints	**Control**	2 (1–2)	2 (1–2)	0.5 (0–1)	1 (1–2)	2 (1–3)	8.5 (5–9)
	OA	2 (1–3)	2 (0–3)	1 (1–2)	2 (0–3)	3 (1–3)	9.5 (7–12)
	P–value	*P = 0.1195*	*P = 0.7087*	***P = 0.0103***	*P = 0.5231*	*P = 0.1973*	***P = 0.0420***
Overall	**Control**	2 (1–2)	2 (1–2)	1[a] (0–1)	1 (1–2)	2 (1–3)	8 (6–9)
	OA	2 (1–3)	2.5 (0–4)	1[a] (0–2)	2 (1–3)	2.5 (2–3)	9.5 (7–12)
	P–value	*P = 0.0818*	*P = 0.1398*	***P = 0.0076***	*P = 0.0514*	*P = 0.3053*	***P = 0.0122***

Synovial Hyperplasia was significantly higher in OA carpi, while Subintimal Edema was significantly higher in OA metacarpophalangeal joints.
[a]the categorical nature of the data produces median values that are equal between groups. P-values <0.05 highlighted in bold.

compared to normal ($p = 0.0158$). Within carpal joints, intimal hyperplasia ($p = 0.0103$), and composite scores ($p = 0.0420$) were significantly higher in OA joints. In the subset of OA joints with gross signs of synovial inflammation ($n = 8$), there was a notable pattern of increased synovial vascularity and shedding of cells from the markedly hyperplastic outermost layer of the intima. In

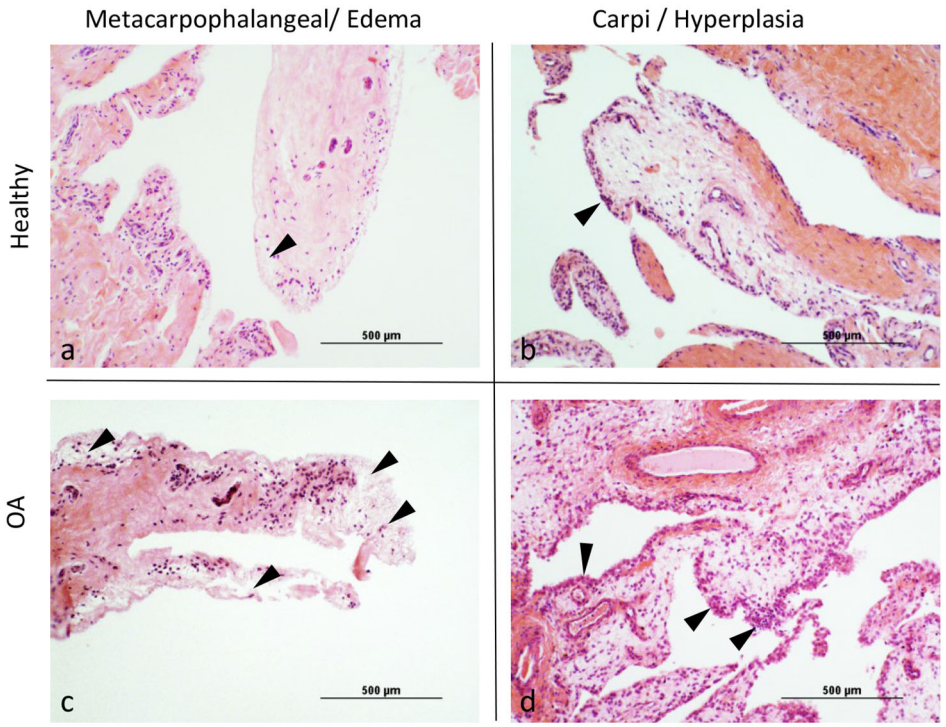

FIGURE 1 | (a–d) Representative images demonstrating (arrowheads) the differences between healthy and osteoarthritic (OA) joints for Intimal Hyperplasia and Subintimal Edema.

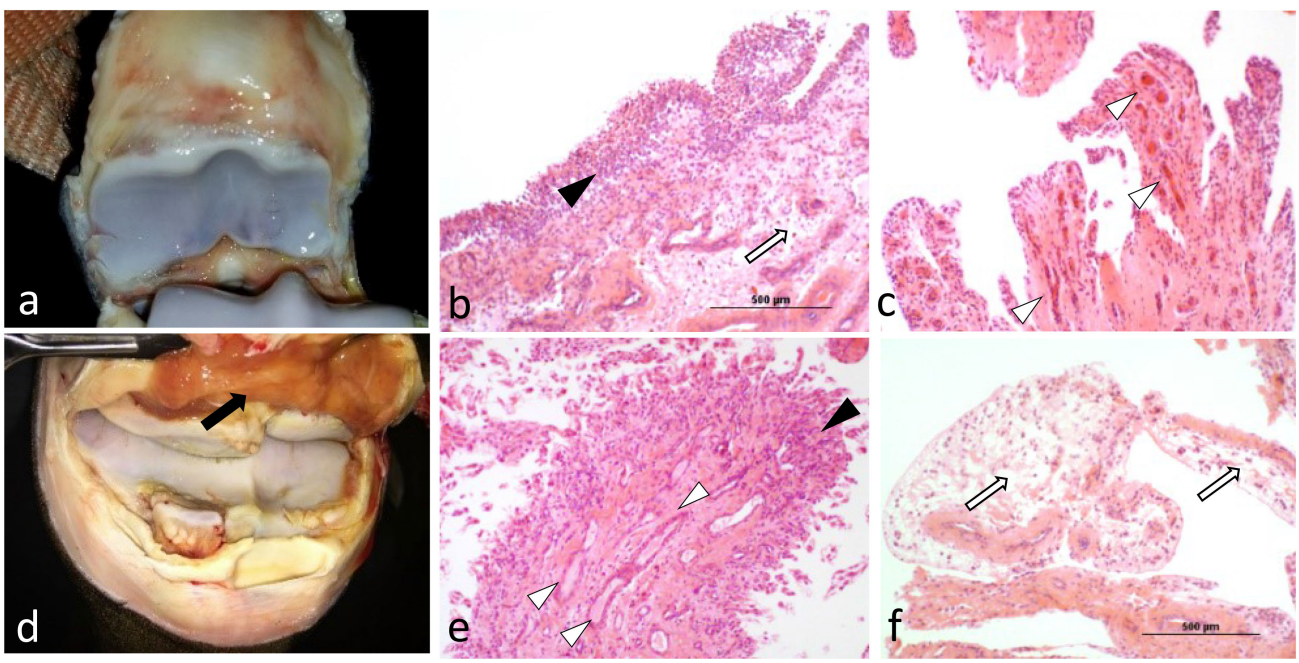

FIGURE 2 | Compared to OA joints with no or minimal signs of gross inflammation **(a)**, OA joints exhibiting gross signs of synovitis (**d**; black arrow), exhibited increased histological changes such as severe cell infiltration and hyperplasia of the synovial intima with shedding of its outermost layer (**b**; black arrowhead), markedly increased vascularization (**c**; white arrowheads), or a combination of both **(e)**. Marked synovial and sub-synovial edema were also frequent findings (**b,f**; white arrows).

this outermost intimal layer, cell nuclei were often decondensed, with decreased hematoxylin uptake typical of hyperactivated cells (**Figure 2**) (62).

Synovial Membrane Immunohistochemistry

The distribution of immunostaining for macrophage markers across the synovial lining differed between healthy and OA joints. In healthy joints, staining was largely limited to the base of synovial villi, while in OA joints the tips of villi were also frequently stained (**Figure 4**). When observed in healthy joints, staining for macrophage markers at the tips of villi was subtle and primarily located at scattered areas of the synovial lining around cell nuclei. In contrast, staining patterns in OA joints were more diffusely distributed in the synovial lining around cell nuclei. Overall, expression of CD14, CD86, and IL-10 were higher in OA compared to normal joints, yet only significantly for CD14 ($p = 0.0157$; **Table 4**). These differences were most apparent in the synovium from MCP joints, and again, only significant for CD14 when comparing healthy and OA MCP joints ($p = 0.0279$). Of note, in both MCP and carpal joints, expression of the M2 marker CD206 was higher in OA joints, although not significantly. Staining for all markers was most intense around blood vessels, especially over cells in the endothelium (**Figure 3**). Overall, CD14, CD86, and CD206 staining was limited primarily to the area immediately adjacent to cells and cell aggregates within the synovial intima and subintima, whereas staining for IL-10 was diffuse throughout the synovial tissue in both healthy and OA joints.

In the subset of OA joints with gross signs of synovitis, staining for CD86 was more markedly intense than remaining OA joints. A similar, but less consistent pattern was observed for CD14, IL-10, and CD206. For 4 horses, we were able to compare OA joints with gross signs of synovitis to the healthy contralateral joints of the same individual (**Figure 4**). Again, while increased expression of all markers in OA joints of these horses varied in intensity, CD86 expression was the most intense and consistently increased. Three of these four samples represented the highest CD86 staining scores among all samples of our study.

DISCUSSION

This is the first study in any species comparing macrophage phenotypes in the synovium from healthy joints to those with naturally occurring OA. Markers widely used to define M1- (CD86) and M2-like (CD206 and IL-10) macrophages were similarly expressed in both groups. Expression for all markers varied with degree of synovial inflammation. While their expression was mildly increased in OA joints with low-grade inflammation (majority), it was markedly increased in grossly inflamed OA joints, with CD86 most highly expressed. *In situ*, similar expression of M1- and M2-like macrophage markers (10, 26, 63) and their increased expression proportionate to inflammatory activation (6, 28, 64) are reported. Current knowledge suggests that, *in vivo,* macrophages are by default homeostatic cells that, following injury, drive inflammation with the purpose of counteracting tissue aggressors and further guide inflammation resolution (20, 65–70). Although no parameters revealed statistical associations with joint condition (healthy or OA), immunohistochemical and histologic findings were consistent with higher synovial fluid concentrations of MCP-1 and lower concentrations of SDF-1 in OA joints and lower IL-10 in OA metacarpophalangeal joints.

Under sustained inflammatory conditions, macrophages have lower expression of pro-resolving molecules such as IL-10 (64). This apparently impaired production by individual cells can be compensated for by increasing the overall numbers of macrophages to achieve inflammation resolution (28, 64). In the chronic inflammation of OA, higher

TABLE 4 | Composite immunohistochemical scores of macrophage markers in healthy and OA synovial membrane (median, 95% Confidence Interval).

Synovial membrane immunostaining					
		CD14	**CD86**	**CD206**	**IL-10**
Metacarpo-phalangeal joints	**Control**	4 (0–5)	4 (0–6)	4 (4–6)	5 (4–6)
	OA	5 (0–7)	5 (0–7)	5 (0–6)	6 (4–6)
	P–value	***P* = 0.0279**	*P* = 0.8593	*P* = 0.2987	*P* = 0.1551
Carpal joints	**Control**	5 (4–6)	6 (4–6)	4 (0–6)	6 (5–6)
	OA	5 (0–6)	6 (5–8)	5.5 (4–7)	6 (5–7)
	P–value	*P* = 0.1135	*P* = 0.2099	*P* = 0.1161	*P* = 0.8826
Overall	**Control**	5 (0–5)	5 (4–6)	5 (0–6)	5 (5–6)
	OA	6 (4–6)	6 (5–7)	5 (4–6)	6 (5–6)
	P–value	***P* = 0.0157**	*P* = 0.3677	*P* = 0.5943	*P* = 0.3651

Osteoarthritic metacarpophalangeal joints exhibited increased expression of all markers, while only CD206 expression was higher in OA carpi. P-values <0.05 highlighted in bold.

