Effects of Three Corticosteroids on Equine Articular Cocultures In Vitro

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Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Master of Science
In
Biomedical and Veterinary Sciences

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April 27th, 2018
Blacksburg, VA

Keywords: osteoarthritis, corticosteroid, osteochondral explant, isoflupredone acetate

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Academic Abstract

The objective was to compare the effects of three corticosteroids at various equimolar concentrations on equine articular explant co-cultures in an inflammatory environment. Synovial and osteochondral explant co-cultures from 6 equine cadavers were exposed to IL-1β (10 ng/mL) and various concentrations (10^{-4}, 10^{-7}, or 10^{-10} M) of MPA, TA, IPA. Concentrations of PGE_2, MMP-13, LDH, and GAG in media were determined at 48 and 96 hours. Results indicated wells with low concentrations of MPA (10^{-7} and 10^{-10} M at 48 and 96 hours), TA (10^{-7} M at 48 hours and 10^{-7} and 10^{-10} M at 48 and 96 hours), and IPA (10^{-10} M at 48 hours) had significantly less PGE_2 than positive control samples. Groups with low concentrations (10^{-7} and 10^{-10} M) of MPA and TA had significantly less PGE_2 than the highest concentration (10^{-4} M) at 48 hours. Significantly less MMP-13 was detected for all concentrations of MPA, TA, and IPA at 96 hours. The LDH assay results indicated cytotoxicity only for samples treated with IPA at 10^{-4} M at 48 and 96 hours. GAG was significantly lower for samples treated with TA 10^{-7} M at 48 hours and MPA 10^{-10} M at 96 hours versus positive controls. These findings suggest corticosteroids at low concentrations mitigated the inflammatory and catabolic effects of IL-1β to a greater extent than high concentrations. Effects of IPA and MPA were similar to TA at clinically relevant low equimolar concentrations.

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Public Abstract

Recent data suggest that isoflupredone acetate is commonly administered intra-articularly for treatment of joint disease (osteoarthritis) in horses. Although much data has been published regarding effects of other corticosteroids on cartilage, to our knowledge there have been no similar studies of the effects of isoflupredone acetate. With increased scrutiny from the general public and more stringent control of medication use by regulatory agencies, determination of information regarding such common intra-articular therapies is imperative. In addition, prior studies have only evaluated the effects of corticosteroids on cartilage, whereas other joint tissues (subchondral bone and synovium) have been shown to be important to the biological responses of joints. Therefore, to more closely simulate the natural joint environment, this study was conducted with a co-culture model incorporating synovial tissue, articular cartilage, and subchondral bone within an inflammatory environment (via stimulation with interleukin-1β). The effects of various concentrations of methylprednisolone acetate, triamcinolone acetonide, and isoflupredone acetate on joint tissues were determined via measurement of selected biomarkers. This study provided the first data regarding biological effects of IPA on joint tissues of horses, and the first comparison of such effects with those of other corticosteroids commonly used intra-articularly for the treatment of joint disease in horses.

Dedication

My entire career and particularly this work is dedicated to my “granny” (Mary Vernice Leger Trahan). Devotion to faith and family was never more perfectly discernible.

In loving Memory.

Acknowledgements

There are innumerable persons that actually deserve acknowledgement for my progression in life and career to this point. First and foremost, my family has kindly accepted my geographical distance no matter where this profession has led me across the continent. For that, I am so grateful for my mom (Pamela Trahan) and brother (Ryan Trahan and his entire little family). To my family here in Blacksburg (Bridgette, Jay and Jacy), y'all are my world and I wish I could have back all the days spent outside of our first real home. The patience, skillset and advice provided me by Dr. Chris Byron throughout my entire surgical residency and beyond has baffled me. Not even the interest compounding on the debt of gratitude I owe him will ever be repayable. Dr. Linda Dahlgren and Dr. Scott Pleasant have also been instrumental in molding me into the person I am today and their efforts require recognition. The laboratory portion of this thesis would not have been possible without the counsel and assistance of three colleagues (Kristel Fuhrman, [soon to be Dr.] Anne Nichols, and Dr. Bruno Menarim.

Thank you all for everything!

Attributions

The research and writing associated with each manuscript (Chapters 3 and 4) would not have been possible without aid from colleagues. Contributions of each coauthor is defined below.

Christopher R. Byron, DVM, MS, DACVS is an Associate Professor of Large Animal Clinical Sciences at the Virginia-Maryland College of Veterinary Medicine. Dr. Byron was integral to the design and logistics of both manuscripts, supervised data acquisition and interpretation, and contributed immensely to manuscript preparation.

Linda A. Dahlgren, DVM, PhD, DACVS is an Associate Professor of Large Animal Clinical Sciences at the Virginia-Maryland College of Veterinary Medicine. Dr. Dahlgren kindly provided laboratory space for experimentation, helped with data interpretation, and contributed to manuscript and thesis defense preparation.

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Stephen R. Werre, PhD a Research Assistant Professor of Veterinary Medicine Experimental Statistics at the Virginia-Maryland College of Veterinary Medicine. Dr. Werre helped performed statistical analysis on all study data and was critical to data interpretation.

Table of Contents

Academic Abstract ii

Public Abstract iii

Dedication iv

Acknowledgements v

Attributions vi

Table of Contents vii

List of Figures x

List of Abbreviations xi

Chapter 1: Thesis Organization 1

Chapter 2: Literature Review 2

Joint Anatomy and Physiology 2

General 2

Arthrogenesis 3

Articular Cartilage 4

Subchondral Bone 10

Joint Capsule/Synovial Membrane 11

Associated Structures 12

Physiology/Biomechanics/Homeostasis 13

Equine Osteoarthritis 16

Definition 16

Osteoarthritis Classification 16

Prevalence 17

Pathophysiology 18
Diagnosis 22
Clinical Signs 22
Radiography 22
Magnetic Resonance Imaging 23
Computed Tomography 23
Arthroscopy 23
Ultrasonography 24
Nuclear Scintigraphy 24
Synovial Fluid Content 25
Biomarkers 25
Histopathologic Assessment 27
Treatment 27
Medical 27
Surgical 32
Use of Intra-articular Corticosteroids for Osteoarthritis 38
General 38
Mechanism of Action 39
In Vivo/In Vitro Models 40
References 44
Chapter 3: Comparison of the Effects of Interleukin-1 on Equine Articular Cartilage Explants and Cocultures of Osteochondral and Synovial Explants 82
Introduction 82

Materials and Methods 85

Results 91

Discussion 101

Footnotes 108

References 109

Chapter 4: Effects of Three Corticosteroids on Equine Articular Cocultures In Vitro 116

Introduction 116

Materials and Methods 119

Results 125

Discussion 131

Footnotes 135

References 136

Chapter 5: Final Comments 142

List of Figures

Figure 2.1: Schematic representation of Synovial Joint 3

Figure 2.2: Articular collagen Orientation 6

Figure 2.3: Osteoarthritis Cytokine Cascade 20

Figure 3.1: PGE_2 Concentration in Media 95

Figure 3.2: TNF-α Concentration in Media 96

Figure 3.3: MMP-13 Concentration in Media 97

Figure 3.4: DMMB Concentration in Media 98

Figure 3.5: BAP Concentration in Media 99

Figure 3.6: LDH Concentration in Media 100

Figure 4.1: In Vitro Well Template 121

Figure 4.2: PGE_2 Concentration in Media 127

Figure 4.3: MMP13 Concentration in Media 128

Figure 4.4: LDH Concentration in Media 129

Figure 4.5: GAG Concentration in Media 130

List of Abbreviations

ACS - autologous conditioned serum

ADAMTS - a disintegrin and metalloproteinase with thrombospondin motifs

ASU - avocado soy unsoponofiables

BAP - bone specific alkaline phosphate

BMSC - bone marrow mesenchymal stem cells

COMP - cartilage oligometric matrix protein

COX - cyclooxygenase

DMMB - dimethylmethylene blue

ESWT - extracorporeal shockwave therapy

GAG - glycosaminoglycan

HA - hyaluronan

IGF – insulin-like growth factor

IPA - isoflupredone acetate

IRAP - interleukin-1 receptor antagonist protein

LDH - lactate dehydrogenase

MMP - matrix metalloproteinase

MPA - methylprednisolone acetate

NO - nitrous oxide

NSAID - nonsteroidal anti-inflammatory drugs

OA - osteoarthritis

OCS - osteochondral + synovial explant

PGE_2 - prostaglandin E_2

PRP - platelet right plasma

PSGAG - polysulfated glycosaminoglycans

TA - triamcinolone acetonide

TGF-β - transforming growth factor-beta

TIMP – tissue inhibitor of metalloproteinase

TNF-α - tumor necrosis factor-alpha

Chapter 1: Thesis Organization

This thesis is compiled and formatted to provide a framework regarding knowledge associated with the anatomic relationship and physiology of intra-articular structures. Following this principle material is a characterization of the clinical management of OA as it applies to the equine patient. A more protracted and specific review of corticosteroid use follows as it is the focus of our laboratory research. Two manuscripts accepted for journal publication (Frontiers in Veterinary Science [published September 20th, 2017] and American Journal of Veterinary Research [in press]) accompany this literature review. The first manuscript was compiled by a joint effort from this author's mentor (CR Byron) and himself (RA Trahan) as a method of comparison of classical (articular cartilage explants) versus novel (osteochondral and synovial explants) in vitro models of articular joint research. The second manuscript documents the responses of various corticosteroids on this novel (osteochondral and synovial explant) model in an inflammatory environment (IL-1 stimulation).

Chapter 2: Literature Review

Joint Anatomy and Physiology

General

Nearly all joints of the equine limb are classified structurally as synovial joints (Figure 2.1), as opposed to cartilaginous or fibrous joints. Least mobile is the fibrous joint, as the dense connective tissue lends stability. An example of a fibrous joint is sutures (synarthroses) of the skull. Slightly more mobile than fibrous joints are the cartilaginous type (amphiarthrosis), and these are found along the midline such as the sternum and pelvic symphysis as well as the physis of immature animals. Synovial (or diarthrodial) articulations function by allowing energy to be transferred and permit mobility from as simple as planar movement to complex compound hinge action, and thus allow locomotion. The simplest anatomic differentiation of joints is based on the gross surface interface. Synovial joints are located at the termination of long bones and are comprised of subchondral bone and articular cartilage contiguous with a thin synovial membrane. Under normal conditions, this cavity is filled with a small amount of synovial fluid and stabilized by a variety of intra/extraarticular structures including tendinous or ligamentous structures, fibrous joint capsule, or menisci.[1,2]