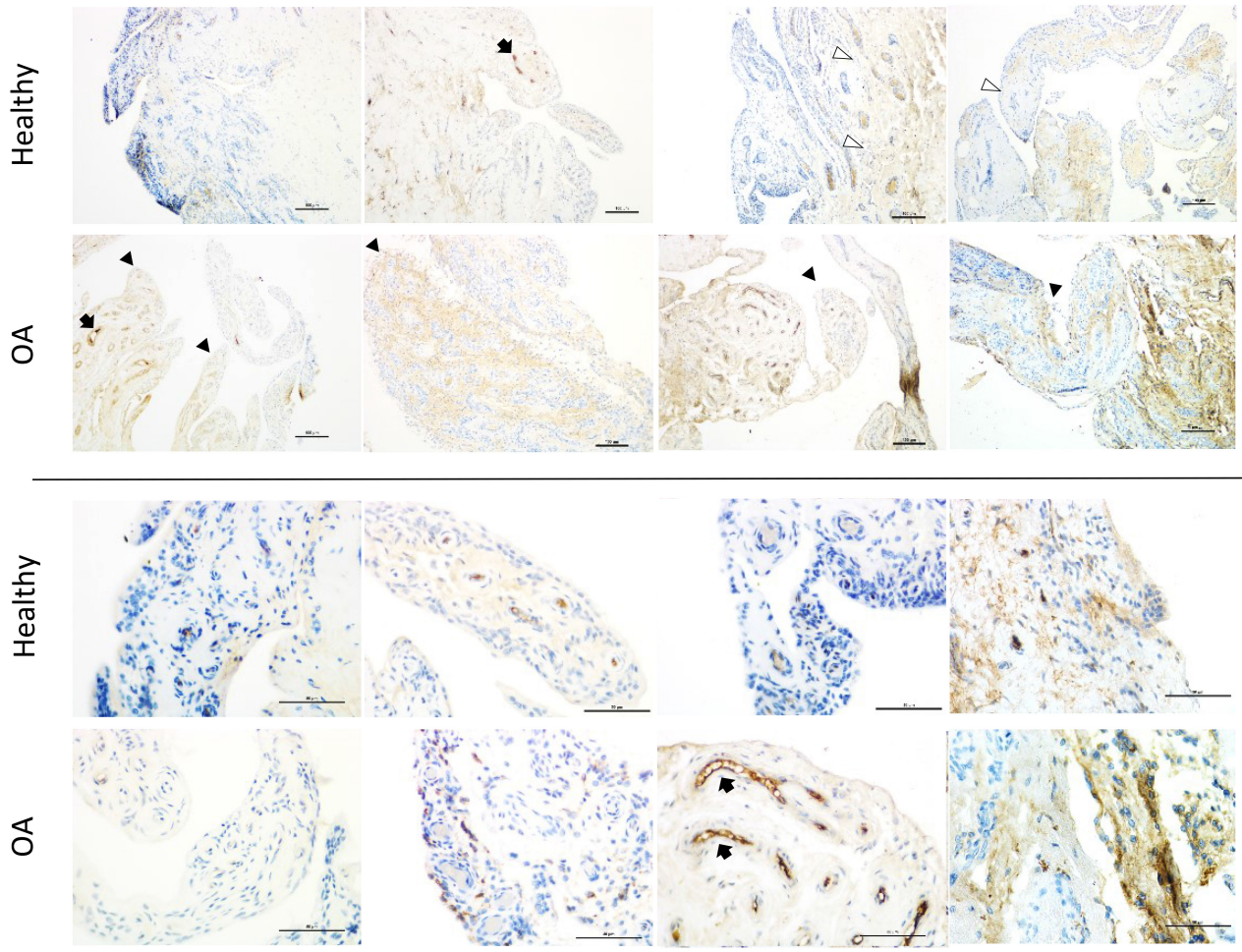

FIGURE 3 | Representative immunohistochemistry sections from healthy and OA equine synovial membrane at low (top 2 rows; scale bar = 100 μm) and high magnification (bottom 2 rows; scale bar = 50 μm) from the same histological section and demonstrating the median staining scores for macrophage markers (CD14, CD86 [M1], CD206 and, IL-10 [M2]). Staining for all markers was most intense on the vascular endothelium (black arrows). In healthy joints, staining was largely limited to the base of synovial villi (white arrowheads), while in OA joints the tips of villi were also frequently stained (black arrowheads). In healthy joints, staining for macrophage markers at the tips of villi was subtle and primarily located at the synovial lining around cell nuclei. In contrast, staining in OA joints was more diffusely distributed in the synovial lining around cell nuclei.

CD14/CD186/CD206/IL10 expression (macrophage activation) and synovial intimal hyperplasia (macrophage recruitment), as observed in our study, is consistent with macrophages forming an isolating barrier at the site of the inflammatory response as shown by the black arrowheads in **Figure 2b** and as previously reported (20, 70). However, combined with decreased synovial fluid concentrations of IL-10, these observations may suggest that regulatory functions of these macrophages could be overwhelmed preventing recovery of joint homeostasis (22, 23, 28, 65–68, 71). Therefore, to efficiently achieve synovial inflammation resolution, recruitment of macrophages to the synovial environment likely needs to be higher than that resulting in intimal hyperplasia. Lower SDF-1 concentrations could be affecting efficient macrophage recruitment.

The concept of macrophage activation as either inflammatory (M1) or regulatory (M2) originated from monocyte-derived macrophages treated *in vitro* with defined and overwhelming cytokine stimuli (21, 52, 72). Clear identification of macrophage phenotypes *in vivo* is significantly more complex than proposed by *in vitro* models (10, 16, 22, 29). Increased CD14 expression during OA, combined with increased macrophage recruitment and activation corroborated by histology, as observed in our study, is consistent with previous reports of increased soluble CD14 in the synovial fluid of OA joints correlated with disease activity and clinical signs (73). Although CD86 and CD206 expression have historically been considered markers of M1- and M2-like macrophages (6, 26, 52, 72), this is an oversimplification of events that occur *in vivo* (16, 22, 74).

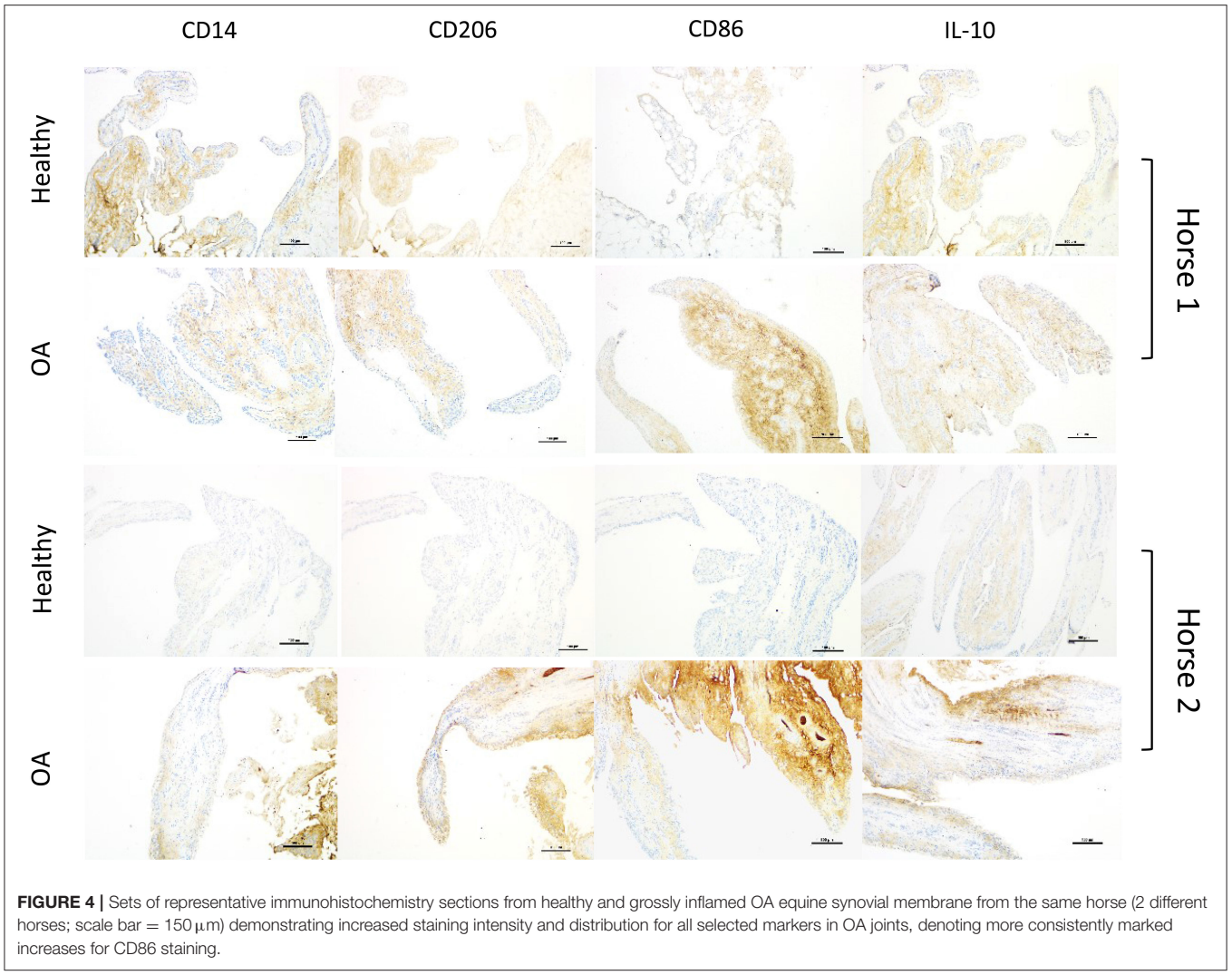

FIGURE 4 | Sets of representative immunohistochemistry sections from healthy and grossly inflamed OA equine synovial membrane from the same horse (2 different horses; scale bar = 150 μm) demonstrating increased staining intensity and distribution for all selected markers in OA joints, denoting more consistently marked increases for CD86 staining.

CD86 is constitutively expressed by early myeloid cells and resting macrophages, including those in the synovial membrane and fluid of normal joints (28). Increased CD86 expression is part of the cellular checkpoints required for monocytic lineage commitment, activation, and inflammation resolution (22, 66). Therefore, as observed in the control joints in this study, and additional reports (22, 28, 64), isolated association of CD86 with an inflammatory phenotype or detrimental effects *in vivo* is likely misleading. Given the key role of CD86 in recruitment, activation, and survival of myeloid monocytes during both inflammation and its resolution, intense CD86 expression during acute (gross) inflammation suggests increased macrophage recruitment.

Similar to CD86, the mannose receptor (CD206) has a pivotal function in host defenses during inflammation, clearance of debris, wound healing and remodeling, and resolution of inflammation. CD206 is also constitutively expressed in mature mononuclear phagocytes and the intensity of its expression is proportionate to demands for anabolic cytokine secretion, efferocytosis, and sensing of damage-associated molecular patterns (68). Thus, the expression of both CD86 and CD206 increase with inflammatory stimuli, as a result of increased macrophage recruitment and response to injury (10), and therefore should be carefully analyzed over time in conjunction with clinical and analytical indicators of health and disease. Although expression of CD86 and CD206 was reported to associate to M1- and M2-like macrophages in the synovial fluid from healthy and OA joints (30), this observation is in disagreement with the profiles of macrophages in the synovium in this and other experimental studies (10, 26, 28, 64).

Like CD86 and CD206, expression of IL-10 in the synovial membrane in our study was directly associated with the degree of synovial inflammation. After injury, macrophage activation leads to increased expression of IL-1, IL-6, and TNF-α, which is followed by proportional increases in expression of IL-10 as a compensatory, negative feedback (75–77). Consequently, the production of these pro-inflammatory cytokines decreases (75). However, if the injurious challenge persists, this cytokine feedback loop is sustained, and may explain the increased synovium expression of IL-10 in our OA joints compared to

healthy joints, especially those grossly inflamed (12, 28, 64, 76). Therefore, marked staining in grossly inflamed joints could suggest that the dynamics of cell recruitment and activation during inflammation (increased CD14, CD206, and CD86), and compensatory negative feedback (IL-10) are being persistently triggered in the vicious cycle of inflammation seen in OA (10, 12, 16, 66, 67, 75, 76, 78, 79). Considering the functions of these markers, their combined higher expression during OA suggests higher macrophage activation and not necessarily a phenotype as traditionally described *in vitro*.

The lower synovial fluid concentrations of IL-10 in OA metacarpophalangeal joints suggests that mechanisms compensating for tissue damage may be impaired or overwhelmed in OA joints. An *in vitro* study challenging monocytes from osteoarthritic and healthy human joints reported that patients with no significant IL-10 increase following challenge were three times more likely to develop OA compared to those responding with a significant increase (77). As a matter of fact, injection of arthritic joints with autologous bone marrow-derived macrophages results in marked clinical improvement, decreased markers of inflammation, and increased synovial fluid concentrations of IL-10 and IL-10^{+} macrophages (28, 80, 81). Inflamed equine joints treated with IL-10-expressing macrophages were comparable to healthy joints histologically, whereas saline-treated controls remained severely inflamed (28). Combined, these studies reinforce the important role of IL-10-producing macrophages in driving resolution of inflammation and promoting joint homeostasis (28, 80, 81).

In response to injury, resident synovial macrophages form a protective immunological barrier in the synovial lining, similar to the hyperplastic synovium, secluding intra-articular structures. Exchange of solutes and cells from the sub-synovial to intra-articular space is restricted and could explain higher IL-10 staining in the synovium from OA joints with lower synovial fluid IL-10 concentrations than healthy joints (20). During overwhelming inflammation, this tight-junction barrier is lost, allowing free exchange of cellular and molecular components between intra-articular and sub-synovial spaces (70). Importantly, each of these mechanisms can be affected by the stage of the inflammatory response (acute-chronic/mild-severe), which was not accounted for in our study design.

Increased overall synovial fluid MCP-1 concentrations in OA, concomitant with clinical signs of joint inflammation, is consistent with the literature (82–84). During synovial inflammation, MCP-1 contributes to recruitment and accumulation of circulating monocytes in the synovial membrane (76, 85). Although it has been suggested that MCP-1 has an important role in vicious cycles of inflammation (20, 86), this response is considered to be a homeostatic response to joint damage. As such, MCP-1-deficient mice are unable to home macrophages to sites of injury and are prone to impaired healing, infection, and chronic inflammation (76, 87, 88). Therefore, in the face of decreased SDF-1 and IL-10, increased concentrations of MCP-1 may be a compensating mechanism for recruitment of myeloid-derived macrophages to the injured joint (89, 90). Lower SDF-1 concentrations in synovial fluid from OA vs. healthy joints in our study is inconsistent with previous studies. SDF-1 has multifaceted roles in synovial tissue biology, including homeostatic and pro-inflammatory functions (91, 92). SDF-1 is reportedly expressed proportionate to disease activity, with higher concentrations in inflamed joints (91–93). Our results showing lower synovial fluid SDF-1 concentrations in OA joints is comparable to two other studies from our lab, where inflammation decreased synovial fluid SDF-1 (28, 64). SDF-1 is known to substantially improve tissue repair and plays a major role in recruiting and homing of myeloid cells involved in tissue repair and inflammation resolution (94), such as IL-10-producing myeloid-derived suppressor cells, critical for resolution of joint inflammation (81). The unbalanced production of all three substances can be related, and disturbances in their concentrations can reflect impaired macrophage recruitment or function. Further studies exploring the relationship of these findings are warranted.

Traditionally, IL-1β and TNF-α have been considered the main drivers of disease processes in OA (95–98). However, these two classic inflammatory cytokines were detected in less than half of our samples with no significant differences between healthy and OA samples, similar to previous reports (99, 100). Limitations in the detection of IL-1 and other cytokines in synovial fluid are widely reported, even in samples from patients experiencing marked inflammation (36, 37, 101, 102). Recent proteomic analysis of synovial fluid and genome-wide transcriptomic analysis of cartilage comparing samples from OA and healthy joints did not identify IL-1 or TNF-α as central targets (103, 104). PGE_2 has also been used as an important marker of joint inflammation (11, 105, 106). However, PGE_2 also plays anti-inflammatory and anabolic roles, such as inhibition of inflammatory cytokines and neutrophil infiltration to the site of injury, chondrocyte protection, and activation of pro-resolving macrophages (23, 52, 107, 108). PGE_2 generated during the early inflammatory response can induce inflammation resolution by upregulating the synthesis of potent mediators of resolution (23). Therefore, PGE_2 is involved in both inciting and resolving inflammation, and concentrations in synovial fluid vary with the stage of response to injury, and may explain the lack of differences between healthy and OA joints in our study.

Although differences between normal and OA joints were observed for both carpal and metacarpophalangeal joints, differences were more often identified in metacarpophalangeal joints. One potential reason for this observation is that, due to a more distal location and higher range of motion, metacarpophalangeal joints are more exposed to higher mechanical loads and stress, and thus the response to trauma and tissue microdamage may be more marked. In fact, metacarpophalangeal joints are the most commonly affected site of injury in many equine disciplines (109–111).

Even though our experimental design was aimed at minimizing variability, synovial histological parameters can vary with joint and site within the joint, and could have contributed to a degree of variability among samples, preventing statistical inference. While assessing the expression of macrophage markers in synovial fluid cells using flow cytometry would have contributed to our findings in the synovial membrane, recent reports are in agreement with the

pattern of expression identified in our study (28, 112). Our study was not designed to infer causality of our findings in the development and progression of OA, and therefore the meaning of our observations is interpreted based on the literature and additional studies from our lab. Quantifying soluble CD14 in the synovial fluid could have reinforced the role of macrophage activation in joint inflammation and disease progression, yet such observations have already been reported, and similar to our study, were associated with increased MCP-1 concentrations in osteoarthritic synovial fluid (84). Immunoblots comparing the activity of the TLR-4 – NFκB-IL-10 axis between the synovium of healthy and OA joints, as well as quantification of other pro-resolving mediators in the samples of this study, would have provided additional information for understanding the mechanism by which drivers of joint homeostasis become overwhelmed. Futures studies comparing synovium single cell transcriptome analysis and synovial fluid lipid profiling between normal and OA joints will further define the role of synovial macrophages in joint disease.

CONCLUSION

Combined with previously reported studies, our results suggest that synovial macrophages are strictly neither M1 nor M2, but represent a hybrid state of activation that overall displays a regulatory response and that ultimately targets resolution of the inflammatory process (22, 28, 64, 70, 113). The majority of parameters investigated in our study, pragmatically called pro- or anti-inflammatory, are building blocks of a complex immune response and must be carefully interpreted, with attention to the phases of inflammation, including its resolution. Secretion of pro- and anti-inflammatory/pro-resolving mediators increase proportionally, and almost simultaneously after macrophage activation in response to injury, decreasing to baseline after resolution (23, 67, 78). In OA joints, increased synovial fluid MCP-1 associated with synovial intimal hyperplasia suggests recruitment of macrophages to the synovium in response to injury. Nonetheless, decreased concentrations of pro-resolving mediators such as IL-10 and SDF-1 implies that pro-resolving mechanisms compensating for tissue damage leading to resolution may be impaired or overwhelmed. Furthermore, inflammation resolution is an active process, largely orchestrated by macrophages, and requires lipid mediators produced during the acute inflammatory response. Thus, the idea of inhibiting inflammation as a therapy may need to be revisited (23). 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, rather than simply blocking inflammation through the use of non-steroidal anti-inflammatory drugs and corticosteroids. Developing approaches to maximize the homeostatic response by healthy macrophages in OA joints has the potential to resolve joint inflammation and re-establish an anabolic synovial environment and overall joint health.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Virginia Tech Institutional Animal Care and Use Committee. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

BM, DR, SW, and LD contributed substantially to study conception and design. BM and DR collected samples. BM was primarily responsible for data acquisition, analysis, and interpretation. KG, AO, and YN assisted BM with data collection and assembly. SB supervised the synovial fluid cytology performed by BM and KG. SW performed statistical analysis and consulted on its interpretation. BM and LD were responsible for manuscript preparation. All authors reviewed the final manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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A Comparative Review of Autologous Conditioned Serum and Autologous Protein Solution for Treatment of Osteoarthritis in Horses

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Many alternative treatments aimed at modulating osteoarthritis (OA) progression have been developed in the past decades, including the use of cytokine inhibitors. IL-1β is considered one of the most impactful cytokines in OA disease and therefore, its blockage offers a promising approach for the modulation of OA. Interleukin-1 receptor antagonist (IL-1Ra) is a naturally occurring anti-inflammatory protein belonging to the IL-1 family that competes with IL-1β for occupancy of its receptors, without triggering the same downstream inflammatory response. Because of its natural anti-inflammatory properties, different methods have been proposed to use IL-1Ra therapeutically in OA. Autologous conditioned serum (ACS) and autologous protein solution (APS) are blood-derived products produced with the use of specialized commercial kits. These processes result in hemoderivatives with high concentrations of IL-1Ra and other cytokines and growth factors with potential modulatory effects on OA progression. Several studies have demonstrated potential anti-inflammatory effect of these therapies with promising clinical results. However, as with any hemoderivatives, clinical outcomes may vary. For optimal therapeutic use, further research is warranted for a more comprehensive understanding of the product's composition and interaction of its components in joint inflammation. Additionally, differences between ACS and APS treatments may not be clear for many clients and clinicians. Thus, the objective of this narrative review is to guide the reader in important aspects of ACS and APS therapies, *in vitro* and *in vivo* applications and to compare the use of both treatments in OA.

Keywords: osteoarthritis, horse, autologous conditioned serum, IL-1Ra, autologous protein solution, growth factors, anti-inflammatory cytokines

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INTRODUCTION

Osteoarthritis is a common cause of lameness observed in horses (1), and has been described as a disease with a common end stage of progressive degeneration of the articular cartilage (2). Subchondral bone and soft tissues are also affected (2). This disease may occur early in the career of equine athletes or later in older horses (3), being responsible for up to 60% of lameness cases (2, 4, 5).

Biological therapies such as platelet-rich plasma (PRP), autologous protein solution (APS), and autologous conditioned serum (ACS) provide a more physiological alternative to conventional treatments capable of modulating inflammation in OA (6–8). These products are derived from the patient's own blood and are rich in anti-inflammatory cytokines and growth factors that reduce inflammation and promote anabolism in tissues (7). These therapies have demonstrated significant clinical and histological improvement in horses with OA (8, 9). Although the proof-of-principle for hemoderivatives is widely known, there are still several limitations for its use. Patient's own biological variability, lack of standardization of the protocols for preparation and application are a few of these limitations. Those variables result in inconsistent concentration of bioactive factors leading to conflicting results (9–11). In addition, although APS and ACS are distinct hemoderivatives, their similarities in cytokine composition may be confusing. The objective of this review is to describe important aspects of ACS and APS therapies, to characterize these products and to compare their modulatory effect in osteoarthritis.

INFLAMMATION AND ROLE OF IL-1RA IN OSTEOARTHRITIS

The inflammatory process plays a paramount role in the pathogenesis of OA, involving the subchondral bone, cartilage and synovial tissues. These tissues release pro-inflammatory cytokines, as IL-1ß and TNF-α, which initiate and propagate inflammation (12). These cytokines promote synovial inflammation and lead chondrocytes to release metalloproteinases (MMP 1, 3, and 13), aggrecanases 4 and 5 (ADAMTS-4 and 5), reactive oxygen specie (ROS) and cytokines such as cyclooxygenase 2 (COX-2) (12). These, in addition to prostaglandin 2 (PGE_2), IL-6 and IL-8 propagate OA (13–16), resulting in cartilage matrix degradation (17–19). Both IL-1β and TNF-α are increased in synovial fluid, synovial membrane, cartilage and subchondral bone during OA (12).

IL-1β binds to two different receptors: type I IL-1 receptor, which leads to downstream regular IL-1 activity; and the type II receptor which is a decoy receptor. Blockage of type I IL-1 receptor results in downregulation of MMPs, ADAMTS, and pro-inflammatory cytokines that propagate OA (20). IL-1Ra is a natural protein that antagonizes the effects of IL-1 by binding to both type I and II receptors without triggering the expected downstream effects of IL-1 (21, 22). IL-Ra is produced by many types of cells including chondrocytes, monocytes and synovial fibroblasts during inflammation (23, 24). In osteoarthritis, as well as other degenerative diseases, the concentration of IL-1Ra available in affected tissues is too low to inhibit the negative effects of IL-1. Different *in vivo* studies suggest that the concentration of IL-1Ra should be 10–1,000-fold higher than the concentration of IL-1 to effectively block IL-1 receptors with significant therapeutic effect (25). Since the discovery of IL-1Ra in 1986 (20), multiple methods of enhancing the concentration of this protein in hemoderivatives have been identified (26–28). The enhancement of endogenous IL-1Ra could be an efficient therapeutic approach in diseases where IL-1 plays an important role in the pathophysiology (22). Recent biochemical modulatory therapies aimed at restoring the anabolic-catabolic balance within the joint, (thereby modulating progression of OA), have been developed to increase IL-1Ra and other anti-inflammatory cytokines and improve clinical outcomes (21, 29).

AUTOLOGOUS CONDITIONED SERUM

The first autologous conditioned serum (ACS) preparation method (Orthokine®) (29), was developed and patented in 2003 by Peter Wehling and Julio Reinecke. This is the most used current method of up-regulating IL-1Ra from whole blood. Although this product has been slowly validated, it gained fast clinical acceptance primarily in human and veterinary sports medicine (20). Autologous conditioned serum is produced by incubating whole blood for 24 h at 37°C with medical grade glass beads coated with $CrSO_4$, generating an enriched serum. After incubation, the blood clots and serum are centrifuged, collected and filtrated before administration (29).

During incubation, platelets degranulate and mononuclear cells are stimulated to produce various cytokines and growth factors such as; IL-1Ra, IL-4, TNF-α, IL-10, IL-6, and basic fibroblast growth factor (FGFb) among various other anabolic factors stated on **Table 1** (27, 28, 35, 36). It was initially stated that this process would not result in the concomitant increase of pro-inflammatory cytokines as IL-1 ß and TNF-α (29). However, more recent studies showed that the concurrent increase in pro-inflammatory cytokines may happen in whole blood incubation (36).

Despite the aforementioned studies, the ratio between anti-inflammatory cytokines and pro-inflammatory cytokines (specifically IL-1Ra/IL-1β) should still be sufficient to justify therapeutic use (20). In fact, considering an IL-1Ra concentration of 3 ng/mL in an ACS kit (Orthokine®), 2 mL of Orthokine® can be injected into the patients' knee, resulting in a minimum ratio of IL-1Ra/IL-1β of 170:1. This is much higher than the minimum 10:1 ratio necessary to be clinically efficient (28).

Monocytes are believed to be responsible for the cytokine increase within the ACS, with both surface area and the chemical-cell interaction playing important role in the type of cytokine produced (20). With regard to surface area, Magalon et al. (37) demonstrated that bead diameter results in varied monocyte induction. For instance, beads of 3 mm and polished beads of 3.5 mm produced higher anti and pro-inflammatory cytokine concentrations compared with other diameters (4 and 2.5 mm coated with $CrSO_4$) (37).

Different studies suggested that the chemical-cell interaction would be essential for cytokine expression in monocytes (28, 38). Initially, it was observed that cellulose acetate beads, used for adsorptive apheresis of granulocytes and monocytes, induced IL-1Ra release in peripheral blood with no concurrent release of TNF-α or IL1β (38).

Interestingly, anti-inflammatory cytokines and IL-1Ra/IL-1ß ratio increased in equine whole blood samples incubated in glass tubes without beads (30). This increase in cytokines

TABLE 1 | Comparison between autologous conditioned serum and autologous protein solution.

Type of hemoderivative	Commercial kits	Amount of blood	Anticoagulant	Equipment necessary	Protocol	Platelet concentration	Leucocyte concentration	Growth-factors/ anti-inflammatory cytokines	Pro-inflammatory cytokines	IL-Ra/IL-β	Number of treatments	References
Autologous conditioned serum (ACS)	IRAP II® Ortokine®	60 mL	No anticoagulant used	Kit, incubator, centrifuge and freezer	Kit is incubated at 37°C for 24 h. Then, the kit is centrifuged for 10 min and serum is collected. Serum is stored at −20°C.	Not reported (30) or lower than whole blood (31) (not statistically significant)	Not reported (30) or lower than whole blood (31) (not statistically significant)	IGF-1, IL-10, sTNF-R1, TGF-β, IL-1Ra, IL-6, IL-4, FGFb,VEGF, HGF,PDGF-AB, osteoprotegerin, interferon-gamma, oncostatin M	TNF-α, IL-1 β, MMP-3	5 (30) to 113 (31)	4	(9, 30, 31)
Autologous protein solution (APS)	Pro-Stride® nSRIDE®	55 mL	ACD-A	Kit and centrifuge (separator and concentrator)	Blood is placed on the separator and centrifuged for 15 min and platelet-rich solution is collected. The product is transferred to the concentrator and centrifuged again for 2 min.	Higher than whole blood (31)	Higher than whole blood (31)	sTNF-R1, TGF-β, IL-1Ra, PDGF-AB/BB, IGF-1, EGF	TNF-α, IL-1β, MMP-3	48.22 (31)	1	(8, 31–34)

sTNF-R1, soluble tumor necrosis factor receptor 1; TGF-β, transforming growth factor beta; FGFb, basic fibroblast growth factor; HGF, hepatocyte growth factor; IL-1Ra, interleukin 1 receptor antagonist protein; PDGF-AB/BB, platelet-derived growth factor AB/BB; IGF-1, insulin-like growth factor; EGF, epidermal growth factor; MMP-3, metalloproteinase 3; osteoprotegerin, osteoclastogenesis inhibitory factor; interferon, gamma, oncostantin M.

was not statistically different compared with serum obtained from commercial kits (30). This indicates that $CrSO_4$-treated glass beads may not be necessary to produce an enriched serum (37). On the other hand, in Meijer et al. (28), the incubation of whole blood with medical grade borosilicate beads did induce significant increase in IL-1Ra concentrations compared with standard consumer beads. Thus, as in any other biological therapies, the methods and kits used for ACS production, influence the end product cytokine profile, which could potentially impact its clinical properties. This also indicates that other cytokines present in ACS may play a role in the inflammatory modulation of this therapy (28).