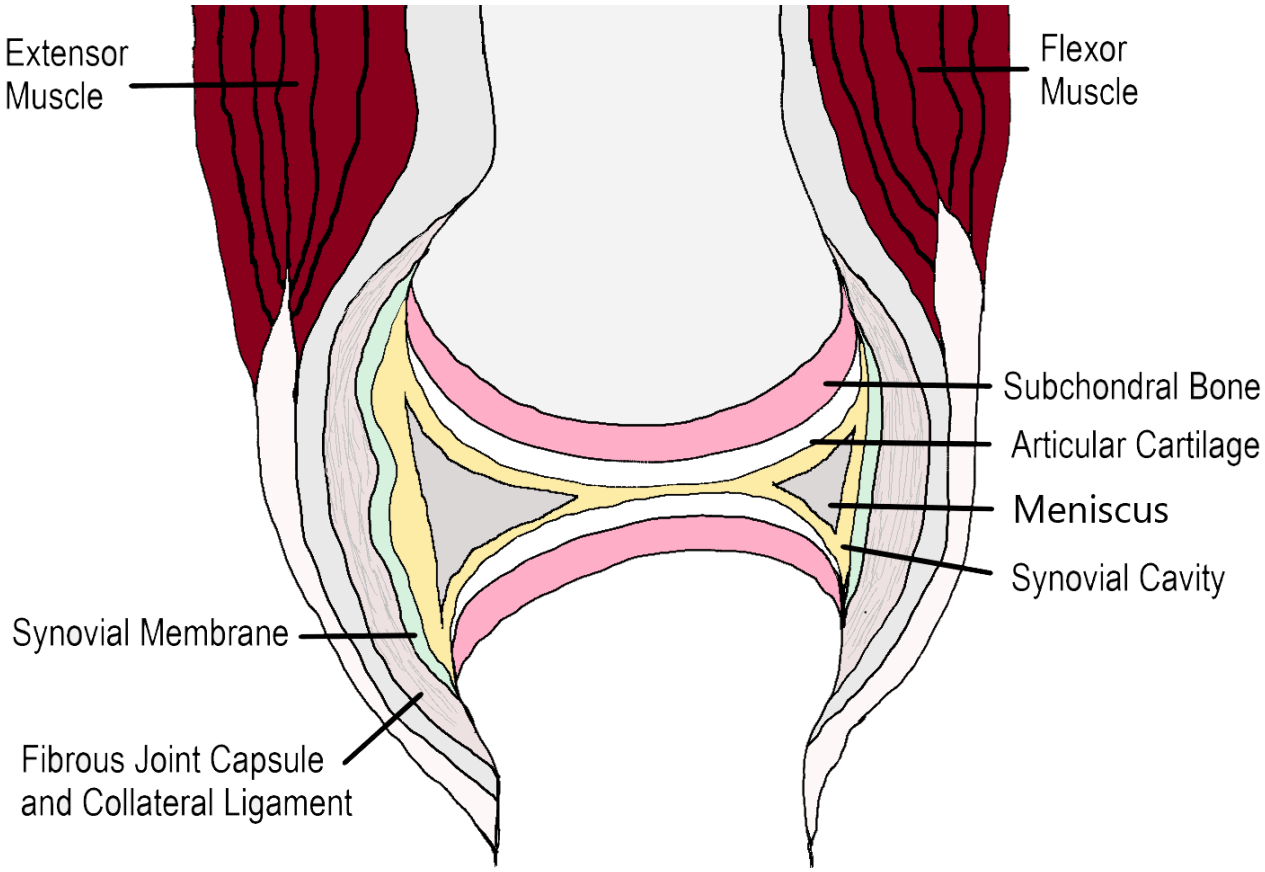

Figure 2.1: Schematic lateral representation of a synovial joint and associated structures.

Arthrogenesis

Development of fetal long bones originates as osteoprogenitors in the mesoderm, which creates primordial cartilaginous representations of individual adult bones. Each cartilaginous model retains an encapsulation of mesoderm (termed perichondrium, later to form periosteum) as well as thickened layers of mesoderm (termed interzone) at bony junctions. This interzone is later to form synovial joints. In horses, the interzone has fully formed by day 40 of embryologic life.[3] Genetic expression of differentiation factors within interzone cells allows for phenotypic appearance of synovial tissues.[4] Experimental studies in an avian model demonstrated that the embryologic outcome of

removal of the "elbow" interzone resulted in absence of an elbow joint and fusion of the humerus, radius and ulna.[5] The articular cartilage scaffold grows lengthwise due to interstitial growth, which occurs via cellular division and deposition of extracellular matrix. Cartilaginous scaffolds are transformed into bones subsequent to endochondral ossification. During this complex process, a primary ossification center forms at the center of the diaphysis and expands in all directions. As this is occurring, the perichondrium also differentiates into periosteum to provide an external "bone collar" to be further molded into the diaphyseal cortical bone. Osteoblasts are specialized mesenchymal stem cells that are utilized for formation of new bone units (osteon). As this primary center of ossification is expanding from the center of the bone, secondary ossification centers at the proximal and distal margins (epiphysis) of long bones are also created. The separation of these ossification centers creates a hyaline cartilage physis ("growth plate"). As the secondary ossification center expands, cancellous ("spongy") bone fills the region between the physis and the thin remnant of the hyaline cartilage model at the epiphyseal margin.

Articular Cartilage

Hyaline cartilage lines the superficial surface (articular margin) of bone within a synovial cavity and is critical to friction reduction, mobility and shock absorption. The articular cartilage has historically been the structure evaluated to determine the health of a synovial structure. Devoid of vascularity, as well as nervous and lymphatic supply, articular cartilage is nourished via solute diffusion from synovial fluid.[6] Articular cartilage is composed primarily of extracellular matrix with a minority contribution of

chondrocytes (1-12% of volume). Water is the major contributor to extracellular matrix (70% volume in adults, 80% in immature animals). When dry matter weight is considered, extracellular matrix is composed of 50% collagen, 35% proteoglycan, 10% glycoprotein, 3% minerals, 1% lipids and 1% miscellaneous substances.[6] Biomechanically, cartilage is viscoelastic and this has been demonstrated in laboratory settings with fluid and creep phases. When static load is applied, there is a rapid initial loss of water (fluid phase) followed by continued compression of collagen and much slower further exudation of water (creep phase). This creep phase will initially reach an equilibrium, which has been estimated to require 4-16 hours of load.[7]

When evaluated microscopically, four contiguous yet distinct layers of articular cartilage are visible (superficial, intermediate, deep, calcified) (Figure 2.2). Although variable at different locations within joints, volume of each includes: superficial zone (10-20%), intermediate zone (40-60%) followed by the deep zone (30%), and calcified layers. These layers are distinguished by the concentration and morphology of chondrocytes and by the collagen orientation. Beginning at the deepest layer, the calcified layer is primarily composed of mineralized cells and is the anchor point of the large diameter collagen fibrils of the Arcades of Benninghoff. This interesting formation was described during the early 20th century and has been confirmed with polarized microscopy techniques.[8] These collagen arcades extend from the calcified layer coursing perpendicular to the tidemark. This orientation continues through the deep layer and begins diverging in the intermediate layer to ultimately reside parallel to the articular surface in the superficial layer. The junction between the calcified and non-calcified cartilage (deep layer) is considered the “tidemark” and is readily visible histologically

and arthroscopically.[9] The deep layer is filled with collagen fibrils and features the first appearance of and largest chondrocytes oriented perpendicular to the joint surface. The intermediate zone can be distinguished by fewer and smaller chondrocytes. Morphologically, they are diversified, as there are populations of round as well as ovoid chondrocyte types. In the superficial zone, collagen and chondrocytes are more densely packed than any of the deeper layers. The collagen fibril and chondrocyte axes are predominantly oriented parallel with the joint surface.

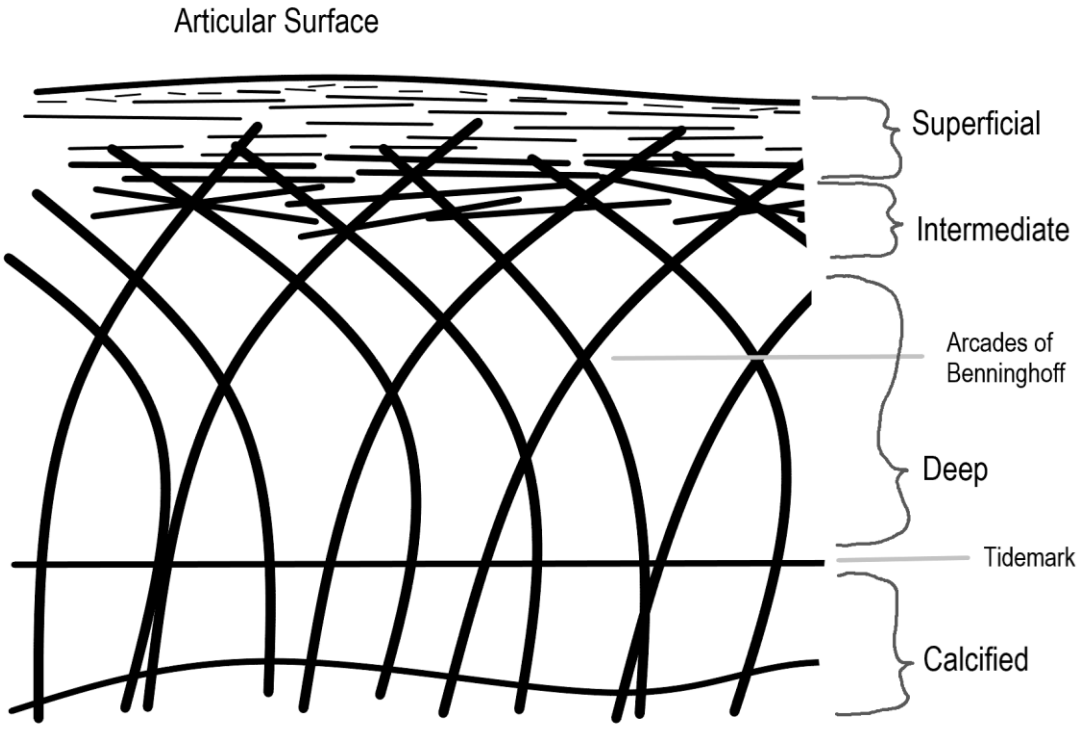

Figure 2.2: Articular collagen orientation.

Chondrocytes constitute only a small percentage of the overall composition of articular cartilage but they are important to synovial homeostasis as they produce the components of extracellular matrix such as collagens, proteoglycans and glycoproteins.

Chondrocytes communicate physiologic and metabolic information through cell-cell contact via cytoplasmic processes.[10] Chondrocytes draw lineage from the mesenchyme and are considered differentiated fibroblasts that are found within extracellular matrix lacunae. Although chondrocytes have been classically described as the only cell type within articular cartilage, recent research in multiple species (including horses) has shown that a limited amount of articular cartilage progenitor cells (ACPCs) are present.[11,12] This cell type has been harvested from the superficial layer of articular cartilage that has healing characteristics attributed to bone marrow derived mesenchymal stem cells; characteristics include delayed senescence, continued chondrogenic potential after in vitro expansion, and lack of association with mineralizing (type X) collagen. These cells may be critical to furthering our understanding of regeneration or repair of damaged articular cartilage and have shown promise as a novel regenerative technique.[13] Injuries to the articular surface present a long-known quandary due to the avascular and aneural nature of this tissue. Limited intrinsic repair mechanisms exist, so many of our therapeutic goals include extrinsic repair methods such as infiltration of mesenchymal cells or extraneous healing factors. Subsistence and solute building blocks provided by synovial fluid are exchanged by gradients created during loading/unloading and it has been calculated that cartilage thickness be confined to 6mm or less for these processes to occur.[14]

As the framework of articular cartilage, the main function attributed to collagens is to resist tensile forces and maintain structure under these forces. Collagens consist of three separate but identical proteins (α_1–chains) wound in right-handed triple helix orientation. These proteins are an aggregation of roughly 1000 amino acids combined

within chondrocytes as procollagen. Terminal amino (N) and carboxy (C) propeptides are cleaved by proteinases once delivered to the appropriate extracellular space and cross-linking is implemented at the amino and carboxy telopeptides.[2] There have been more than two dozen MMPs identified and numbered or identified on the basis of which substrate they were first identified to degrade (collagenases, stromelysins, and gelatinases). MMP-1, -2, -3, -8, -9, and -13 have all been implicated in OA of multiple species. Another enzyme closely related to MMPs that has the capacity to cleave aggrecan is known as "a disintegrin and metalloproteinase" (ADAM). Articular cartilage is predominantly composed of the fibrillar type II collagen (90-95% dry weight)[15] with minor contributions from types VI, IX, XI, XII and XIV.[16,17] Of the 16 described collagens, the fibril forming collagens include types I, II, III, V and XI.[16] Mechanical strength within articular cartilage has been attributed to type II collagen fibril formation. This has been classically compared to the strength gained of concrete after reinforcing with steel rebar. At the superficial aspect of cartilage, increased amounts of smaller diameter collagen (31 nm) oriented parallel with the joint surface are found and may be thought of as similar to armor plating.[2] There are a subset of distinctly sized collagen fibrils located within the superficial layer known as the lamina splendens. These fibrils are firmly attached to the synovium and are associated with marginal transition.[18] Within the intermediate zone, the diameter increases (40-100nm) and the pattern is more randomly oriented. Observation within the deep zone reveals collagen oriented perpendicular to the joint surface with the largest diameter. These fibrils extend past the calcified zone and are anchored within the subchondral bone.