The most common commercial kits require a period of 24 h for preparation of ACS. In those kits, the increased concentration of IL-1Ra is time-dependent, reaching a maximum concentration in 24 h (28). Prolonged periods of whole blood incubation does not increase concentrations of cytokines or growth factors in ACS (39). Notably, the use of certain medications e.g., reserpine and phenylbutazone have been demonstrated to interfere with platelet function and clotting, which may potentially interfere with ACS cytokine content (40, 41).

Use of Autologous Conditioned Serum as a Therapy for Osteoarthritis

The therapeutic effects of IL-1Ra have been of interest to researchers as a potential modulatory treatment for OA. Numerous *in vitro* and *in vivo* studies involving different animal models have tested the efficacy of this therapy (9, 20, 30, 42, 43). Conditioned serum (CS) stimulated chondrogenic differentiation in adipose-derived stem cells and induced these cells to reduce lymphocyte proliferation and activation *in vitro* (44). It is possible that intra-articular CS administration influence resident stem cell behavior and immunomodulation (44). *In vivo,* IL-1Ra reduced osteophyte formation and cartilage fibrillation in rabbits (17).

In a randomized block study using an equine model, animals were injected at days 14, 21, 28, and 35 after OA induction and subsequently compared with placebo (9). A significant decrease of synovial proliferation and overall joint inflammation was observed in treated animals compared with placebo. Similar clinical improvement was observed in human patients with knee osteoarthritis (10). Still, ACS is yet to be proven to prevent cartilage degradation (9). The administration of ACS may stimulate the endogenous production of IL-1Ra, since this cytokine significantly increased over time after ACS injection (9). In another study, it was determined that natural inflammatory mediators such as IL-1β and IL-6 can potentially activate IL-1Ra transcription in chondrocytes and macrophages *in vitro* (23). The positive results obtained experimentally supported the clinical use of ACS in equine sports medicine (45).

AUTOLOGOUS PROTEIN SOLUTION

Over a decade after the initial investigation of ACS (32), a newer hemoderivative called autologous protein solution (APS) was developed. This method proposes to increase the anti-inflammatory and anabolic concentrations of hemoderivatives of

clinical use. Autologous protein solution consists of a platelet-rich plasma (PRP) separated from the blood. The PRP is then processed through a special kit intended to stimulate white blood cells (WBC) to produce anti-inflammatory cytokines concentrating its content in a smaller volume of plasma.

For the preparation of this product, blood is collected and mixed with anticoagulant citrate dextrose solution (ACD-A), transferred to a separation device and centrifuged for 15 min (8, 46). Then, the intermediate cell solution (containing platelets and white blood cells) is transferred to an APS concentrator, where the solution is mixed with polyacrylamide beads and centrifuged again for 2 min. The autologous protein solution is then collected and ready for use (32, 46).

In horses, APS presented significantly more white blood cells, platelets and significantly less erythrocytes compared with the whole blood (31). Different cytokines were detected in APS such as TNF-α, TGF-β, IL-1β, IL-6, IL-10, MMP-3, and IL-1Ra among others (**Table 1**) (8, 31, 33, 46). In fact, a positive ratio of IL1Ra:IL-1β was observed in APS for horses (31). Increased values of interleukin 1 receptor I and tumor necrosis factor receptor II (sIL-1RI and sTNF-RII; **Table 1**) have been observed in APS in humans (32).

Cytokines in APS seem to correlate to the cellular content within the product. For instance, a negative correlation has already been determined between WBC and TGF-β concentrations (31). Platelets may also negatively correlate to TGF- β and sTNF-1R (31). Interestingly though, a positive correlation between leukocytes and IL-1Ra concentrations, but not IL-1ß has also been observed (47).

The general condition of the patients did not seem to interfere significantly with the cytokine composition of APS. Similar concentrations of anti-inflammatory cytokines and anabolic growth factors were observed in APS from patients with OA and controls (32).

Use of Autologous Protein Solution as a Therapy for Osteoarthritis

Although not extensively studied as other hemoderivatives, APS has been successfully used as a symptom modifying option for the treatment of OA. This treatment seems to be safe and its side-effects (i.e., discomfort post-injection) are commonly associated with other intraarticular injections (34).

The use of APS has provided optimistic results for OA treatment in different species (8, 48, 49). In a prospective, blinded, placebo-controlled clinical trial performed in dogs, animals presented improvement in pain, lameness assessment and weight bearing in the affected limb 12 weeks after APS treatment, compared with placebo treated joints (49).

In fact, one single injection of APS demonstrated significant improvement in Western Ontario and McMaster Universities osteoarthritis index (WOMAC) scores in OA patients after 2 weeks post-injection compared with baseline (34). Pain scores improved up to 90% in 6 months post-treatment (34). In addition to the clinical improvement, APS treatment reduced bone-marrow lesions 12 months post-treatment (50). However, results should be interpreted with caution since OA varied among the patients and some were actually medicated with non-steroidal anti-inflammatory drugs (NSAIDs), which may have influenced the results (50).

In horses with naturally occurring OA, APS significantly improved lameness, pain-in-flexion, gait analysis and range of motion up to 14 days after treatment compared with baseline and controls (8). In equine joint fluid, there was a significant decrease in protein concentration in treated horses compared to untreated controls (8).

Promising results with APS use were justified due to its favorable cytokine content. A IL-1Ra:IL-1 ratio >1,000 in APS seemed to predict the inhibition of inflammation in human OA patients (47). Additionally, IL-1Ra and sTNF-RII content in APS was correlated with improvement in pain scores after treatment (47). Whilst such correlations are still in their infancy, further studies, focusing on the integration of APS cytokines on the multiple inflammatory pathways in the different stages of OA, are warranted.

DISCUSSION

Although positive results were observed with clinical use of ACS and APS, others demonstrated that ACS did not have a direct effect on cartilage metabolism compared with unstimulated serum. This may justify the lack of disease modifying effects observed clinically with the use of these products (36, 42).

Concentration of Cytokines in Joints After Treatment

The conflicting results observed *in vivo* may be attributed to the reduction of therapeutic cytokine concentrations in the synovial fluid after injection, suggesting that new intra-articular therapies should focus on the prolonged presence of the cytokines in the joint space. For this reason, some authors opted for a protocol of multiple serial ACS injections separated by short periods of time (42).

It is still unknown how long the cytokines remain present in the joint and to what extent they exercise an effect (20). In fact, most of the cytokines in ACS are released within 6 h and increased hours of incubation at body temperature are known to reduce bioactivity of these cytokines (36). For instance, IL-1Ra has a short half-life of only 4–6 h. Thus, it is uncertain if the IL-1Ra concentrations within the OA joints actually correlate with anti-inflammatory effects of ACS as the ratio of IL-1Ra/IL-1β in synovial fluid of patients has not been correlated with disability and pain (51). Similarly in horses, in joints treated with a single injection of APS, synovial concentrations of IL-1 β, TNF-α, and IL-1Ra were not significantly different compared with control groups (8). A standard protocol was suggested by Lasarzik et al. (52), which described better results with 2-day treatment interval for ACS injections. This was based on the evidence that in horses with OA, ACS induced significant decrease of IL-1β and increase of IL-1Ra in synovial fluid, despite these effects lasting no more than 48 h (52). With regards to APS use, this issue has not been raised and the protocols described, currently involved the use of only one intra-articular treatment (8, 53). Nevertheless, it might be interesting to study the effects of a single vs. multiple APS injections in the modulation of OA.

Therapeutic Effect of Autologous Conditioned Serum and Autologous Protein Solution in Chronic Osteoarthritis

It is possible that the blockage of IL-1 receptors is insufficient to abolish cartilage degradation in more advanced cases of OA (11, 51, 54). Indeed, the use of ACS did not significantly delay or prevent surgical intervention in end-stage osteoarthritis in humans compared with placebo after 10 years of treatment (11). For APS, treatment did demonstrate significant clinical improvement up to 3 years after injection mainly in patients with mild to moderate knee OA (48). In horses, the use of APS was clinically more efficient when no severe lameness, asymmetry in gait or subchondral bone sclerosis was observed in arthritic joints (8). Animals were up to 30-times more likely to present therapeutic improvement with APS treatment when affected with mild OA (8). Similarly, in humans, patients with better cartilage conditions responded better compared with patients with more significant cartilage loss (11).

Such findings suggest that the inhibition of IL-1Ra in chronic cases of OA may not be sufficient to improve clinical outcome. In cases in which disease was identified early though, clinical improvement was reported (9, 42). Osteoarthritis and rheumatoid arthritis are complex inflammatory processes that involve multiple pathways and cytokines that change dynamically during the course of the condition (55, 56). Thus, the impact of hemoderivatives in these multiple pathways may need to be further explored (57).

Modulatory Effects of Autologous Conditioned Serum and Autologous Protein Solution in Joint Tissues

The modulatory effects of ACS and APS therapies have been validated experimentally despite the lack of a comprehensive understanding of their effects on multiple pathways in OA (8, 9, 31, 48). A recent study suggested that both ACS and APS seems to have more efficient anti-inflammatory and chondroprotective effects compared with triamcinolone in an *in vitro* OA model. These hemoderivatives significantly reduced expression of IL-1ß and TNF-α and showed a trend in upregulation of aggrecan (ACAN) and collagen type II (COL2-A1) expression in cartilage (31). In the synovium, both treatments upregulated IL-10 gene expression and significantly decreased the release of PGE_2 in synovium compared with triamcinolone (31). The effects of ACS and APS seemed to be dose dependent (31).

Studies such as Velloso Alvarez et al. (31), revealed overall similar effects of ACS and APS in a co-culture model, although the cytokine profile of these hemoderivatives is essentially different. The effects of the different cytokine profiles may be more easily observed in simpler models as monocultures (46). In chondrocytes, the concentrations of IL-1Ra and IL-10 released by cells treated with APS were actually higher than ACS treated cells, although no difference was observed in gene expression. The significant difference in cytokine release obtained in monocultures vs. co-culture models can indicate that the pool of cytokines in both hemoderivative products may influence tissues differently, but that the overall effect in joints may be similar.

Additionally, detailed investigation of the ACS cytokines other than IL-1Ra and their individual (or synergic) effect in different tissues within the joint, may reveal other potential treatment routes equally or more important than IL-1 blockage. For instance, in horses with mild osteoarthritis, clinical improvement was statistically associated with content of not only IL-1Ra but also IGF-1 in ACS (57). *In vivo* studies will be paramount to better establish the importance of such cytokine interactions and their relevance in a clinical scenario.

Limitations and Requirements for Treatment Use

As with other biological therapies, the optimization of protocols for preparation and application of ACS and APS in different OA scenarios is warranted. Considering the autologous nature of the product, cytokine variability is an inevitable issue with this treatment, which can lead to inconsistent modulatory and clinical effects (57). The development of an allogeneic preparation of these products, as suggested for other hemoderivatives, could provide a more consistent cytokine profile (58).

Autologous protein solution currently offers a more user-friendly approach since it requires shorter preparation and no incubation times, unlike ACS which requires a minimum laboratory setting (i.e., centrifuge, incubator, and freezer). Autologous protein solution can be prepared using a portable centrifugation equipment and is a very basic and undemanding technique (8). The intra-articular injections can be applied as a quick, point-of -care single treatment in an ambulatory-based practice (8). Conversely, in case the practitioner is already familiar with the ACS technique and equipment, the continuation of its use would be pertinent. There is insufficient evidence-based research to support superiority of APS compared with ACS at this point.

FINAL CONSIDERATIONS

Despite the limited number of clinical studies using ACS and APS and the lack of knowledge in relation to its composition, these therapies provide a symptom-modifying option of treatment in early OA cases (8, 9). These hemoderivatives have shown clinical improvement with no adverse effects compared with placebo in humans (48, 50) and horses (8, 9, 45), being widely used in sports medicine (45). Additionally, these treatments seem to give clients the impression of a more "natural" therapy for horses, which may be preferred over the traditional anti-inflammatory treatments. Also, both ACS and APS may offer alternatives for clinicians when other therapies are prohibited due to drug testing within equestrian competitions.

Recent studies presented in this review provided important information regarding to the lack of disease modifying effect associated with the use of these therapies. This information may help researchers direct investigations into learning how conditioned serum and protein solution composite can modulate

inflammation and enhance improvement in lameness in selected cases.

The promising anecdotal results with the use of APS and the practicality of the treatment compared with ACS may lead clinicians to prefer its use (8, 48). However, both APS and ACS could benefit from additional clinical research and some refinement in product preparation and application for clinical use. The direct comparison of both products, in horses with different severities of naturally occurring OA, will further support the use of ACS and APS clinically.