As previously stated, approximately 1/3 of the dry weight of articular cartilage is composed of proteoglycans and the major proteoglycan (85% of dry weight) is aggrecan.[2] This molecule is responsible for resistance of compressive musculoskeletal forces. Aggrecan is constructed as peripheral glycosaminoglycans surrounding a core protein with amino- and carboxy- terminal sides (molecular mass, > 2500 kDa.[19] This core protein has three globular domains (G1, G2, G3). The function of the G2 domain has yet to be determined. The G3 domain is located at the carboxy terminal end and attaches the aggrecan molecule to the extracellular matrix.[20] Located between the G2 and G3 domains are a multitude of glycosaminoglycan sidechains attached to the core protein that make up the majority of the molecules size. Glycosaminoglycans are highly hydrophilic nonbranching polysaccharides, and those utilized for construction of aggrecan consist of chondroitin-4-sulfate (repeating glucoronic acid and n-acetylglucosamine), chondroitin-6-sulfate and keratin sulfate (repeating galactose and n-acetylglucosamine). The hydrophilic and highly viscous nature of these compounds is attributed to the sulfated acetylglucosamine. The interaction between water and these two molecules (chondroitin and keratin sulfate) provide the reserve water that is released when the articular surface is loaded. This also creates a hydrostatic/osmotic pressure gradient later utilized when water is resorbed. As animals mature, more chondroitin-6-sulfate is detected compared to chondroitin-4-sulfate.[2] Single monomers of aggrecan are utilized as aggregates of molecules noncovalently bonded to hyaluronan molecules at the G1 (hyaluronic acid binding region) domain. These aggregates can consist of more than 100 monomers and can equate to a size of 200 million daltons.[21] A smaller subset (5% of dry weight) of proteoglycans are known as nonaggregating proteoglycans and are mainly found within

the superficial zone of articular cartilage and are thought to be associated with cartilage healing.[22] Experiments with enzymatic digestions of either the collagen network or proteoglycans have clarified the functional interactions of each within articular cartilage. Partial digestion of the collagen network leads to loss of the tensile properties of articular cartilage, whereas PG removal reduces the viscous and compressive properties of the tissue.

Subchondral Bone

As an extension of epiphyseal bone, subchondral bone provides substructure shape and support to articular cartilage. The subchondral bone is composed histologically of the same cell types as diaphyseal and metaphyseal bone. Metabolic activity is governed by osteoblast (bone synthesis) and osteoclast (bone resorption) interplay. Osteocytes are another cell type observed within subchondral bone; these are residual osteoblasts entrapped within the osteoid matrix that osteoblasts secreted during the production of osteons. These cells are associated with regulation of nutrient and waste exchange and communicate via cytoplasmic extensions. Mineral (calcium, phosphorus and magnesium) storage is also a function of subchondral bone. As opposed to diaphyseal bone, haversian systems (osteons) run parallel to the joint surface within subchondral bone. This histologic difference is thought to support permissible deformation (up to 10x more deformable than cortical shaft in humans[23]). In contrast to the articular cartilage, subchondral bone has a rich vascular and nervous supply, as well as higher metabolic activity and turnover rate. Subchondral bone depth is typically thickest along the weight bearing surface of joints and reduced in depth at the periphery; this is continuously

modified according to Wolff's law. Plainly stated, if more load is placed on a particular bone or region of bone, then the internal architecture will adapt to resist the loading. Although articular cartilage and subchondral bone are two distinctly different tissues, there is crosstalk between the two tissues and both are affected in pathologic conditions such as OA. There is suggestion that clinical syndromes such as sclerosis of the subchondral bone may actually be a primary cause of OA, rather than a consequence.[24] Inclusion of subchondral bone within in vitro articular models of multiple species (bovine[25] and equine[26]) has been shown to be chondroprotective as determined via histologic and fluid biomarker assessment.

Joint Capsule and Synovial Membrane

The joint capsule is composed mainly of fibrous (type I collagen) tissue and affixes to structures such as collateral ligaments to provide mechanical stiffness while also allowing range of motion. The synovial membrane is integral to the intra-articular environment and anatomic description (intimal and subintimal layers) has been defined by physiologic function. The subintimal layer is composed of loose connective tissue, adipose and fibrous tissue, well differentiated vascular capillary network and nervous supply. The intimal layer is devoid of a basement membrane and consists largely of synoviocytes tasked with one of two functions: 1) phagocytosis (type A) via phagocyte-like cells or 2) synovial fluid content regulation (type B) via fibroblast-like cells. When viewed microscopically, there is a villous appearance that increases surface area a great deal and has been postulated that molecules up to 10kDa can freely diffuse across the synovial membrane.[6]

Synovial fluid is classified as a non-Newtonian thixotropic fluid.[27] This description can be clarified by stating the more motion an individual joint has, the less viscous its joint fluid is. As a plasma ultrafiltrate secretion of the synovial membrane, synovial fluid has three main functions: 1) provide lubrication to the intra-articular environment, 2) provide nutrition to the articular cartilage, and 3) protect the intra-articular environment from microbes or foreign material. These functions are clarified when composition is considered. Proinflammatory cytokines such as TNF-α, eicosanoids (PGE_2), interleukins, MMPs, as well as anti-inflammatory products such as hyaluronan and growth factors are secreted as needed for regulation of articular anabolism and catabolism and may be dysregulated in the face of pathologic conditions. Nonsulfated glycosaminoglycan, hyaluronan, and lubricin are three products secreted by the synovial membrane that contribute to the viscosity of synovial fluid.[28] Although the synovial fluid is considered an immune-privileged site (similar to the central nervous system), a modest population of lymphocytes and macrophages also reside within the synovial fluid but are typically less than 500 cells/μL.[29,30]

Associated Structures

Structures that are relevant to pathology and are critical to the stability of synovial joints include menisci, peri- and intra-articular ligaments (collateral and cruciate) and musculotendonous units. Menisci are crescent shaped fibrocartilages (observed in femorotibial and temporomandibular joints) that provide alignment of incongruent bones and even load distribution within the articular environment. Much like the fibrous joint capsule, periarticular ligaments are composed of a minor component (25% dry weight) of

proteoglycan and the majority (75% dry weight) is type I collagen.[31] These extra-articular structures undergo the same metabolic activity with respect to hypertrophy and atrophy in response to use and disuse.[32]

Physiology, Biomechanics and Homeostasis

Normal values for equine synovial fluid volume have been published for many joints.[33] There is still a great deal of subjectivity as to classifying effusion or joint capsular distention and the regulatory mechanisms associated with intra-articular volume and pressure are poorly documented. In a neutral position, most synovial joints are subatmospheric (-2 to -6 cm H_2O[34]) in pressure, whereas at extreme ambulatory angles pressures may increase to a level similar to that in vehicle tires (30 psi in human knee joints).[2]

Articular mechanics can be described in relation to three important components: kinematics, kinetics and lubrication. Kinematics refers to the interaction of the articulating surfaces in relation to each other. Kinetics relies on kinematics and is characterized as the forces and loads created during motion. Mechanical motion (kinematics) within joints is most commonly translational or sliding (combination of translational and rolling) and is particularly related to the appositional geometry. To determine kinetics one must also account for extra-articular forces, including body weight and muscular/tendinous pull. Analysis of kinetics can become quite complex and laboratory modelling is a focus of human and animal research.

As with any appositional surface, lubrication is important for friction reduction and prevention of damage. Lubrication within the articular environments occurs in two ways: boundary and fluid-film. Boundary lubrication is found at the gliding surface, is most useful within constructs with little load, and is most simply defined as any liquid or semi-liquid substance separating two solid materials (such as with piston rings in a combustion engine). Predominant boundary lubricants of distinct structures within the articular environment include hyaluranon (at the synovial membrane) and hyaluranon and lubricin (at the articular cartilage).[35,36] Boundary lubrication is not believed to be independently effective at providing friction-free articular surfaces under natural loads,[14] thus fluid-film models are thought to be the primary model at work. Although boundary lubrication may not be the predominant technique, decreased levels of these substances are observed in pathology.[37,38]

Although several models of fluid-film lubrication exist (squeeze film, hydrostatic, hydrodynamic, and elastohydrodynamic), the elastohydrodynamic example is most widely accepted as the most representative for the articular environment.[2] This method of lubrication consists of a combination of the squeeze film and hydrodynamic models. To describe this model, we must keep in mind that the articular cartilage is porous as well as conformable, and water is attracted to aggrecan molecules within the cartilage. When a joint is loaded (i.e. force is applied), water is squeezed from within the cartilage to between the articular surfaces. If a nonparallel force is then applied, the water then becomes a wedge-like leading edge toward the “front”, and at the trailing “back” edge the water is resorbed.[14] Hydrodynamic lubrication alone requires at least one surface that is non-conformable. Squeeze film lubrication is only applicable to non-conformable models

with no motion between surfaces (although there is load) where the fluid may be completely squeezed out from between surfaces. The hydrostatic model is similar to squeeze film except fluid is restricted from escaping from between the surfaces. These models are all likely found within the body but are restricted by location (anatomic configuration within and biomechanical forces placed upon individual joints).

Joints should be considered a composite organ not dissimilar to the liver, kidney, or heart, requiring separate but interrelated components with widely varied metabolic potential.[39,40] To function appropriately, synovial structures vary from anabolic and catabolic states and malfunction of one component will likely have deleterious effects on the other structures.

Equine Osteoarthritis

Definition

OA has classically been associated with articular cartilage loss or degradation, but there is much interplay between intra- and periarticular tissues, and the reduced functionality of these tissues contribute to the pathologic state. In 2011, the Osteoarthritis Research Society International reached a consensus statement regarding the definition of OA, which included the progressive nature of the disease that is associated with "failed repair of joint damage". Ultimately, the clinical signs of pain, stiffness and loss of function may result from a flaw in any of the previously described intra- and extra-articular structures (including synovial membrane, fibrous joint capsule, subchondral bone and ligamentous structures)[41] and may be confined to single, multiple, or generalized joint involvement. A litany of biomechanical, biochemical and genetic factors have been implicated in the original injury or lack of repair function. Treatment of OA should be devised on an individual basis and based on the etiology of that individual patient.[42] OA was initially defined for equine patients as primary ("degenerative joint disease", unidentified cause) or secondary (known etiology or previous traumatic event).[43]

Osteoarthritis Classification

In 1988, McIlwraith and Vachon described 5 clinical forms of equine OA.[44] These groups were reclassified by McIlwraith in 1996 to include three subtypes: Type 1 (primary) - observed commonly in the carpus, fetlock, distal tarsus and distal

interphalangeal joints; Type 2 – secondary to and associated with identified injury or previous damage such as a fracture, septic joint or developmental orthopedic disease; Type 3 – incidental or non-progressive articular cartilage damage.[45]

Prevalence

OA is recognized as the most common musculoskeletal abnormality of a multitude of animal species, including humans but obtaining accurate prevalence data is difficult due to a multitude of reasons (differing study populations, inconsistency in definition across species and populations, and selective evaluation of joints). Prevalence of OA in human studies is well documented for particular geographic regions, races and anatomic sites.[46-49] Prevalence is wide-ranging depending on previously described differences but a representative statistic includes 37% of participants >60 years of age having radiographic evidence of knee OA.[50] To better quantify these numbers, in 1995 it was estimated that 21 million people in the United States were clinically affected with OA; that estimate was increased to 27 million by 2008 and is expected to be 67 million by 2030.[50,51] The financial burden attributed to arthritic and rheumatic conditions of Americans amounted to $128 billion dollars annually.[52] Multiple publications on equine operations estimated or quantified that 8-60% of lameness was attributed to joint disease or OA and spans the spectrum of young equine athletes to older horses.[53-59] One third of 50 Thoroughbred racehorses 2 to 3 years of age and less than 60 days into their racing careers had evidence of OA in metacarpophalangeal joints.[60] The U.S. horse population was recently estimated to include 7.3 million animals,[61] leaving millions of potentially affected animals. The USDA-APHIS estimated in 1998 the economic burden due to all

lameness conditions ranged from $678 million to $1 billion due to a combination of veterinary care, treatments, loss of use and death.[62]

Pathophysiology

There are three mechanisms hypothesized for OA: 1) inherently defective cartilage (primary OA); 2) OA secondary to subchondral bone structural changes; and 3) abnormal forces being applied to and causing damage to healthy cartilage.[63-65] An alternate way of considering the pathogenesis of OA is to examine mechanical factors and biologic and molecular mediators. The classical representation (mechanical) of OA is associated with an "overuse" injury as what may be observed frequently with human and equine athletes of all ages. To understand this type of injury we must consider that joint lubrication and proteoglycan production is related to articular loading and above a certain threshold the repetitive loading becomes detrimental to proteoglycan synthesis, alters collagen structure, and leads to water influx within articular cartilage.[66-67] Unfortunately, the overuse threshold has not been quantitated to this point. In the previous scenario, the cartilage is now mechanically weaker, upregulates inflammatory cytokines and proteases likely to further degrade the cartilage and other articular tissues, thus ultimately creating a permanently inferior cartilage. As with all organs, metabolic homeostasis is important. OA may be described as the catabolic pathway overwhelming the anabolic repair mechanisms. Primary defects of collagen have been documented in both human (Ehlers-Danlos syndrome) and equine (hereditary equine regional dermal asthenia) patients.