AUTHOR CONTRIBUTIONS

LCG was responsible for the literature search and preparation of the manuscript. MJM contributed with the literature search and edition of the manuscript. All authors contributed to the article and approved the submitted version.

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Targeting Soluble Epoxide Hydrolase and Cyclooxygenases Enhance Joint Pain Control, Stimulate Collagen Synthesis, and Protect Chondrocytes From Cytokine-Induced Apoptosis

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Objective: To determine the symptomatic and disease-modifying capabilities of sEH and COX inhibitors during joint inflammation.

Methods: Using a blinded, randomized, crossover experimental design, 6 adult healthy horses were injected with lipopolysaccharide (LPS; 3 μg) from *E. coli* in a radiocarpal joint and concurrently received the non-selective cyclooxygenase (COX) inhibitor phenylbutazone (2 mg/kg), the sEH inhibitor *t*-TUCB (1 mg/kg) or both (2 mg/kg phenylbutazone and 0.1, 0.3, and 1 mg/kg *t*-TUCB) intravenously. There were at least 30 days washout between treatments. Joint pain (assessed *via* inertial sensors and peak vertical forces), synovial fluid concentrations of prostanoids (PGE_2, TxB_2), cytokines (IL-1β, IL-6, TNF-α) and biomarkers of collagen synthesis (CPII) and degradation (C2C) were measured at pre-determined intervals over a 48-h period. The anti-apoptotic effect of COX and sEH inhibitors was determined *via* ELISA technique in primary equine chondrocytes incubated with TNF-α (10 ng/ml) for 24 h. Apoptosis was also determined in chondrocytes incubated with sEH-generated metabolites.

Results: Combined COX and sEH inhibition produced significantly better control of joint pain, prostanoid responses, and collagen synthesis-degradation balance compared to each compound separately. When administered separately, pain control was superior with COX *vs.* sEH inhibition. Cytokine responses were not different during COX and/or sEH inhibition. In cultured chondrocytes, sEH inhibition alone or combined with COX inhibition, but not COX inhibition alone had significant anti-apoptotic effects. However, sEH-generated metabolites caused concentration-dependent apoptosis.

Conclusions: Combined COX and sEH inhibition optimize pain control, attenuate loss of articular cartilage matrix during joint inflammation and cytokine-induced chondrocyte apoptosis.

Keywords: synovitis, arthritis, osteoarthritis, lameness, mobility, equine model

INTRODUCTION

Inflammation of the synovial lining (i.e., synovitis) and cartilage damage are prominent features of osteoarthritis (OA), strongly correlating with pain sensitization and disease severity. Synovitis can be present in all stages of OA, tends to worsen with radiographic severity and may contribute to the progression of cartilage damage (1–5). In equine and human OA, local and systemic release of cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 are associated with pain (lameness) as well as greater loss of cartilage volume and joint space narrowing (6–9). As actively secreting cells, chondrocytes are especially susceptible to endoplasmic reticulum (ER) stress, an important mechanism leading to chondrocyte apoptosis (10–12) which also correlates with synovitis in OA (13).

Lameness and pain continue to be the primary complaint associated with OA in horses and humans. In horses and humans, cyclooxygenase (COX) inhibitors are first-line therapies to control OA pain yet this strategy is only partially effective and can cause serious adverse effects in both species (14, 15). In addition, COX inhibitors can induce ER stress and apoptosis (16), although evidence regarding their chondrotoxic or chondroprotective roles in humans remains conflicting (17). Recently, the arachidonic acid-derived metabolites 8,9-, 11,12-, and 14,15-dihydroxyeicosatrienoic acids (DiHETs), which are converted by soluble epoxide hydrolase (sEH) from the corresponding epoxyeicosatrienoic acid (EETs) regioisomers (**Figure 1**), were found to be significantly associated with the prevalence and progression of knee OA in older adults (18). This finding is important because a growing body of evidence suggests that, by preventing conversion of EETs to DiHETs, sEH inhibitors promote inflammatory resolution (19), antinociception (20–25), prevent ER stress and apoptosis (26–28), and support organ and tissue repair (29). In addition, in mouse models, sEH inhibitors lack addictive effects (30) and do not affect motor ability (25), result in similar or even greater antinociception compared to COX inhibitors (25) and morphine (22), display antinociceptive synergy with COX inhibitors (31) and can prevent COX inhibitor-induced intestinal ulceration (32). Thus, sEH inhibition alone or combined with COX inhibition could represent a significant development in the management of OA by conferring both symptom- and disease-modifying effects.

Our laboratory has previously demonstrated that sEH is involved in joint pain and physical disability using equine models of joint inflammation (33) and naturally-occurring models of chronic pain (laminitis) (34, 35) that, similar to human (36) and equine (37) OA, is characterized by inflammatory and neuropathic changes (38). We have also determined that the sEH inhibitor *t*-TUCB (*trans*-4-{4-[3-(4-Trifluoro-methoxy-phenyl)-ureido]-cyclohexyloxy}-benzoic acid) and several others developed for the human enzyme are equally potent against equine sEH (35) and that *t*-TUCB has a good plasma pharmacokinetic profile in horses, achieving therapeutically relevant concentrations within the equine joint (33). Recently, pharmacologic inhibition of sEH ameliorated hyperalgesia, edema, and expression of pro-inflammatory cytokines in joint tissues of a mouse model of rheumatoid arthritis (39). Another study in laboratory Beagles with naturally-occurring OA showed a statistically significant, albeit modest, improvement in subjective measures of pain and mobility (40). Further, incubation of cultured canine chondrocytes with EETs attenuated IL-1β-induced IL-6 and TNF-α secretion and reduced cytotoxicity (40). However, no study has examined the symptomatic (pain) and disease modifying effects of sEH and COX inhibition in the context of painful joint diseases.

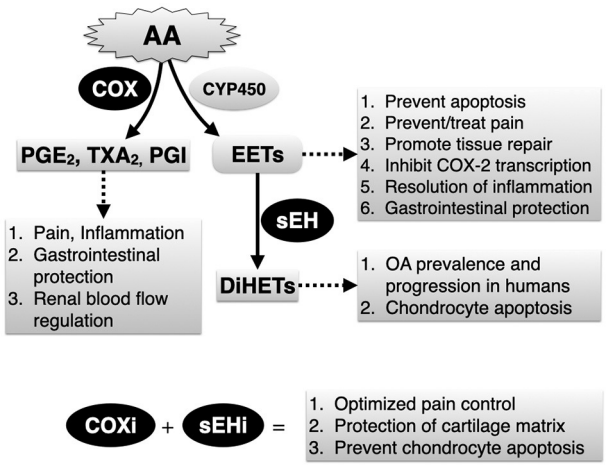

FIGURE 1 | Simplified depiction of arachidonic acid (AA) metabolism *via* the cyclooxygenase (COX) and the epoxygenase (CYP450) pathways. Soluble epoxide hydrolase (sEH) is a critical yet relatively unexplored enzyme that breaks down endogenously produced and beneficial epoxyeicosatrienoic acids (EETs), generated by epoxygenases to their corresponding and potentially harmful dihydroxyeicosatrienoic acid (DiHETs). Main biology related to COX and sEH activities, and expected therapeutic outcomes related to combined COX inhibitors (COXi) and sEH inhibitors (sEHi) based on results of current study are listed.

The goals of the present study were to assess the symptomatic and disease-modifying capabilities of sEH and COX inhibitors on articular tissues. We hypothesized that combined COX and sEH inhibition would attenuate joint pain on ambulation and the breakdown of articular cartilage matrix associated with synovitis, and would prevent cytokine-induced chondrocyte apoptosis to a significantly greater degree than inhibiting COX or sEH separately.

MATERIALS AND METHODS

Experimental Animals

Six adult horses (5 castrated males, 1 sexually intact female) aged 8.5 ± 3 years (range 5–13 years old) and weighing 462 ± 50 kg (range 397–536 kg) were used. Horses were considered healthy and free of radiocarpal joint disease on the basis of complete veterinary work-up that included general physical and orthopedic examinations, complete blood cell counts and plasma chemistry profile. Horses were housed as a group on paddocks for the duration of the study with water available *ad libitum* and grass hay fed once/daily. The University of Minnesota Institutional

Animal Care and Use committee reviewed and approved the study protocol.

Test Compounds

The non-selective COX inhibitor, phenylbutazone, was obtained from Vedco Inc. (EQUI-PHAR, Phenylbutazone injection 20%; Saint Joseph, MO, USA) or from Sigma-Aldrich, Inc. (Saint Louis, MO, USA), and the 8,9-, 11,12-, and 14,15-DiHETs were obtained from Cayman Chemical (Ann Arbor, MI, USA). The sEH inhibitor, *t*-TUCB, was kindly provided by Dr. Bruce Hammock (University of California-Davis), synthesized and characterized according to established methodology (41–44). Equine recombinant TNF-α was purchased from R&D Systems (Minneapolis, MN, USA).

Equine Radiocarpal Synovitis Model and Drug Treatments

Horses were sedated (xylazine, 0.2–0.5 mg/kg IV; AnaSed, Akorn Inc., IL, USA) and synovitis was induced by injecting 2 ml of a freshly prepared solution of lipopolysaccharide (LPS) from *E. coli* O55:B5 (1.5 μg/ml in 0.9% NaCl; total 3 μg; catalog number L5418, Sigma-Aldrich, St. Louis, MO, USA) into one randomly assigned radiocarpal joint for the first injection, with subsequent injections alternating between joints, as in a previous study from our laboratory (33). There were at least 30 days washout between subsequent LPS injections (and treatments). Following a randomized crossover experimental design in which each horse served as its own control, treatments consisted of the non-selective COX inhibitor phenylbutazone (PBZ, 2 mg/kg; 0.01 ml/kg of a 200 mg/ml commercial solution), the sEH inhibitor *t*-TUCB (1 mg/kg) or a combination of both drugs (2 mg/kg PBZ and 1, 0.3, or 0.1 mg/kg *t*-TUCB). We intentionally chose a relatively moderate dose of phenylbutazone to avoid fully blocking pain or prostanoid production, reasoning that this would allow interactions with the varying doses of the sEH inhibitor to be revealed. *t*-TUCB was dissolved in dimethyl sulfoxide (100% DMSO, Sigma-Aldrich) to final concentrations of 100, 30, and 10 mg/ml and filter-sterilized with 0.2 μm pore size sterilizing-grade membranes prior to administration (0.01 ml/kg). Drugs were administered slowly (30–45 s) as a single intravenous injection using separate jugular vein catheters, at the same time that the joints were injected with LPS. In the phenylbutazone-only treatment, horses also received the vehicle diluent of *t*-TUCB (DMSO, 0.01 ml/kg) to control for possible DMSO anti-inflammatory effects (45, 46) Horses in the *t*-TUCB-only treatment group also received an intravenous injection of 0.9% saline (0.01 ml/kg). Treatment responses were determined by assessing lameness (pain on ambulation) in a straight-line trot on a hard, flat surface. Kinematic parameters were assessed by calculating the vector sum of the head height difference relative to the stride cycle using an inertial sensor system (Lameness Locator, Equinosis LLC, Columbia, MO, USA) (47, 48) and the peak vertical force (PVF) using an in-ground force platform system (AMTI, Watertown, MA, USA). Prior to (i.e., baseline) and at 2, 4, 8, 24, 32, and 48 h after LPS/drug administration horses were trotted to achieve a minimum of 25 total strides for kinematic analysis, and 5 acceptable trials per set of ipsilateral fore and hind limbs on the force plate within 2.8–3.3 m/s and an acceleration <10%. If the horse was unable to bear weight on the LPS injected limb for a trial period, then the maximum decrease in vector sum was inputted (−137), as per the manufacturer's recommendation, and the PVF was recorded as zero for that time.

Synovial Fluid Biomarkers

Baseline synovial fluid was collected from the radiocarpal joint immediately prior to injection with LPS. Subsequent arthrocenteses were performed 8, 24, and 48 h after injection with LPS/treatments. All samples were collected without dilution, centrifuged, aliquoted, and stored at −80°C until further analyses. To determine the effect of COX and sEH inhibition on articular cartilage matrix, prostanoid production, and pro-inflammatory biomarkers during synovitis, we assayed (ELISA) all synovial fluid samples for type II collagen synthesis (CPII) and degradation (C2C) biomarkers (IBEX Technologies, Quebec, CAN), prostaglandin (PG) E_2, thromboxane (Tx) B_2 (Enzo Life Sciences, Farmingdale, NY, USA), interleukin (IL)-β, IL-6, and tumor necrosis factor (TNF)-α (equine specific assays from Genorise Scientific, Inc., Glen Mills, PA, USA). Use of equine synovial fluid with type II collagen biomarkers have been previously validated (49), as have the cytokine biomarkers by the manufacturer; these assays were measured without digestion. Type II collagen synthesis-degradation balance was assessed by calculating CPII to C2C ratios. Measurements were performed as per the manufacturer's recommendations, at necessary dilutions, in duplicate or triplicate. Mean intra-assay coefficients of variations (CV) for the biomarkers were: CPII 3.1%, C2C 3.9%, PGE_2 10.7%, TxB_2 4.3%, IL-1β 5.2%, IL-6 2.4%, and TNFα 2.1%, and inter-assay CVs were: CPII 9.0%, C2C 12.4%, PGE_2 4%, TxB_2 5.8%, IL-1β 12.9%, IL-6 7.0%, and TNFα 8.0%.