The major inflammatory cytokines (figure 2.3) associated with equine OA include interleukin-1 (IL-1) and tumor necrosis factor-alpha upregulated by macrophages, synoviocytes and chondrocytes.[68,69] These cytokines produce a multitude of primary and secondary degradative effects, including production of MMPs, prostaglandins, production of type I and III collagens associated with joint capsule fibrosis, stimulation of osteophyte formation, upregulation of osteoblast-like cells, and inhibition of anti-inflammatory molecules. Evidence for IL-1 being a major inflammatory cytokine has been documented by chondroprotective effects associated with the upregulation of IRAP.[70-72] Although TNF-α concentration is elevated in OA, the detrimental effects that causes appear to be less potent than those of IL-1.[73] Prostaglandins such as PGE_2 have been shown to upregulate the pain response.[74] Matrix metalloproteinases are highly destructive and may alter all components of articular matrix. MMPs are generally categorized by which tissues they alter: collagenases, stromelysins and gelatinases.[75] Closely related molecules known as a disintegrin and metalloproteinases (ADAMs, also known as aggrecanases) are also expressed by chondrocytes and cleave components of aggrecan and may play an integral part in OA.[76-78] ADAMs appear to be similar enough to MMPs that they both are inhibited by TIMP-1[79,80] produced locally within chondrocytes. Alteration of a 1:1 ratio of TIMP:MMP has been indicative of matrix degradation progression.[81,82] Byproducts of nitric oxide synthesis have been observed in elevated quantity in both rheumatic diseases and OA[83-86] and inhibition of this mediator has reduced the severity of lesions in animal models of OA.[87-90] Additional anti-inflammatory or inhibitory cytokines include IL-4, IL-10, IL-13, insulin-like growth factor and fibroblast growth factor.

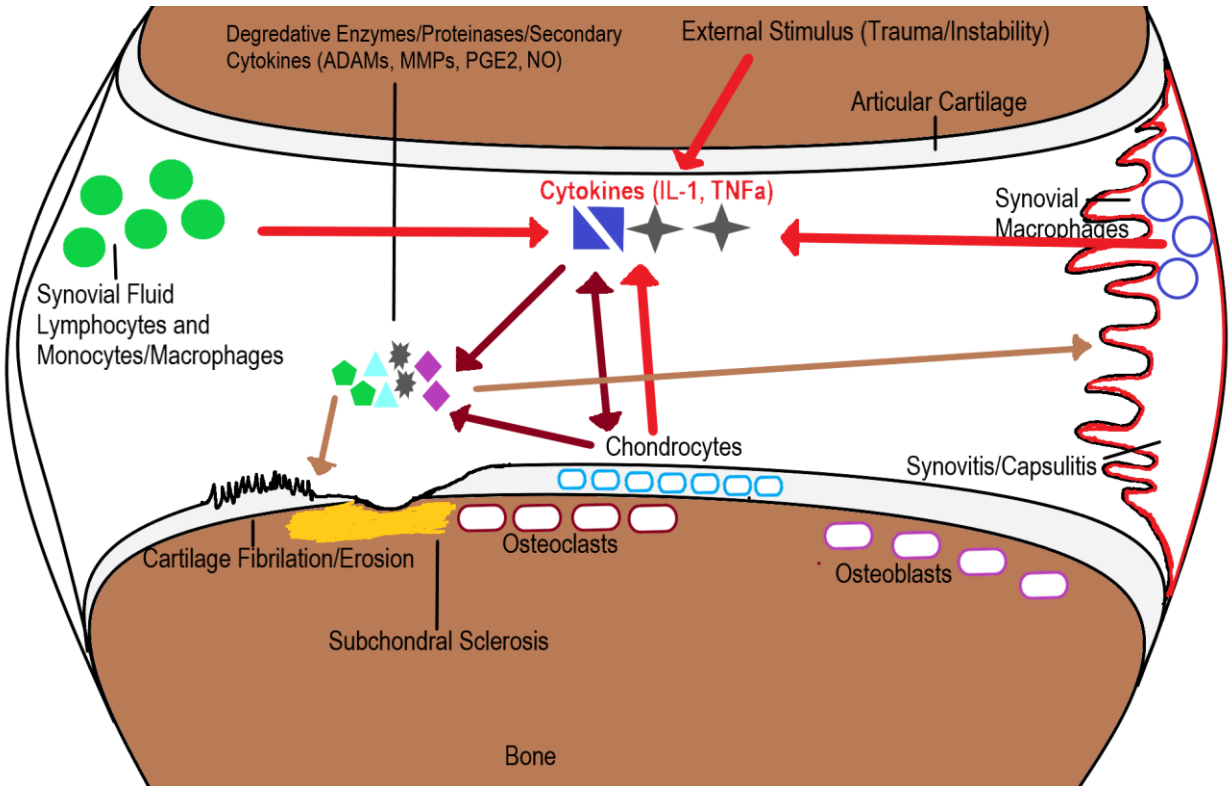

Figure 2.3: Simplified OA cytokine cascade. Thick red arrows indicate proposed initiators and resultant primary cytokines. Thinner maroon arrows indicate secondary pathways to degredative cytokines. Thinnest brown arrows indicate resultant damage.

Pathologic changes within subchondral bone that have been associated with OA in human and equine patients include bony remodeling such as sclerosis. As a part of the mechanical overload theory of OA, subchondral bone sclerosis develops for added strength, but diminished shock absorption is observed in stiffened or sclerotic bone. When athletic function is considered, high-intensity training has been shown to increase the density of and sclerosis in subchondral bone.[57,91-93] Sclerotic bone has reduced deformation potential and the animal therefore relies on surrounding soft tissues (i.e. articular cartilage, fibrous joint capsule, and collateral ligaments) for load sharing. Sclerotic bone is susceptible to microdamage due to reduced deformation and may

ultimately collapse if necrosis occurs subsequent to ischemia; this may eventually lead to pathologic fractures.[94-95]

The homeostatic mechanisms that govern turnover of extracellular matrix in articular cartilage are complex and maintained by mechanical stimuli, chondrocytes and cytokines.[96,97] The collagenases (MMP-1, 8, 13), and particularly MMP-13, received their namesake due to the efficiency of cleaving type II collagen and thus articular cartilage matrix depletion. These collagenases are not only produced by synoviocytes but also locally by chondrocytes.[75,98-101] As a response to matrix degradation, proteoglycan production is upregulated but several studies have shown that the resultant molecules are not of the same biomechanical quality or quantity.[102,103] Collagen crosslinking failure and articular fibrillation has been observed secondary to sclerotic subchondral bone.[104] Oxygen-derived free radicals are an alternative pathway to the destruction of articular cartilage.[105-108]

Likely underemphasized in most research, the synovial membrane is integral to articular health due to its production of a multitude of both inflammatory and anti-inflammatory mediators. Many researchers have observed that synovium releases significant amounts of previously described substances known to degrade articular cartilage: MMPs, prostaglandins and other cytokines.[109-117] It has yet to be determined which cell type (synoviocyte or chondrocyte) consistently contributes the majority of inflammatory mediators, though both are known as abundant sources. Primary synovitis due to biomechanical damage or secondary synovitis due to other damage such as osteochondral fragmentation alters cytokine production ratios, increases localization and liberation of inflammatory cells and reduces lymphatic drainage of the joint space.

Considering the chronic nature of most OA, capsulitis typically produces reduced range of motion due to laydown of biomechanically inferior fibrous tissue.

Diagnosis

Although there is much variability to patient presentations, a thorough physical examination as well as adjunctive diagnostic testing is utilized for a clinical diagnosis of OA. Physical examination parameters that have been suggestive of OA include lameness, crepitus, synovial effusion and periarticular fibrosis. As part of lameness evaluation, particular attention to range of motion/flexion testing may provide a general indication of location. Intra-articular analgesia may or may not mitigate lameness depending structures involved. Pain associated with periarticular structures and subchondral bone are not likely to be abolished with intra-articular analgesia.[118] Many horses with bilateral lameness (common in OA) will not manifest lameness in the less-affected limb until contralateral limb pain is alleviated.

Post-imaging software manipulation, contrast resolution, and widespread availability and reduced cost of digital radiography contribute to its use as a standard screening tool for detection of osseous changes. A variety of radiological findings are supportive of a diagnosis of OA. These include but are not limited to: asymmetrical joint spaces (indicative of articular cartilage thinning), subchondral radiolucency or radio-opacity, free osteochondral fragments, and periarticular osteophytosis or ankyloses. Although radiography is common practice for detection of OA, there are radiographically

inapparent lesions[119,120] and there is a lack of sensitivity for early or focal lesions as well as lack of agreement with arthroscopic findings.[121-124]

The gold standard for noninvasive anatomic evaluation of all peri- and intra-articular tissues associated with joints is magnetic resonance imaging (MRI). MRI provides a means of direct evaluation of cartilage morphology and volume. There is very good correlation between findings from images obtained with high-field magnets and arthroscopy, as well as, necropsy findings as they relate to metacarpophalangeal joint OA.[118] Evaluation of cartilage loss is both sensitive and reliable as a marker of progression of disease.[123,124] Unfortunately, not all equine patients or anatomic locations fit within MRI tunnels and low-field magnets are unable to evaluate cartilage to the same degree as high-field magnets.[125-131]

Computed tomography has been used to document the physiologic and pathologic changes in young Thoroughbred racehorse bone, tendon and cartilage as they progress through race training.[91] Normal Subchondral bone density and patterns of cartilage degeneration of the equine metacarpal bone have been determined.[59,132,133]

When comparing diagnostic arthroscopic and MRI findings in regards to OA in human femoropatellar joints, the arthroscopic assessment was more precise and reflective of the actual condition than MRI.[134] The authors concluded that "arthroscopy should not be generally replaced by MRI".[134] Arthroscopic visualization allows for direct visualization of synovial fluid and observation of synovial integrity. Experimentally induced synovitis has been serially observed and progresses from marked petechiation and hyperemia of the villi progressing to villi fusion, strands of fibrinoid material, and ultimately adhesions.[135] Unfortunately, synovial biopsy has not provided consistently

reliable results even within a single joint[136,137] and is more appropriate when diagnosing synovial infectious processes. Visualization of fibrocartilagenous menisci and attachment sites has been a difficult endeavor using any modality, but clinically relevant portions are visible arthroscopically.[138,139] There is typically good agreement between radiographic and arthroscopic appearance and severity of subchondral bone disease, though for some locations arthroscopy is superior.[140] Arthroscopy is superior for detection of focal articular lesions.[121] In another study, results of arthroscopy were generally in agreement with radiographs, but arthroscopy was more sensitive for diagnosing hemarthrosis, degenerative joint disease (radiographs were not predictive of severity, arthroscopy superior), synovitis, and ruptured intracarpal ligaments.[124] Although more invasive, arthroscopic assessment of synovial joints of the proximal limb provides visualization of lesions that may not be visible by use of other imaging modalities (such as use of MRI for equine femoropatellar joint imaging).