Chondrocyte Cultures

Primary equine articular chondrocytes of second passage (American Research Products, Inc., Waltham, MA, USA) were cultured in high-glucose Dulbecco's Modified Eagle Medium (Gibco Laboratories, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal calf serum and 1% penicillin/streptomycin (Sigma-Aldrich) in an atmosphere with 5% CO_2 at 37°C until reaching ∼80–90% confluency and then passaged to 12-well culture dishes at a density of 10,000 cells/ml for 24 h. Next, the chondrocytes were changed to serum-free medium and incubated with TNF-α (10 ng/ml) along with the COX inhibitor PBZ, the sEH inhibitor *t*-TUCB or both at several concentrations for 24 h. The inhibitors were used at their approximate half-maximal inhibitory concentration or IC_{50} (*t*-TUCB 4 nM, PBZ 4 μM), 80% inhibitory concentration or IC_{80} (*t*-TUCB 40 nM, PBZ 40 μM) and 10-fold > IC_{80} (*t*-TUCB 400 nM, PBZ 400 μM) for the equine enzymes (35, 50). In additional experiments, chondrocytes were incubated for 24 h in serum-free medium with several concentrations (0, 1, 10 ng/ml) of the sEH-generated 8,9-, 11,12-, and 14,15-DiHETs. Controls were treated with 0.9% saline (vehicle diluent for TNF-α) and ethanol (vehicle diluent for PBZ, *t*-TUCB and DiHETs; 0.0001% final concentration in culture medium). At the end of the 24-h incubation period, cells were harvested and apoptosis was

determined in duplicates using ELISA technique as per the manufacturer's instructions (Cell Death Detection ELISA^{PLUS}, Roche GmbH, Mannheim, Germany).

Statistics

Data were analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA). Areas under the curves (AUC) were calculated using the trapezoidal method for some of the variables, as indicated in the Results section. Data from the *in vivo* studies were analyzed using linear mixed-effects model whereby treatment and time were included as fixed factors and horse as random factor if normally distributed, or the Friedman test if not. Normality was determined primarily by visual inspection of QQ plots and the Shapiro-Wilk normality test. Chondrocyte apoptosis data were considered normally distributed by visual inspection of QQ plots and the Shapiro-Wilk normality test, and were analyzed by One- or Two-way ANOVA. In all cases, the two-stage step-up method of Benjamini, Krieger, and Yekutieli was used as multiple comparison test while correcting for multiple comparisons by controlling the False Discovery Rate. The Geisser-Greenhouse correction was applied for data showing different scatter. Since there were missing data for TxB_2, IL-1β, IL-6, and TNF-α, the number of analyzed data points is indicated in the text. Significance level was set at $p < 0.05$. Data are presented as mean±SEM.

RESULTS

Combined sEH and COX Inhibition Resulted in Better Control of Joint Pain During Synovitis Than Inhibiting Each Enzyme Separately

Results of kinematic and kinetic parameters are shown in **Figure 2**. We analyzed these data in two separate periods (0–8 and 24–48 h) because joint pain/lameness induced by LPS is transient, peaking at 8–12 and resolving thereafter even without treatment (33) and the duration of action of the selected dose of the COX inhibitor phenylbutazone to control joint pain in horses is ∼8–12 h (51). In the first 8 h following induction of synovitis, pain on ambulation assessed objectively with inertial sensors was significantly less with combined COX and sEH inhibition than when each enzyme was inhibited separately. Further, sEH inhibition alone was significantly less efficacious than COX inhibition alone. From 24–48 h after synovitis induction, there were no significant differences among treatments. Simultaneous assessment of peak vertical forces using an in-ground force platform system yielded similar results. Thus, based on internally consistent results obtained with two separate, unbiased and objective measures of joint pain during ambulation, our findings suggest that COX inhibition provides superior control of synovitis-associated joint pain than sEH inhibition, and that concurrently inhibiting both enzymes result in significantly better control of joint pain than inhibiting each enzyme separately.

Effect of COX and sEH Inhibition on Inflammatory Biomarkers

We quantified several biomarkers of inflammation to compare the extent that COX and sEH inhibition modified the joint responses to synovitis. We did not divide these data into separate periods for statistical analyzes because joint inflammatory response in the LPS-induced synovitis model peaks at 12–24 h and lasts for at least 48 h (33). As shown in **Figure 3**, synovial fluid concentrations of PGE_2 were significantly lower during treatment with combined inhibition of COX and sEH compared to COX or sEH inhibition individually. All doses of sEH significantly potentiated the inhibition of PGE_2 production by the COX inhibitor. We also determined the synovial fluid concentrations of TxB_2 during treatment with the highest dose of the sEH inhibitor (1 mg/kg *t*-TUCB) alone or when combined with the COX inhibitor ($n = 4/6$ horses). Results showed that TxB_2 levels were not significantly different between COX inhibition alone and the combined COX and sEH inhibition, but was significantly higher than either of these during sEH inhibition alone. Thus, sEH inhibition added a significant inhibitory effect on COX-2-generated PGE_2 but not on COX-1-generated TxB_2. We also assessed cytokine responses within the joint (**Figure 4**) with emphasis on IL-1β ($n = 4/6$ horses), IL-6 ($n = 4$–$6/6$ horses), and TNF-α ($n = 5$–$6/6$ horses), given their demonstrated association with joint pain and cartilage loss in OA (2, 7, 9, 52–54). Unexpectedly, there were no significant differences between COX, sEH or combined COX and sEH inhibition on cytokine release in synovial fluid, except for IL-6, which was significantly higher during sEH inhibition alone compared to COX inhibition alone and the combined COX and sEH inhibition. Notably, TNF-α levels increased 100–200% above baseline with all treatments. Taken together, these findings suggest that combined COX and sEH inhibition produced better control of PGE_2 synthesis in response to synovitis than inhibiting each enzyme separately, whereas there was no added benefit of sEH inhibition upon cytokine release.

Combined COX and sEH Inhibition Provides Superior Protection of the Articular Cartilage Matrix Than Inhibiting COX Alone

Since synovitis and joint inflammation alters the normal collagen synthesis-degradation coupling and contribute to loss of articular cartilage matrix and OA development (1, 4, 13), we sought to determine the effect of COX and sEH inhibition on the collagen-degradation relationship during synovitis. As shown in **Figure 5**, CPII concentrations were not significantly different during inhibition of COX, sEH, or both whereas C2C concentrations were significantly lower during sEH inhibition compared to COX inhibition and the lowest dose of combined COX and sEH inhibition. The resulting synthesis-degradation balance favored synthesis by ∼10-fold during combined COX and sEH inhibition compared to only ∼5-fold when COX or sEH were inhibited separately. The synthesis-degradation ratio was higher during combined COX and sEH inhibition compared to either COX or sEH inhibition alone.

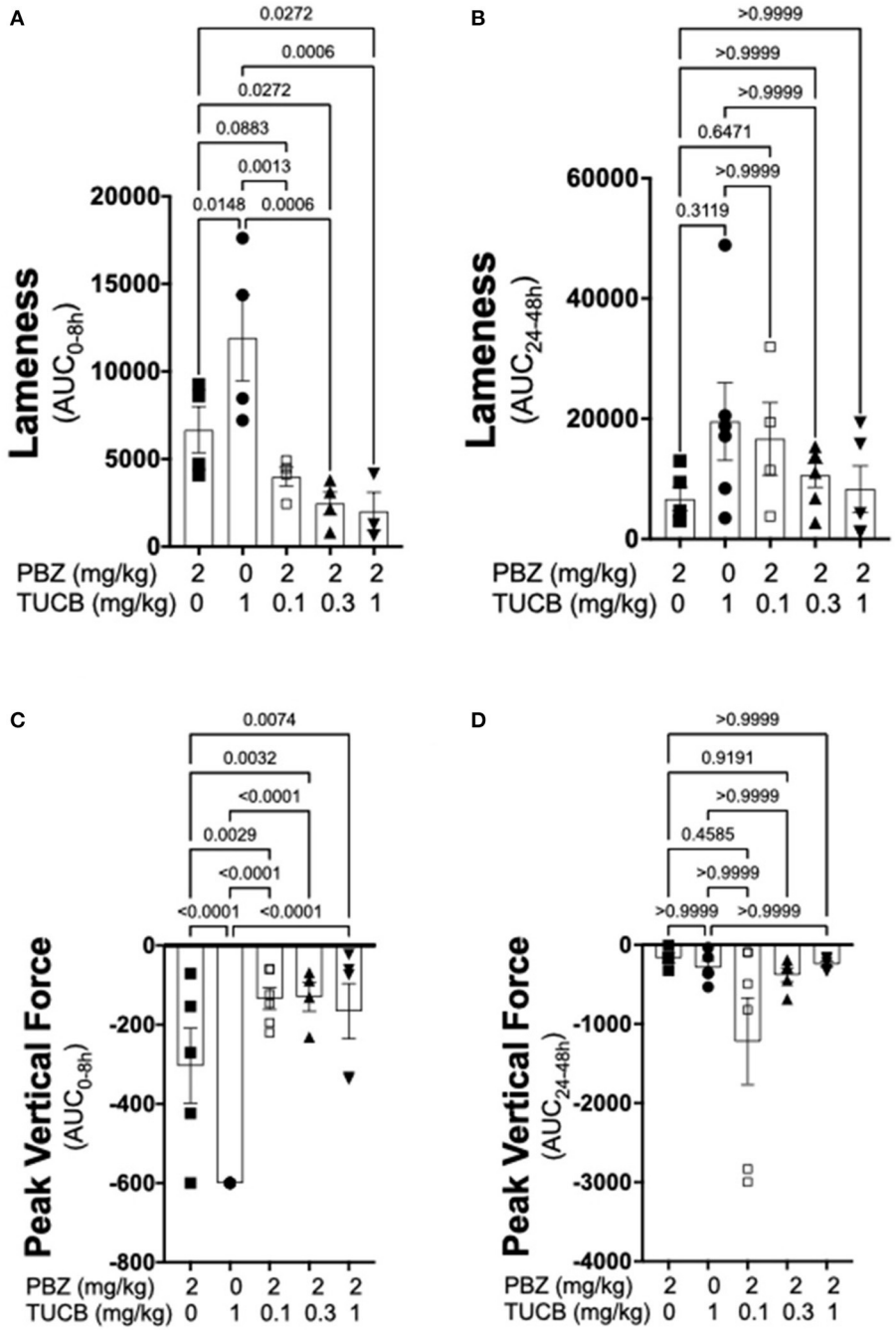

FIGURE 2 | Combined sEH and COX inhibition resulted in better control of joint pain in the early phase of synovitis than inhibiting each enzyme separately. Pain was estimated as a change in lameness that was objectively assessed *via* inertial sensors **(A,B)** as well as forces applied to an in-ground force platform **(C,D)** during the early, 0–8 h **(A,C)**, and late 24–48 h **(B,D)** phases of synovitis induced by intra-articular injection of lipopolysaccharide (LPS; 3 μg) into the radiocarpal joint in horses ($n = 6$). Horses were treated intravenously with the sEH inhibitor *trans*-4-{4-[3-(4-Trifluoro-methoxy-phenyl)-ureido]-cyclohexyloxy}-benzoic acid (TUCB), the non-selective COX inhibitor phenylbutazone (PBZ) or both, at the indicated doses marked below the graphs, at the same time as the intra-articular LPS injection. Data are shown as individual values (symbols) and mean ± SEM (columns) of the 8 h **(A,C)** and 24 h **(B,D)** aggregate area under the curve (AUC) corrected for baseline (% change). *P*-values are shown above each comparison (Mixed-effects model, **(A,C)**; Friedman test, **(B,D)**; Corrected for multiple comparisons by controlling the False Discovery Rate).

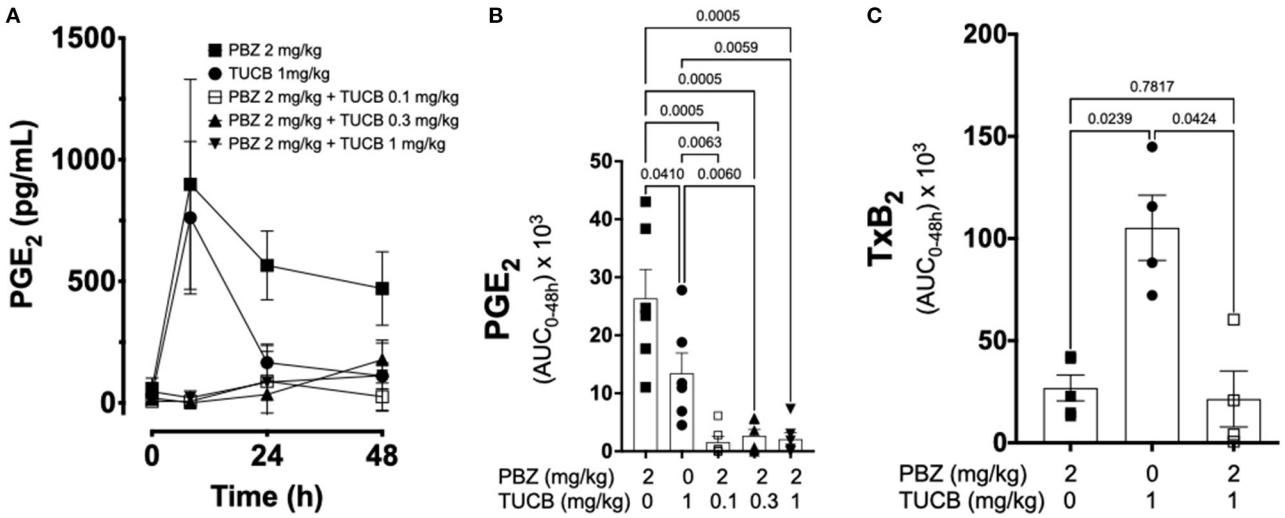

FIGURE 3 | Effect of soluble epoxide hydrolase (sEH) and cyclooxygenases (COX) inhibition on prostanoid response in joint fluid during synovitis. Forty-eight-hour time course **(A)** and aggregate area under curve (AUC) **(B)** for synovial fluid concentrations of prostaglandin (PG) E_2, and thromboxane A_2 AUC **(C)** in an equine synovitis model induced by intra-articular injection of lipopolysaccharide (LPS; 3 μg) into the radiocarpal joint ($n = 6$ horses) as measured using ELISA techniques. Horses were treated intravenously with the sEH inhibitor *trans*-4-{4-[3-(4-Trifluoro-methoxy-phenyl)-ureido]-cyclohexyloxy}-benzoic acid (TUCB), the non-selective COX inhibitor phenylbutazone (PBZ) or both, at the indicated doses marked below the graphs, at the same time as the intra-articular LPS injection. For graph **(A)** data are shown as mean ± SEM. For graphs **(B,C)** data are shown as individual values (symbols) and mean ± SEM (columns), and *p*-values are shown above each respective comparison [Friedman test **(B)**; Mixed-effects model **(C)**; Corrected for multiple comparisons by controlling the False Discovery Rate].

Inhibition of sEH Prevents TNF-α-Mediated Chondrocyte Apoptosis and sEH-Generated Metabolites Cause Chondrocyte Apoptosis

As shown in **Figure 6**, TNF-α-induced apoptosis of primary equine articular chondrocytes was prevented by combined COX and sEH inhibition. This finding did not hold true, however, when the two inhibitors were used at 10-fold their respective IC_{80} in which case the inhibitor combination appeared to potentiate apoptosis. Chondrocytes were also treated separately with COX and sEH inhibitors to determine their individual anti-apoptotic contribution, revealing that COX inhibition with phenylbutazone did not prevent and even appeared to potentiate TNF-α-induced apoptosis at the highest concentration tested. On the contrary, sEH inhibition with *t*-TUCB resulted in a concentration-dependent protection of the chondrocytes from TNF-α-induced apoptosis. Lastly, as shown in **Figure 7**, chondrocytes developed significant apoptosis when incubated with sEH-generated DiHETs but in particular the 8,9- and 14,15-DiHETs.

DISCUSSION

The current study provides the first experimental evidence of the pain and disease-modifying capabilities of sEH and COX inhibitors during inflammation of an articular joint. Known analgesic mechanisms of sEH inhibitors include indirect transcriptional inhibition of COX-2 expression, activation of neurosteroid transcription in the central nervous system (22) and opioid-dependent signaling (55). sEH inhibitors have no direct pharmacologic effect on COX-2 activity (22). As such, combination therapy with COX and sEH inhibitors result in multimodal analgesia that should be more effective than single modality therapy. Consistent with this notion, our results demonstrated that combined COX and sEH inhibition attenuated joint pain and the breakdown of articular cartilage matrix associated with synovitis more effectively than inhibiting COX or sEH separately. However, protection against cytokine-induced chondrocyte apoptosis was not greater with combined COX and sEH inhibition compared to sEH inhibition alone.

We employed objective and unbiased measures of lameness and ground-reaction forces as indirect readouts for synovitis-associated joint pain. Inhibition of both COX and sEH, rather than blocking each enzyme separately, was required for optimum control of synovitis-induced joint pain, and that sEH inhibition alone is less effective in improving joint pain than COX inhibition alone. The significantly lower efficacy of sEH inhibition compared to COX inhibition contrasts with earlier findings in rodent models of inflammatory pain (25), whereas the superior results obtained with combined COX and sEH inhibition is consistent with previous reports in mice (31) and rats (21). The increased production of antinociceptive EETs due to the shift in arachidonic acid carbon flow toward the P450 and sEH pathways caused by COX inhibition (31), which are prevented from degradation by sEH inhibition, likely explain the enhanced analgesia when the activities of both enzymes are blocked. In the absence of sEH inhibition, sEH rapidly converts EETs to DiHETs, inactivating their antinociceptive activities (21, 22, 56). Thus, our findings suggest that sEH inhibition alone

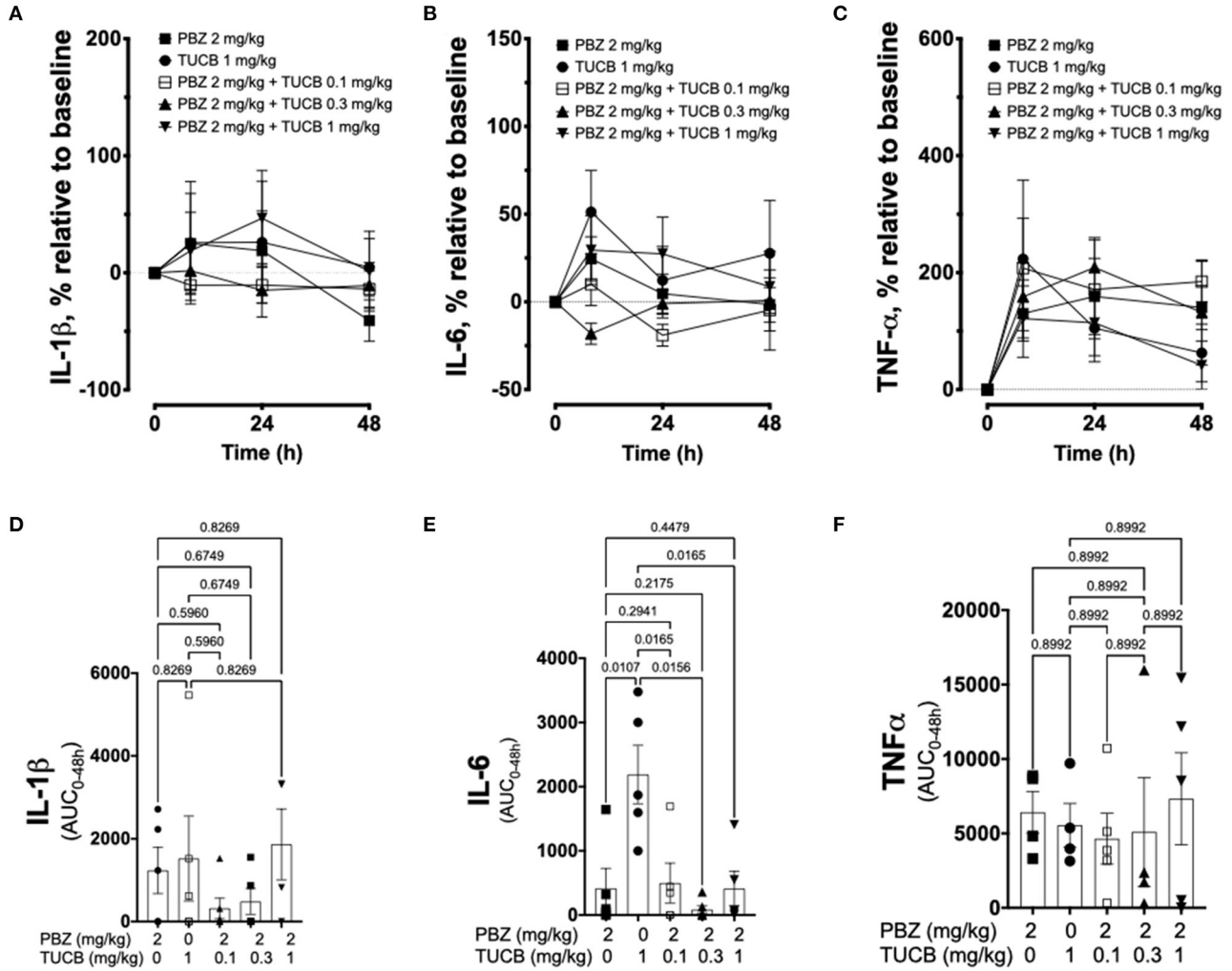

FIGURE 4 | Cytokine response associated with synovitis during inhibition of soluble epoxide hydrolase (sEH) and cyclooxygenases (COX). Forty-eight-hour time course (top row) and aggregate area under curve (AUC, bottom row) for synovial fluid concentrations of interleukin (IL)-1β **(A,D)**, IL-6 **(B,E)**, and tumor necrosis factor (TNF)-α **(C,F)** in an equine synovitis model induced by intra-articular injection of lipopolysaccharide (LPS; 3 μg) into the radiocarpal joint ($n = 6$ horses) as measured using ELISA techniques. Horses were treated intravenously with the sEH inhibitor *trans*-4-{4-[3-(4-Trifluoro-methoxy-phenyl)-ureido]-cyclohexyloxy}-benzoic acid (TUCB), the non-selective COX inhibitor phenylbutazone (PBZ) or both, at the indicated doses marked above (top row) or below (bottom row) the graphs, at the same time as the intra-articular LPS injection. Top row: Data are shown as mean ± SEM. Bottom row: Data are shown as individual values (symbols) and mean ± SEM (columns), and *p*-values are shown above each respective comparison (Mixed-effects model corrected for multiple comparisons by controlling the False Discovery Rate).

does not provide superior relief of synovitis-associated joint pain compared to COX inhibition alone, but pain relief is greatest when both enzymes are inhibited concurrently.

In our study, combined COX and sEH inhibition was significantly more effective in blocking COX-2-generated PGE_2 than COX inhibition alone. While PGE_2 has biologically relevant activities such as increased vascular permeability that facilitate edema formation and leukocyte infiltration that could be important in the setting of joint infections, these are likely not desirable in OA. Furthermore, there is significant correlation between OA and PGE_2-responsive signaling pathways in human articular cartilage (57). These results are consistent with the multimodal effect of the inhibitor combination on COX-2 activity, including direct pharmacologic inhibition by phenylbutazone and transcriptional inhibition by *t*-TUCB (22). Since phenylbutazone is a non-selective COX inhibitor, we also measured COX-1-generated TxB_2 to determine if the COX and sEH inhibitors interacted to affect COX-1 activity. The sEH inhibitor had no significant inhibition of COX-1 as indicated by (i) similar TxB_2 levels between combined COX and sEH inhibition and COX inhibition alone, and (ii) significantly higher TxB_2 during sEH inhibition alone. Taken together, these results demonstrate that sEH inhibition added a significant inhibitory effect on COX-2, but not on COX-1, during synovitis, which should mitigate the activation of PGE_2-responsive signaling pathways in chondroncytes (57).

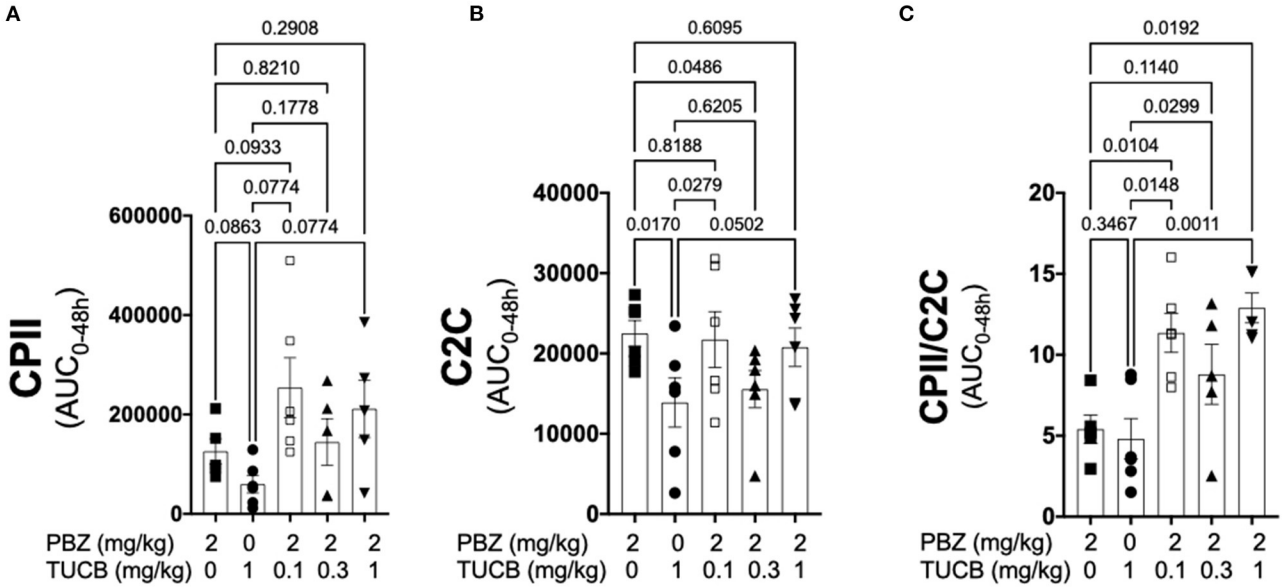

FIGURE 5 | Combined inhibition of soluble epoxide hydrolase (sEH) and cyclooxygenases (COX) during joint inflammation favored collagen synthesis over degradation. Synovial fluid concentrations of the biomarker of type II collagen synthesis CPII **(A)**, type II collagen degradation C2C **(B)** and the synthesis-degradation ratio **(C)** calculated based on 48-h aggregate concentrations (AUC) in synovial fluid from an equine synovitis model induced by intra-articular injection of lipopolysaccharide (LPS; 3 μg) into the radiocarpal joint ($n = 6$ horses) as measured using ELISA techniques. Horses were treated intravenously with the sEH inhibitor *trans*-4-{4-[3-(4-Trifluoro-methoxy-phenyl)-ureido]-cyclohexyloxy}-benzoic acid (TUCB), the non-selective COX inhibitor phenylbutazone (PBZ) or both, at the indicated doses marked below the graphs, at the same time as the intra-articular LPS injection. Data are shown as individual values (symbols) and mean ± SEM (columns), and *p*-values are shown above each respective comparison (Mixed-effects model corrected for multiple comparisons by controlling the False Discovery Rate).