Ultrasonography is quite valuable for assessing periarticular soft tissue structures, as it was originally utilized to evaluate chronic proliferative synovitis.[141,142] Articular cartilage echogenicity and depth can also be determined to a certain extent and osteophytes or enthesiophytes may also be characterized. Though multiple publications exist describing axial skeletal ultrasonography techniques for therapeutic guidance,[143-148] there is a paucity of literature correlating this imaging modality to others.

The previously described modalities have varying degrees of ability to image anatomic detail but none provides physiologic assessment. Nuclear scintigraphy provides metabolic information, particularly of the subchondral bone. Using animal OA models, focal radiopharmaceutical uptake was most intense within the subchondral bone or

osteochondral junction.[149] Progression of OA in the human femoropatellar joint is well predicted via scintigraphic imaging, which be used to diagnose clinical disease prior to development of radiographic changes.[150-153]

Assessment of synovial fluid content is commonplace and reduced viscosity is often observed in equine OA. Semiquantitative testing is available for determination of hyaluronan content (mucin clot test). This test is simple but sensitivity is lacking. There is also considerable patient variability in hyaluronan content documented in normal and abnormal joints.[154,155] There is significant variation among animals, and cellular counts and total protein content may not differ between osteoarthritic joints and normal joints.

Osteoarthritic biomarkers are a promising area of research aimed at earlier diagnosis (i.e. prior to onset of irreversible articular changes) and serial assessment of disease. Biomarkers are broadly classified as directly (produced only by intra-articular tissues) or indirectly related to catabolic or anabolic process at play in OA. Biomarkers may be quantified from synovial fluid or peripheral blood samples. Biomarkers values are typically elevated in pathology and thought to reach the peripheral bloodstream principally via diffusion across the synovial membrane.

Direct biomarkers are enzymes or products originating typically from articular cartilage or bone cells and are active only in these tissues. Direct articular biomarkers include breakdown products of type II collagen and fragmentation of aggrecan molecules.[156] Carboxypropeptide of type II collagen (CPII) has been measured within the peripheral blood stream at a higher concentration in horses with osteochondral fragmentation.[9] A particular epitope of chondroitin sulfate (CS-846) is detected in increased quantity in the fluid of osteoarthritic joints and to a lesser extent in the

peripheral blood of osteoarthritic patients.[9,157] Quantification of type II collagen fragments is possible and has been useful in predicting OA and osteochondritis dissecans (OCD) in equine, small animal and exotic patients.[158-160] Total GAG content of synovial fluid is pathologically elevated and has been useful at predicting traumatic arthritis and OCD in equine patients[161] and is more accurate than radiographs at predicting severe-acute, moderate-chronic, and severe-chronic joint abnormalities compared to normal joints.[162] In one study, increased serum GAG content was useful to differentiate exercised exercised horses with induced OA from control cohorts.[163] Although keratin sulfate is one proteoglycan associated with aggrecan, detection has not been a reliable predictor of joint disease.[9] Elevated amounts of BAP isoform correlates well with total GAG synovial fluid content and visible joint damage.[164]

Indirect biomarkers are produced by intra-articular tissues when OA is present but may be nonspecific as to their origin. Examples include matrix metalloproteinases, pro- or anti-inflammatory cytokines, proteolytic enzymes and growth factors associated with a multitude of traumatic or inflammatory conditions of the joint. Matrix metalloproteinases are critical to remodeling of articular extracellular matrix and degradation of collagen and proteoglycans. Many matrix metalloproteinases are documented and the most pertinent to equine OA include MMP-13, MMP-9 and MMP-2.[112,165-168] Elevation of the synovial content of the cytokine IL-6 was shown in one study to be most sensitive and specific for joint disease; IL-1β and TNF-α were not as predictable as screening tools for joint disease.[169] In this same study, elevation of the eicosanoid PGE_2 was also an excellent marker of joint disease. Elevation in synovial fluid hyaluronic acid concentration has been correlated with radiographic progression of OA, rheumatoid arthritis and

experimentally induced arthritis.[170-174] Exercise increases synovial fluid and peripheral serum content of several biomarkers in horses, and even early osteoarthritic patients do not have the same biomarker expression pattern.[175]

Histopathologic assessment is rarely utilized for the diagnosis of OA but there are distinct difference observed between changes in high motion and low motion joints. Although there is typically synovitis, cartilage erosion, subchondral sclerosis and capsular fibrosis associated with high motion joints, low motion joints typically do not have evidence of synovitis and have full thickness cartilage necrosis but not erosion. Other features of low motion joints include subchondral lysis (osteoclastic resorption) and ankylosis.[176]

Treatment

Injuries to the articular surface may manifest as a consequence of a multitude of clinical scenarios (chronic instability, incongruity, acute trauma, or lubrication failure) and important factors include location (in relation to weight-bearing area), depth/area of defect and age of patient. Defects in cartilage may range from focal fibrillation (collagen fiber condensation and fraying) to full thickness eburnation (ulceration). Defects that do not extend past the tidemark more commonly reach a stagnant non-healing state[177,178] but when purposefully extended past the tidemark have improved healing. Current dogma is that this improved healing is due to an influx of mesenchymal cells and other healing factors from within the subchondral bone (extrinsic repair). Intrinsic repair relies on the inherent repair capability of chondrocytes. Small cartilage lesions undergo a specific type of intrinsic repair termed “matrix flow” in which chondrocytes fill the defect in a

centripetal fashion. Similar to integument injuries, larger full-thickness lacerations heal first by fibrous infiltration which is primarily composed of type III, then type I collagen. This fibrous infiltrate is known as fibrocartilage or hyaline-like cartilage and is deficient in type II collagen.[179] Fibrocartilage is biomechanically inferior to hyaline cartilage.[180] Although 3mm diameter experimental lesions were observed to heal appropriately,[181] 15mm^2 lesions degenerated after 5 months of improvement.[182] Extrinsic repair methods (such as stem cells and osteochondral explants) are under investigation in equine patients.

When considering medical treatment modalities for arthropathies, the two main goals include reducing pain (typically manifested as lameness) and reducing the progression of joint deterioration. A multitude of systemic and local therapies have been utilized in one or both of these capacities, including oral supplements, NSAIDs, corticosteroids, HA, PSGAGs, extracorporeal shockwave therapy, and biological or regenerative therapies.

The veterinary nutritional supplement industry accounted for retail sales of $1.2 billion in 2007 and was anticipated to eclipse $2 billion by 2012.[183] Half of all owners purchase dietary supplements for their horses and 34% of all supplement sales are for those marketed to aid in joint function.[183] Oral joint supplements are also widely used in human and small animal patients, with the majority to provide the elementary molecules important to cartilage and synovial fluid homeostasis. The most common preparations include glucosamine, chondroitin sulfate and hyaluronan, with no uniform sourcing, formulation or concentration. Radioisotope tracking has shown bioavalibility of both glucosamine and chondroitin sulfate but therapeutic levels have not been established.[184-188] There is encouraging anecdotal and in vitro evidence for the use of glucosamine and chondroitin sulfate in horses[189] as well as well controlled studies in people showing

success in joint disease therapy.[187,189-193] Trials utilizing avocado and soy unsaponifiables have shown benefit in horses with experimentally induced OA.[194] Products are commercially available (Cosequin ASU®, Nutramax Laboratories® and CJ Formulation, Platinum Performance®) that include ASU but do not provide the same daily dose (2g/day) as provided in the prior described experimental study (6g/day).[194]

Nonsteroidal anti-inflammatory drugs are the most commonly utilized medications for OA in humans and horses alike. NSAIDs act through blockade of cyclooxygenase of the arachidonic acid cascade at the local or regional level; recent research has also shown blockade of glutamate and substance P receptors at the spinal level of laboratory animals and humans, though this has not been confirmed in horses.[195] Current dogma describes that prostaglandin and thromboxane production are inhibited by cyclooxygenase blockade, which uninhibited would ultimately alter cartilage metabolism and increase inflammation and pain. With the discovery of cyclooxygenase isoforoms (COX-1 and COX-2), better understanding of the functionality of each have been experimentally ascertained. COX-1 is essential to renal and gastrointestinal mucosal health. COX-2 is inducible and associated with inflammatory events, particularly associated with macrophages and synoviocytes within joints. It has been shown in a murine model that complete COX-2 suppression inhibited healing of gastric ulceration, which indicates that total blockade is also not ideal. Although phenylbutazone is a relatively inexpensive medication, the potential side effects as they relate to gastrointestinal and renal pathology have led to the development of preparations with an increased COX-2 selectiveness, including carprofen and firocoxib. Topical NSAID applications such as liposomal

suspensions of diclofenac have been shown beneficial in clinical and experimental OA and reduce systemic toxicity.[196,197]

Hyaluronan is an integral element of synovial fluid and articular aggrecan molecules. Supplementation of exogenous hyaluronan is commonly performed in people and veterinary patients in the form of intra-articularly and systemically administered preparations. In vitro anti-inflammatory properties include downregulation of the lymphocyte response and inhibition of inflammatory cell phagocytosis (granulocytes, macrophages). Dose dependent inhibition of free radial scavenging and macrophage prostaglandin release has also been observed.[198] Exogenous administration of hyaluronan theoretically stimulates endogenous production of high molecular weight hyaluronan thought to be important for normal function and retention within the synovial space. Retrospective analysis has demonstrated 500kDa as a minimum molecular weight for clinical effectiveness.[45] In addition to increasing molecular weight, synthetic cross-linking of HA increases articular retention time, reduces susceptibility to free radicals and is mainly utilized for fluid viscosity improvement (viscosupplementation) in human patients. Intra-articular deposition of a high molecular weight, cross-linked HA product (Hylan G-F 20) was effective in human clinical studies[199-201] but was not beneficial in an acute equine synovitis model.[202] The authors did note that the experimental design and severity of the equine model may have negatively influenced the results. Determination of equine clinical dosing utilizing force place analysis indicated that at least 20 mg/joint was needed for lameness improvement.[203] Human clinical patients showed reduced pain and effusion scores when three doses were administered at one week intervals. In a recent equine experimental OA study, high molecular weight HA (Hyvisc®, Boehringer

Ingelheim) did not improve clinical parameters but did improve cartilage fibrillation and synovial parameters.[204] Intravenous use of HA has resulted in symptom modification, and improvements in histologic and synovial fluid characteristics in an experimental equine model.[205] When utilized prophylactically, improvements in speed index, number of starts and prize winnings in both Quarter Horse and Thoroughbred racing populations were observed.[206]

Polysulfated glycosaminoglycan is a polysulfated polysaccharide composed of chondroitin sulfate of bovine trachea origin. PSGAGs exert substantial anti-inflammatory effects, such as inhibition of IL-1, MMPs, and PGE_2, and as a result alter the degradation and synthesis of proteoglycan.[207] Intra-articular PSGAGs improved clinical signs of acute synovitis.[208-211] When utilized during an equine experimental OA model via the intramuscular route, modest improvements were observed in lameness at 56 days.[204] Repeated intramuscular administration of PSGAGs in equine induced OA model showed PSGAGs to be inferior to ESWT as indicated by lameness assessment but no histopathologic differences were observed.[212] Specific to equine patients, septic arthritis has been documented when intra-articular PSGAGs have been administered.[213] In this model, 33 colony-forming units of staphylococcus aureus were deposited in addition to various therapies (PSGAGs, MPA, or HA). Septic arthritis was observed in all horses administered PSGAGs (8/8), but only 3/8 MPA medicated and 4/8 HA medicated joints. Septic arthritis has not been observed if amikacin is provided in addition to PSGAGs.[214] Pentosan polysulfate, an agent similar to PSGAG, is not of animal origin but is sourced from beechwood hemicellulose and clinical efficacy has been documented in both human and small animal patients.[215]