To better understand the effects COX and sEH inhibition on other pro-inflammatory biomarkers relevant to OA, we also determined the synovial fluid concentrations of several cytokines and only found a significant difference with IL-6 during sEH inhibition alone. This result contrasts with prior studies in mice showing that sEH inhibition significantly attenuated serum TNF-α protein during systemic endotoxemia (19) and joint tissue TNF-α and IL-1β messenger RNA levels in whole joint tissues in a model of rheumatoid arthritis (39). It is difficult to reconcile these disparities given the different methodologies and species studied. However, our results are likely more relevant to humans because the equine synovial joint shares greater similarity with human joints than that of mice, both histologically and in terms of biomarkers (58). In adddition, we determined cytokine protein levels in synovial fluid, which is an important compartment due to its direct contact with the chondrocytes that are lost during OA. An intriguing result was the significant increase in IL-6 concentration during sEH inhibition alone. The IL-6 biology is complex and may involve both pro- and anti-inflammatory activities, the degree of which depend on the magnitude of IL-6-*trans*-signaling *via* its soluble receptor (sIL-6R) vs. "classical" signaling *via* its membrane bound receptor (mIL-6R) (59). Investigating the reasons for the increased IL-6 concentrations during sEH inhibition was beyond the scope of our study, but it may represent a response to increased levels of gp130, the transducer molecule for soluble sIL-6R and mIL-6R. Increased gp130 binds and inactivates the IL-6/soluble IL-6R complex as well as insulate its signaling molecules, driving the concentration of IL-6 to elevate sufficiently for signaling to occur (59). A decrease in IL-6 signaling protects against cartilage matrix damage and OA development (54). Further, the increased IL-6 levels within the joint may explain the lower antinociceptive efficacy of sEH inhibition alone compared to the other treatments because IL-6 is involved in peripheral nociceptor sensitization and pathological pain (60).

We also sought to determine whether or not COX and/or sEH inhibition could protect the articular cartilage matrix during synovitis because (i) type II collagen degradation by matrix metalloproteinases (MMP) correlates with symptomatic radiographic and pre-radiographic OA (61), (ii) cytokines that increased during synovitis in our study (e.g., IL-1β, TNF-α) and toll-like receptor ligands (e.g., LPS) are known to induce MMP expression (62), and (iii) sEH activity has been linked to OA prevalence and progression (18). Inhibition of sEH alone significantly suppressed type II collagen degradation (C2C) compared to inhibition of COX alone, although the resulting synthesis-degradation balance was similar to COX inhibition because it also slightly attenuated collagen synthesis (CPII). Since sEH inhibitors are known to block activation of NF-κB (63), a transcription factor involved in MMP expression (62), the observed significant decrease in collagen degradation suggests that sEH inhibitors might decrease MMP activity. Most importantly, combined COX and sEH inhibition significantly favored synthesis over degradation compared to inhibiting

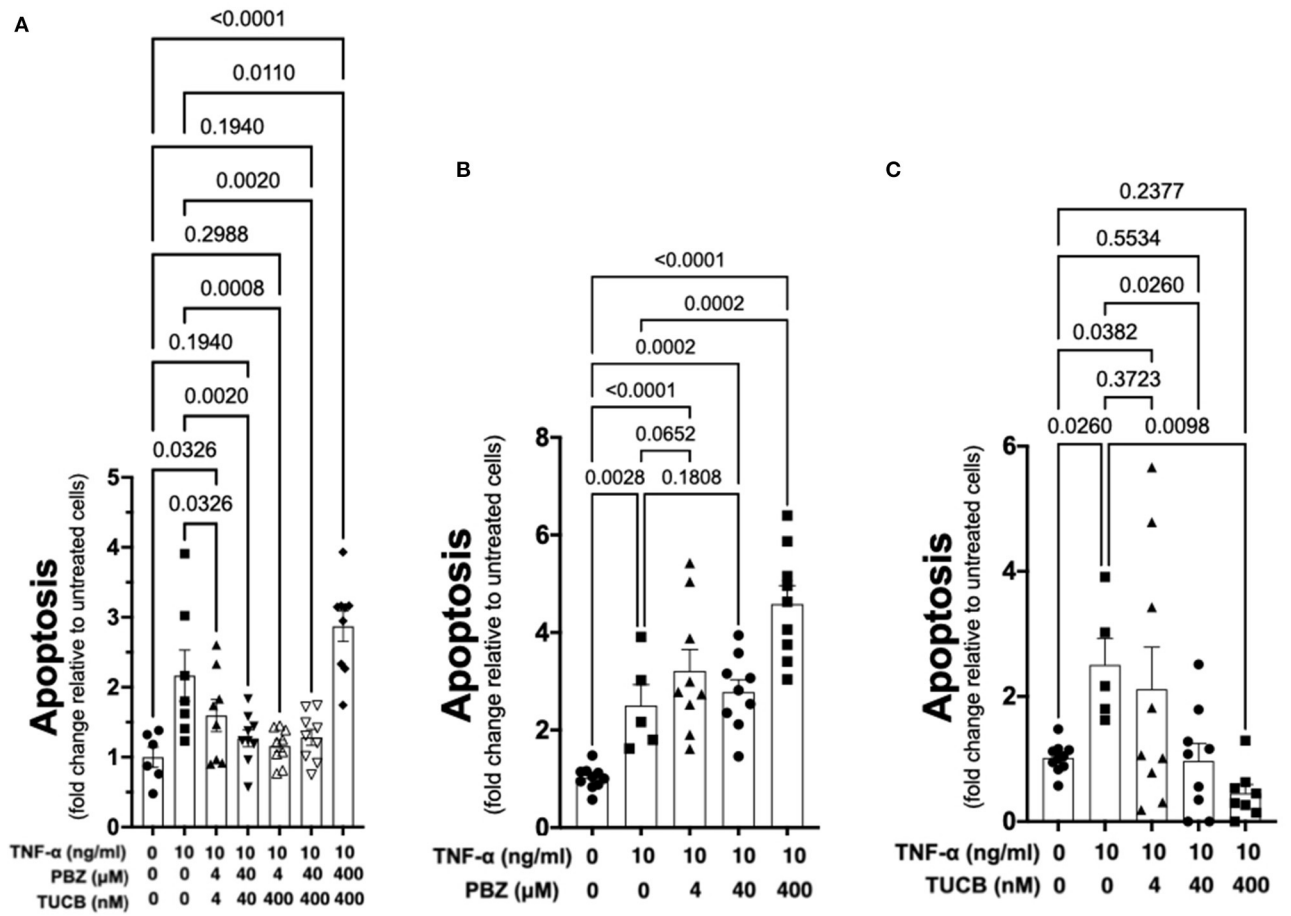

FIGURE 6 | Soluble epoxide hydrolase (sEH) inhibition prevents cytokine-induced chondrocyte apoptosis. Primary equine articular chondrocytes were incubated with TNF-α (10 ng/ml) for 24 h along with several concentrations of the cyclooxygenase (COX) inhibitor phenylbutazone (PBZ) combined with the sEH inhibitor *trans*-4-{4-[3-(4-Trifluoro- methoxy-phenyl)-ureido]-cyclohexyloxy}-benzoic acid (TUCB) **(A)**, with PBZ alone **(B)** or with TUCB alone **(C)**. PBZ and TUCB were used at their approximate half-maximal inhibitory concentration or IC_{50} (4), IC_{80} (40), and 10-fold > IC_{80} (400) for the equine enzymes. Negative controls were treated with 0.9% saline (vehicle diluent for TNF-α) or ethanol (vehicle diluent for PBZ and TUCB; 0.0001% final concentration in culture medium). Apoptosis was determined using ELISA technique as per the manufacturer's instructions. Concentrations are listed below each graph. Data are shown as individual values (symbols) and mean ± SEM (columns), and *p*-values are shown above each comparison (One-way ANOVA corrected for multiple comparisons by controlling the False Discovery Rate).

COX or sEH alone. Since the CPII-C2C ratio is indicative of progression/non-progression of OA (64), a therapeutic approach with combined COX and sEH inhibition has the potential to mitigate degradation of articular cartilage matrix and slow or prevent OA progression.

The medical treatment of OA focuses primarily on alleviating pain symptoms, and no currently available therapy also targets the joint pathology and progressive cartilage damage. In our study, targeting sEH in addition to COX during synovitis and joint inflammation resulted in significantly better pain control, lower synovial fluid concentration of PGE_2 and improved collagen synthesis-degradation balance. These are important findings because PGE_2 is pro-nociceptive, activating PGE_2-responsive signaling pathways in chondrocytes that sensitizes them to apoptosis (57, 65, 66), and destruction of the articular cartilage matrix also predisposes chondrocytes to apoptosis in response to cytokines such as TNF-α (67). Since TNF-α is strongly associated with cartilage loss in OA (7, 52) and was poorly controlled with COX and sEH inhibition in our synovitis model, we tested the effects of COX and sEH inhibition on TNF-α-induced apoptosis of primary equine articular chondrocytes. The combined inhibition of COX and sEH had a significant anti-apoptotic effect, which was confered by the sEH inhibitor since it displayed concentration-dependent anti-apoptotic effect whereas the COX inhibitor either did not prevent or even increased chondrocyte apoptosis. Further, the sEH-generated DiHETs caused significant chondrocyte apoptosis, consistent with their previously reported association with knee OA in older adults (18). An increased DiHET production during COX inhibition due to the higher arachidonic acid carbon flow toward the P450 and sEH pathways (31) might explain apoptosis by COX inhibitors (16). Taken together with the findings of Valdes and colleagues linking sEH metabolites and OA (18), we suggest that blocking the conversion of EETs to DiHETs with sEH

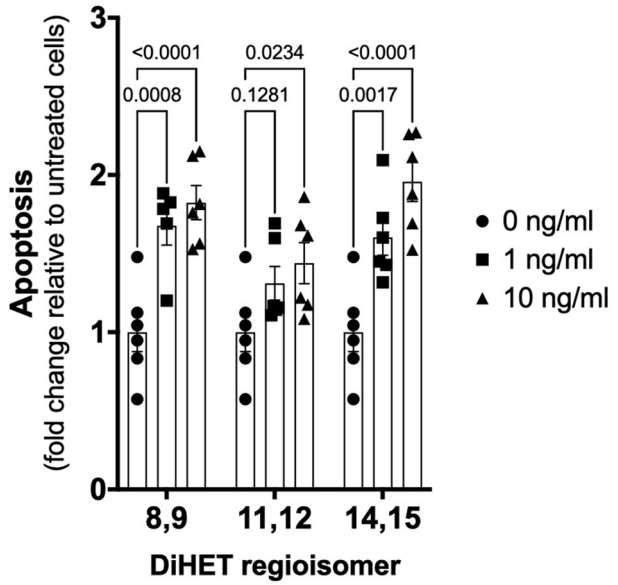

FIGURE 7 | Soluble epoxide hydrolase (sEH)-generated metabolites from arachidonic acid cause chondrocyte apoptosis. Primary equine articular chondrocytes were incubated with the sEH-generated dihydroxy-eicosatrienoic acid (DiHETs) 8,9, 11,12, and 14,15 regioisomers for 24 h at the indicated concentrations. Controls (0 ng/ml) were treated with vehicle diluent (ethanol; 0.0001% final concentration in culture medium). Apoptosis was determined using ELISA technique as per the manufacturer's instructions. Data are shown as individual values (symbols) and mean ± SEM (columns), and *p*-values are shown above each comparison (Two-way ANOVA corrected for multiple comparisons by controlling the False Discovery Rate).

inhibitors will prevent chondrocyte death in naturally-occuring OA, mitigating cartilage damage. Such therapy would represent a significant advance in the medical care of OA.

Our study has several possible limitations worth considering. The majority of our horses were castrated males and only one was a sexually intact female, thus the study was not balanced by sex or gonadal status. While there has been no study examining gonadal status or sex predilection for OA in horses, the condition tends to be more prevalent in older women than in men and is attributed to changes in sex hormones associated with menopause (68). The reproductive cycle of female horses can be influenced by photoperiod (69), a notable difference from that of human females. Testosterone levels in our male castrated horses were presumably lower than if they were gonadally intact. Higher testosterone levels were associated with less pain in severe knee OA in men and women and less disability in women (70). Our sample size was relatively small and the LPS-induced synovitis model used produces a transient but moderate to severe inflammation and joint pain (33). This is unlikely to be the type/degree of synovitis encountered in many naturally-occurring OA cases, and thus our results might represent the more extreme forms of OA. On the other hand, the LPS-induced synovitis model is relevant because systemic and local concentrations of LPS have been associated with macrophage activation in the knee joint capsule and synovium as well as with the severity of structural abnormalities and symptoms of knee OA in humans (71). Finally, the treatment sequence was not modeled in the statistical analyzes to control for a possible increased permeability of the microvascular endothelium in the more frequently injected joints. However, this is unlikely to be an important factor based on our previous experience using a similar experimental design in this synovitis model (33), which ensures that the same joint is injected no >60 days apart allow the synovial fluid protein levels and leukocyte counts to return to pre-injection values even after multiple injections.

In summary, our study provides multiple lines of evidence suggesting that combined COX and sEH inhibition provides an effective mode of therapy in this large animal model. Our findings indicate that this therapeutic strategy would enhance symptomatic pain control and attenuate loss of articular cartilage matrix and apoptotic death of chondrocytes, ultimately resulting in reduced cartilage damage and disease progression.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by University of Minnesota Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

AG and TT carried out the analysis and interpretation of the data. LT, DG, ED, CB, EW-H, and AG collected and assembled the data. TT and AG drafted the manuscript. LT, TT, DG, ED, CB, and EW-H revised the article for important intellectual content. TT and AG obtained funding for conceived and designed the experiments. All authors provided final approval of the article prior to submission.

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