Extracorporeal shockwave therapy (ESWT) has been anecdotally shown to improve signs of OA of the scapulohumeral and proximal and distal interphalangeal joints.[216-218] In experimentally induced carpal OA, ESWT was superior to PSGAGs as determined by improvement in lameness scores[219] and induced elevation in biomarkers indicative bone remodeling.[220]

Biologic and regenerative therapies include products such as autologous conditioned serum (ACS; also known as IRAP), PRP, and fat-derived or bone marrow-derived mesenchymal stem cells. ACS has shown to be disease modifying in relation to OA[221] and may be indicated in cases for which corticosteroids are undesirable. Platelet rich plasma has the convenience of rapid production and administration. Stem cells have typically been surgically deposited within articular defects utilizing varying scaffold types, though there are few studies of deposition of stem cells intra-articularly without utilizing scaffolding (thus no surgical component).[222-224] In two models, deposition of 20 millon BMSCs with the inclusion of HA has shown success: medial femoral condyle microfracture and grade 3 meniscal tears subsequent to arthroscopic debridement.[225,226]

Ongoing research into the surgical management of joint disease include varied techniques with the ideal goal of producing a normal articular surface. Procedures to be highlighted include synovectomy, arthroscopic debridement or reconstruction of osteochondral fragments, arthrodesis, as well as multiple methods of articular resurfacing. Joint replacement is a fairly common procedure in human patients and is becoming more commonplace in small animals due to reduced costs and improved implant lifespan. Unfortunately, in equine patients, significant morbidity, costs, and

increased weight bearing requirement (leading to ultimate implant failure) has precluded clinical use of this modality.

Arthroscopy has supplanted arthrotomy as the gold standard of articular visualization, especially in cases with localized clinical pain but nonspecific or absent diagnoses following complex imaging modalities such as magnetic resonance imaging and computed tomography. An advantage of arthroscopy is the ability to lavage debris and pro-inflammatory cytokine-rich fluid out of the joint via arthroscopic cannulas. Arthroscopic lavage was more effective compared to lavage with 14 gauge needles alone.[226-228] Partial thickness lesions of articular cartilage (such as fibrillation) are typically debrided to a base of healthy cartilage, leaving as small a diameter margin as possible without typically converting the lesion to full thickness. However, partial thickness lesions do not heal and have remained unchanged for at least 2 years.[177] When full thickness lesions are encountered or created, the articular margin should be vertically oriented, calcified cartilage debrided, and the subchondral bone plate exposed to uncover extrinsic healing factors. Arthroscopic removal of osteochondral fragments is the most common equine orthopedic procedures performed. In general, removal of fresh fragments in acute cases is of more benefit compared to chronic cases. In the case of sizeable intra-articular fractures, anatomic reconstruction of the articular fracture gap is of utmost importance and is typically the most important prognostic indicator for future athletic use of horses.

Subtotal or complete synovectomy has been performed in a multitude of species including small animals, lagomorphs, people and horses. Experimental synovectomy was initially performed to ascertain the importance of synovium to joint homeostasis.

Resection in the normal horse has not shown untoward effects but synovium in horses regenerates at a slower pace than it does in other species.[229-231] In human patients with rheumatoid arthritis, the synovial membrane is believed to be the primary inciter of cartilage degeneration. Although there is clinical improvement after synovectomy, the short duration of clinical of improvement limits utilization of the technique. It is believed that removal of chronically hypertrophied synovium reduces pro-inflammatory cytokine production in joints of horses and clinical improvement is appreciated particularly in the carpal and metacarpo- and metatarsophalangeal joints.[229] Resection of the synovial membrane is most commonly achieved with motorized synovial resectors but may also be performed via chemical or radioisotope methods.

Articular resurfacing is another surgical option for treatment of advanced OA. Articular cartilage lesions of 5 mm diameter or greater do not heal spontaneously[182] and resurfacing methods should be considered. Resurfacing can be achieved by either stimulating endogenous repair or by utilizing a grafted or transplanted tissue. These are not mutually exclusive procedures and are commonly used in concert. Methods utilized for stimulating endogenous repair include abrasion arthroplasty (debridement to subchondral bone plate), spongialization (debridement past the subchondral bone plate into cancellous bone), osteostixis (targeted drilling to cancellous bone through lesion) and subchondral bone microfracture (multiple discrete regions of subchondral bone penetration). Considering that bone marrow is a much better source of stem cells and growth factors fundamental to cartilage repair compared to the articular cartilage itself, it stands to reason that exposure of the subchondral bone would increase the possibility of appropriate healing.[232,233] Growth factors considered important for these procedures

include IGF-1, bone morphogenic proteins (BMPs) 2 and 7, and TGF-β. Abrasion arthroplasty is currently recommended if the lesion does not enter cancellous bone. Subchondral bone microfracture may be used in association with abrasion arthroplasty if sclerotic bone is encountered with petechial hemorrhage and unexposed cancellous bone. Microfracture provides further access for growth factors and reparative cells, does not disturb biomechanical stability and provides additional attachment via bone spiculation. Although histologic grading was not significantly improved, when compared to debridement alone, the addition of microfracture of large articular defects (up to 2cm diameter) healed with greater volume and had a greater percent of type II collagen.[9,179] Results of microfracture were improved with the addition of IGF-1 and IRAP.[234] Osteostixis is not currently recommended due to an increased rate of subchondral bone cyst formation and inferior histologic grading compared to microfracture.[235,236] Spongialization is not currently recommended due to destabilization of the subchondral bone plate.[237] Although there are no long-term results published for equine cartilage resurfacing, microfracture in people resulted in similar histologic grades and patient outcomes in short and long term evaluations.[238,239] Ongoing research into equine grafting techniques can be divided into one of three implant types: osteochondral tissue, chondrocytes, and stem cells. Osteochondral grafting in equine patients was successful in the short-term but failed in the long-term due to incongruity and morbidity at the harvest sites.[240-242] Chondrocyte implantation (FDA-approved in humans) is typically autologous in origin and requires two surgical procedures with a multiple-week cellular expansion time.[243] Costs and technical difficulties limit this use in horses. A more recent technique, cartilage autologous implantation system (CAIS), utilizes the distal femoral trochlea as a

harvest site, to create a minced cartilage graft held in place with fibrin glue in a single surgical procedure; this has shown promising results and may be the most likely current technique to gain widespread acceptance.[244,245] Frozen foal allografts have also been successful in a limited number of cases.[246] Substantial research is ongoing into the use of mesenchymal stem cells to improve success of such implant treatments. Laboratory cellular expansion is necessary, time consuming, and costly but improved healing has been reported.[246] Original work in equine femur trochlear ridge defects showed that BMSCs produced significant healing early on but the technique was not advantageous after 8 months.[247] A recent study demonstrated BMSCs to be superior to both foal and adult chondrocytes in producing cartilage-like neo-tissue.[248] A pilot experimental study has recently been undertaken utilizing a synthetic graft made of polycarbonate urethane and titanium used in the medial femoral condyle of normal horses indicating that the implant did allow for bony incorporation but there was significant morbidity and mortality.[249]

Without the opportunity for widespread clinical use of joint replacement in horses, arthrodesis of a high motion joint is typically the last option limited to a life-salvage procedure in the nonathletic patient. However, minimally mobile joints are occasionally fused in athletic patients with some success. Joints amenable to fusion in athletic patients may include carpometacarpal, proximal interphalangeal, distal intertarsal, and tarsometatarsal joints. Fusion of a joint typically alleviates discomfort originating from motion and has been accomplished by multiple methods, including internal surgical fixation, surgical drilling, and chemical or laser ablation. Internal fixation is required when attempting arthrodesis of high motion joints such as the two proximal carpal joints,

metacarpophalangeal and distal interphalangeal joints. Laser ablation (superheating and vaporization of synovial fluid) of the distal tarsal joints is promising as radiographic evidence of ankyloses was observed in a shorter time (5-12 months) than may be expected with other modalities.[250] Chemical means of assisting fusion (most commonly via alcohol injection) must be used with particular caution as synovial communication may lead to unintended arthrodesis of associated structures.

Use of Intra-articular Corticosteroids for Osteoarthritis

General

Intra-articular corticosteroids have been used since the 1950's and are the most researched intra-articular medication in horses. Their mechanisms of action is blockade of the arachidonic acid cascade through inhibition of phospholipase A_2. Corticosteroids bind corticosteroid receptors (type I and type II). Type I corticosteroid receptors (known as mineralcorticoid receptors [MR]) and Type II corticosteroid receptors (known as glucocorticoid receptors [GR]) can be differentiated by binding affinity, as the former has an affinity for aldosterone and endogenous glucocorticoids and the latter has poor affinity for mineralocorticoids but do bind endogenous and exogenous glucocorticoids. When endogenous cortisol concentrations are elevated, the binding affinity of type II is elevated; otherwise type I is the primary receptor.[251] All cells in the body express both type I and type II receptors, although type II receptors predominate in the brain; genes regulating GR density have been determined in humans.[252] Corticosteroid receptors may be expressed on the cellular membrane or within the cellular cytoplasm. Circulating corticosteroids enter the cell via passive diffusion or rate limiting active transport and bind corticosteroid receptors located within the cytoplasm. Inactivated cytoplasmic receptors are typically bound to heat shock proteins (HSP 70 or 90).[253,254] In summary, corticosteroids interact with cells via three methods: 1) binding to cell membrane receptors which create the most rapid onset responses, 2) intracellular steroid-receptor complexes that interact with cytoplasmic transcription factors, and 3) steroid-receptor complexes enter the nucleus and regulate genes via DNA sequence binding.[255,256]

Mechanism of Action

The anti-inflammatory properties of corticosteroids are primarily via upregulation of lipocortin-1 (annexin A1) synthesis; however, they also selectively inhibit transcription of cyclooxygenase-2 and reflect upregulation of all three previously described cellular interactions[257-259] including regulation of up to 2000 separate genes.[255] Lipocortin-1 is a phospholipid-binding protein that reduces the inflammatory response by direct inhibition of eicosanoid production and reduced leukocyte function (reduced expression of TNF-α, IL-1, IL-2, platelet activing factor). Obstruction of the arachidonic acid cascade at the level of phospholipase A_2 inhibits production of prostaglandins, thromboxanes, and leukotrienes. Leukocyte function is markedly reduced via reduced capacity for margination, migration, phagocytosis, and chemotaxis.[260] Ultimately, inflammation is altered by inhibition of fluid accumulation (edema), deposition of fibrin and collagen, and reduced fibroblastic and capillary proliferation. Responses of bone to corticosteroids include vitamin D_3 antagonism and reduced bone formation and increased bone resorption, observed as osteoporosis subsequent to chronic use in people[261]; this has not been well documented in other species. Physiologic doses of glucocorticoids stimulate collagen synthesis but supraphysiologic doses have the opposite effect and reduce proteoglycan content of cartilage and chondrocyte metabolism.[209,262]

Dozens of corticosteroids are available for animal and human use with the primary differences related to duration of action, anti-inflammatory potency (expressed relative to hydrocortisone activity) and mineralocorticoid activity. The biological half-life and duration of action are positively correlated with the anti-inflammatory potency. Synthetic modifications of cortisol have included 1,2 double bonding (increased

glucocorticoid activity and duration of action), C-16 methylation (mineralocorticoid activity elimination) and C-9 fluorination (increased glucocorticoid potency). As a general rule, duration of action is increased with acetates compared with succinate esters. Commercial corticosteroids that are currently available to practicing equine veterinarians include (but are not limited to) methylprednisolone acetate (Depo-Medrol®, Zoetis Inc.), triamcinolone acetonide (Kenalog®, Bristol-Myers Squibb Company), and isoflupredone acetonide (Predef 2x®, Zoetis Inc.). Use of isoflupredone acetate has been documented for disorders in multiple species including the following: successful adjunctive treatment of bovine heifers with experimentally induced bronchopneumonia,[263] recognition of in vivo minerocorticoid and adverse metabolic derangements in lactating cows[264] and characterization of use for promoting porcine preweaning growth.[265] Equine specific literature associated with IPA includes a report detailing the use of fluoroprednisolone for a multitude of musculoskeletal maladies,[266] the successful implementation of IPA for in vivo management of recurrent airway obstruction[267] and postbreeding endometritis,[268] and pharmacokinetic characterization after intra-articular deposition.[269-271]

In Vivo and In Vitro Use

The first recognized use of hydrocortisone for equine (and bovine) musculoskeletal conditions was in 1955 with observed clinical improvement.[272] In the two decades subsequent to this observation, a sequence of publications reported on the effects caused by differing steroidal compounds.[273-277] There were reports of continued in vivo use through the 1970s with beneficial effects reported,[278-282] though recent commentary has advised caution regarding these results due to lack of appropriate control

populations.[283] Comparison of methylprednisolone to injection of peripheral blood into normal carpal joints reportedly induces less synovial effusion and no toxic effects.[284] Results of another study using normal horses and repeated large doses (120mg) of MPA injected into both radiocarpal and intercarpal joints indicated chondrocyte necrosis, reduced glycosaminoglycan staining, hypocellarity, and cartilage fibrillation compared with contralateral control limbs; however, evaluated variables did improve when evaluated at 4 and 8 weeks.[285] Trotter et. al. reported on repeated MPA use in normal middle carpal joints; results indicated no alteration of clinical or radiographic parameters but there was decreased GAG content of cartilage in treated joints.[286] When administered to horses with experimentally induced OA, MPA-medicated joints have more extensive cartilage erosion and periarticular proliferation than controls.[287] Though these findings may be consistent with the description of "steroid-and exercise-induced arthropathy"[288], this has not been widely reported in equine patients and the prior studies only utilized one particular corticosteroid. A carpal chip fragment model was refined and extensively utilized for comparison of differing steroid compounds in vivo.[289-291] In the initial study,[289] betamethasone esters were deposited in one limb 12 and 35 days after creation of bilateral carpal chip fragments. Neither exercise nor injection of corticosteroid created negative effects as observed via cartilage histology or histochochemical analysis. After refinement of the experimental model, MPA and TA were compared,[290,291] indicating worse histopathologic grading and reduced synovial fluid volume associated with the MPA group that was not observed with the TA group. Positive effects observed within the TA group included reduction in lameness, reduced synovial fluid protein content, increased synovial HA and GAG concentrations, and improved cartilage histopathologic

grades. When looking at bony remodeling subsequent to osteochondral fragmentation and TA deposition, there was no deleterious effects observed associated with subchondral bone healing in the face of exercise.[292]

In vitro study of OA and testing of compounds to treat OA has increased significantly over the past decade due to a multitude of factors, including development and advancement of laboratory equipment and protocols and humane considerations surrounding animal use. Benefits of in vitro testing include a controlled and repeatable environment and ability to evaluate short timeframes; this results in sufficient statistical power to determine outcomes at a reduced cost compared with in vivo testing. In vitro models range from culture of single isolated cell types to complex co-culture models with or without inclusion of inflammatory mediators. Deleterious effects of MPA (ie, reduced chondrocyte proteoglycan content) were observed in normal articular cartilage pellets in a non-inflammatory environment.[26] A study utilizing canine and equine articular cartilage explants in an inflammatory (monocyte-conditioned medium) environment indicated that MPA mitigated cartilage degradation.[293] Utilizing a bovine model, significant time- and dose-dependent chondrotoxicity was observed after exposure to MPA.[294] Chondrotoxicity was further exacerbated when lidocaine was included in addition to MPA. This finding is particularly important in regards to the ubiquitous use of local anesthetics in equine lameness examinations. To mitigate the negative effects of MPA in joints, many clinicians have included HA.[295] Results of two in vitro studies have shown minor but beneficial effects of the MPA and HA combination.[296,297] In an inflammatory environment (lipopolysaccharide), TA is protective of cartilage explant morphology.[298] Neither anabolic nor catabolic gene expression (mRNA) was significantly different

between MPA and TA groups when quantified within articular cartilage exposed to IL-1β stimulation,[299] though this study was particularly short in duration. Inclusion of tissue types other than articular cartilage has allowed for cautious extrapolation of the contribution of other tissues within the articular environment. Experiments that evaluated the inclusion of synoviocytes with equine articular cartilage in an inflammatory environment (stimulated with either IL-1α or MMP-13) contributed to the fundamental understanding that synoviocytes play a major role in the production of GAGs and release of inflammatory mediators.[300] Results of a subsequent human articular tissue coculture (synovium and articular cartilage) experiment agreed with this previous finding.[301] In that study, human OA cartilage and synovium were cultured long term (21 days); results indicated that inclusion of synovium reduced GAG production that was mitigated by the addition of TA. The effects of subchondral bone have been studied in a bovine model.[25] When subchondral bone was included within the well either unattached or attached to cartilage there was a significant chondroprotective effect.

Some consideration should be given to the relationship between corticosteroid use and laminitis in horses. There has been no definitive link associating corticosteroid use and laminitis, but total body doses are suggested not to exceed 18 mg of triamcinolone or 200 mg of methylprednisolone.[302] These recommendations are formulated based partly on a report of 1200 treated horses with no incidence of laminitis when the total dose of TA did not exceed 18mg.[303] Two publications have documented a laminitis incidence of 0.5% in 205 horses and 0.15% in 2000 horses; ponies accounted for all but one of the laminitic cases.[304,305] In both of these studies, corticosteroid doses exceeded the currently recommended levels.

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Chapter 3: Comparison of the Effects of Interleukin-1 on Equine Articular Cartilage Explants and Cocultures of Osteochondral and Synovial Explants

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Introduction

Osteoarthritis-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 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 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 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).

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[a] 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[b]. Cartilage explants without subchondral bone (diameter, 7.9 mm) were also collected with a coring reamer[b] 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[c]. Then, articular tissues were transferred to 12-well coculture plates[d] 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)[e].

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 OCS with and without IL-1 [10 ng/mL[11,19]; rhIL-1β][f] 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 PGE_2, TNF-α, MMP-13, DMMB, BAP, and LDH.

PGE_2 Assay

The concentration of PGE_2 in spent media was determined by use of a commercial colorimetric assay[g] 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 2N sulfuric acid was added to each well. Absorbance was measured at 450 nm[h] and PGE_2 concentrations determined by comparison to a standard curve with 4-parameter logistic regression.

TNF-α Assay

The concentration of TNF-α in media was determined with a commercial assay[i] in accordance with the manufacturer's instructions. Briefly, plate wells were coated with anti-TNF-α 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-α 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[h] and TNF-α 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[j] 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[h] 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)[k] at 65°C for 4 h. The 1,9-dimethylmethylene blue assay[l] 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[h]. 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[m] 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[h]. 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[n]. 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[h]. 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[o]. 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 PGE_2 concentration at 48 and 96 h, respectively (Figure 3.1). Stimulation of cartilage explant cultures with IL-1 resulted in a 2.6- and 3.0-fold increase in the PGE_2 concentration at 48 and 96 h, respectively. In IL-1-stimulated OCS explant culture, PGE_2 concentration was significantly ($P = 0.03$) higher than the concentration for unstimulated OCS explants at 48 h. In IL-1-stimulated cartilage explant culture PGE_2 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 PGE_2 concentrations were not significantly different for cartilage at 48 h and OCS cultures at 96 h. Comparisons of fold changes in PGE_2 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.

TNF-α

Stimulation of OCS explant cultures with IL-1 resulted in a mean 1.1-fold increase in the media TNF-α concentration at 48 h and a 1.3-fold decrease in TNF-α concentration at 96 h (Figure 3.2). Stimulation of cartilage explant cultures with IL-1 resulted in a 2.9- and 2.7-fold increase in the TNF-α concentration at 48 and 96 h, respectively. However, differences between IL-1-stimulated and unstimulated culture TNF-α concentrations were not significantly different for cartilage or OCS explant

cultures at 48 or 96 h. At 48 h, the fold increase in TNF-α 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-α 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.

MMP-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 3.3). 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 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.

DMMB Assay

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 3.4). 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.

BAP

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 3.5). 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.

LDH

Concentrations of LDH were not significantly different between IL-1-stimulated and unstimulated explants for either culture type at 48 or 96 h (Figure 3.6). Likewise, no significant differences in fold change comparisons were found.

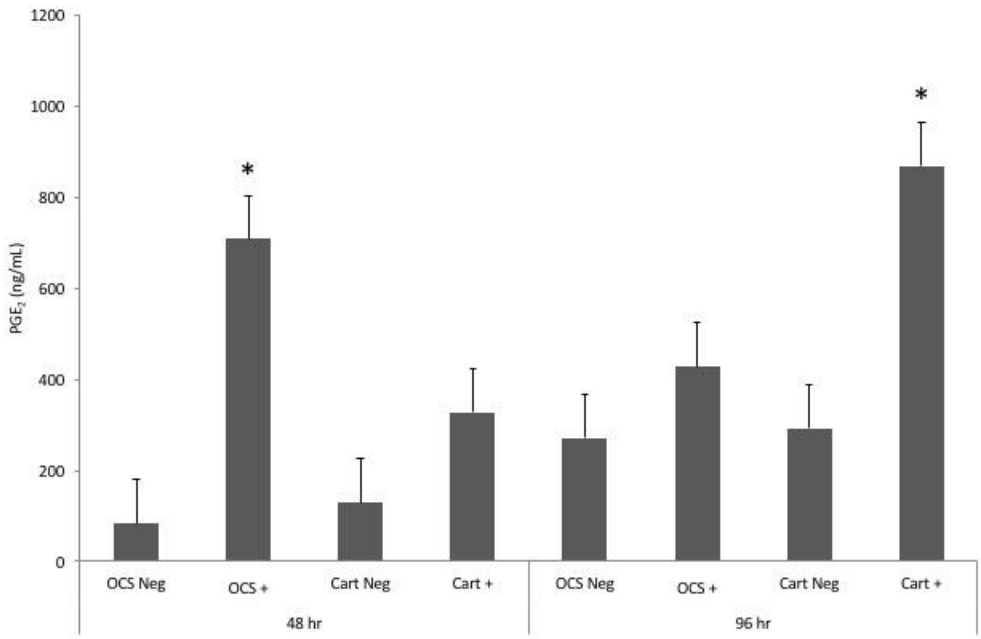

Figure 3.1. Mean ± SE concentrations of PGE2 in media samples of cultures containing OCS or cartilage explants alone (Cart) that were unstimulated (Neg) or stimulated with IL-1 (10 ng/mL; +) at 48 and 96 h after initiation of treatments. *Within a culture type and time, concentration for IL-1-stimulated culture is significantly different from than that for the unstimulated culture.

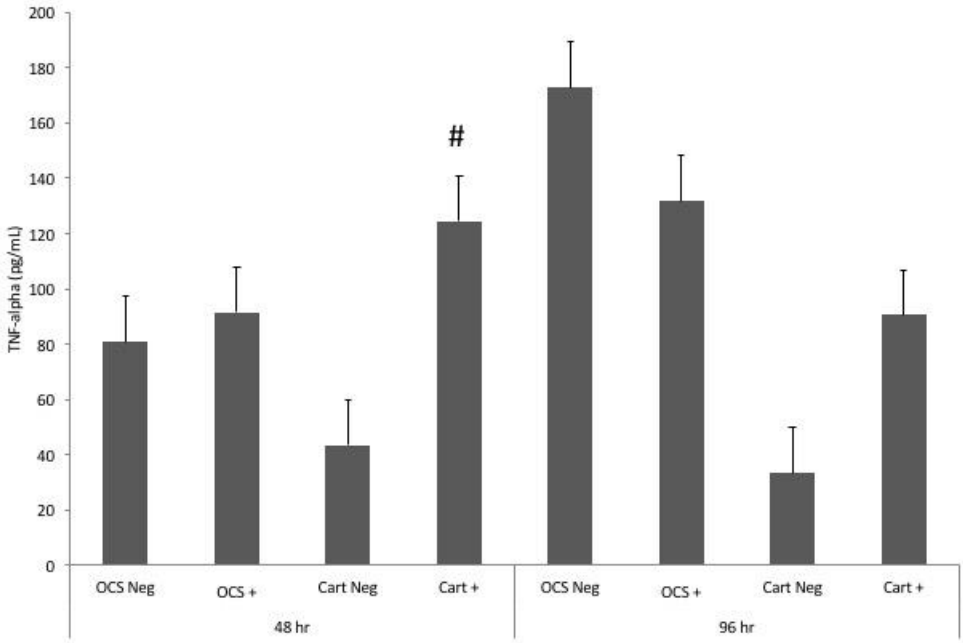

Figure 3.2. Mean ± SE concentrations of TNF-α in media samples of cultures containing OCS or cartilage explants alone (Cart) that were unstimulated (Neg) or stimulated with IL-1 (10 ng/mL; +) at 48 and 96 h after initiation of treatments. #Within a time, the fold increase in TNF-α concentration between unstimulated and stimulated cultures is significantly different between culture types.

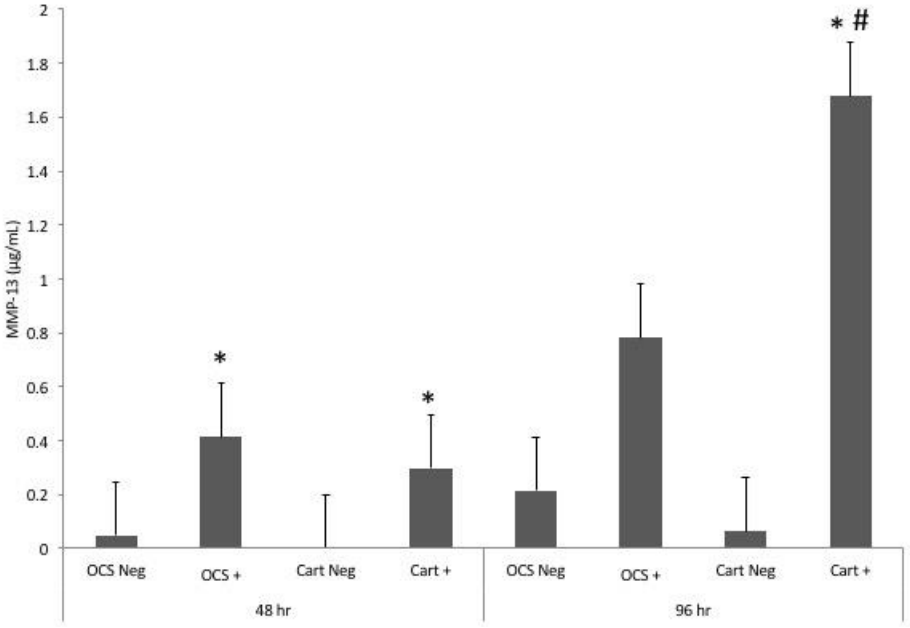

Figure 3.3. Mean ± SE concentrations of MMP-13 in media samples of cultures containing OCS or cartilage explants alone (Cart) that were unstimulated (Neg) or stimulated with IL-1 (10 ng/mL; +) at 48 and 96 h after initiation of treatments.

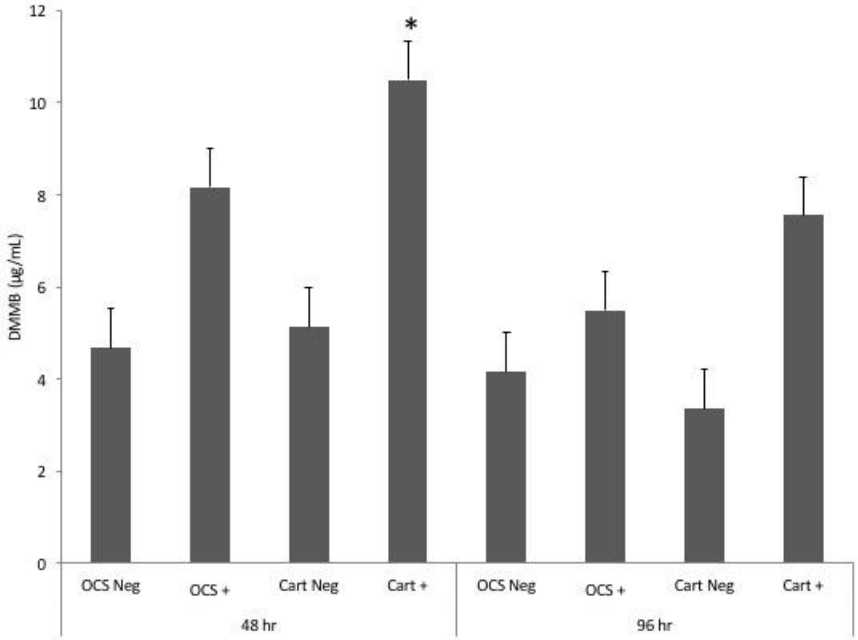

Figure 3.4. Mean ± SE concentrations of DMMB in media samples of cultures containing OCS or cartilage explants alone (Cart) that were unstimulated (Neg) or stimulated with IL-1 (10 ng/mL; +) at 48 and 96 h after initiation of treatments.

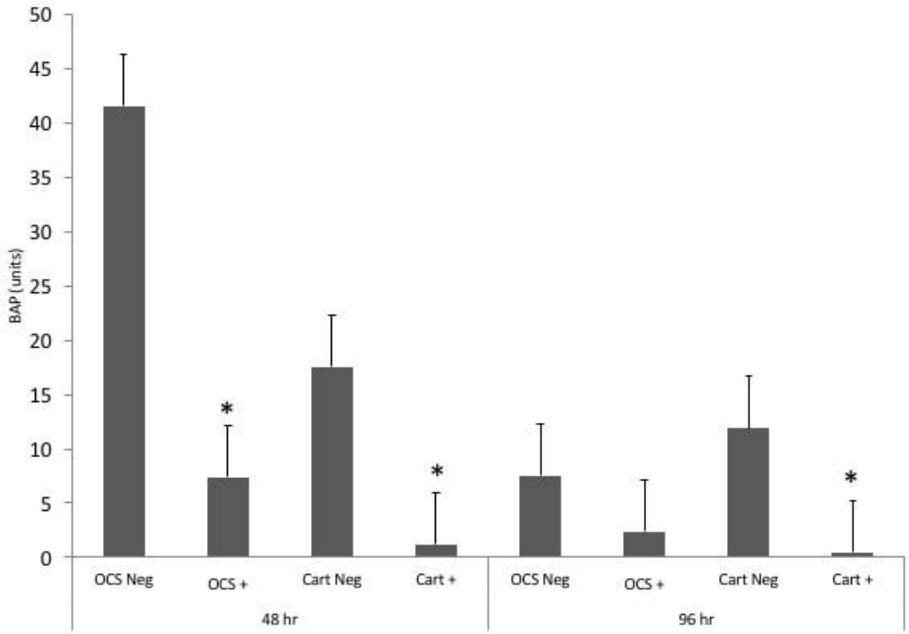

Figure 3.5. Mean ± SE concentrations of BAP in media samples of cultures containing OCS or cartilage explants alone (Cart) that were unstimulated (Neg) or stimulated with IL-1 (10 ng/mL; +) at 48 and 96 h after initiation of treatments.

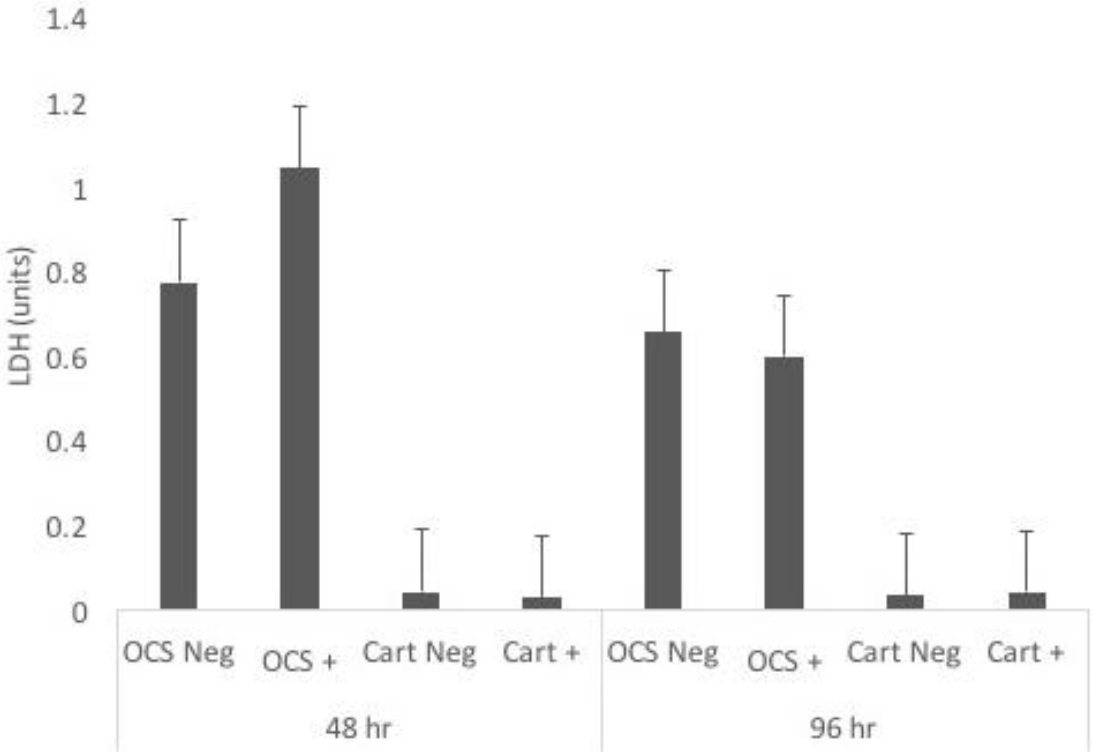

Figure 3.6. Mean ± SE concentrations of LDH in media samples of cultures containing OCS or cartilage explants alone (Cart) that were unstimulated (Neg) or stimulated with IL-1 (10 ng/mL; +) at 48 and 96 h after initiation of treatments.

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-α), 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 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-α expression at 48h, 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-α expression between cartilage and OCS explant cultures at 48 h. The modest increase in expression of TNF-α in these culture systems is not unexpected. Human cartilage and synovial tissue obtained from osteoarthritic joints have low expression of TNF-α when culture alone or together in a coculture system.[16] Other authors found that synovial fluid concentrations of TNF-α 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-α concentrations increase in joints with osteochondrosis dissecans or acute trauma. On the basis of these results, it seems that the TNF-α response to inflammation and joint damage is variable. Our results indicated a mild decrease in TNF-α 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-α.

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.

Footnotes

a. Punch biopsy 6mm diameter, Integra Miltex, Plainsboro NJ,.

b. TEKTON Hollow Punch Set Catalog# 6586, Michigan Industrial Tools, Grand Rapids, MI.

c. Thermo Fisher Scientific, Waltham, MA USA.

d. Corning Transwell Catalog# 3462, Corning Inc., Corning, NY.

e. Corning cellgro, Mediatech Inc., Manassas, VA.

f. rhIL-1β Catalog #201-LB, R&D Systems, Minneapolis, MN.

g. Parameter Prostaglandin E2 Catalog# KGE004B, R&D Systems, Minneapolis, MN.

h. Molecular Devices SpectraMax M5, Sunnyvale, CA.

i. Thermo Scientific catalog #EH3TNFA, Thermo Scientific, Waltham, MA.

j. RayBio Human MMP-13 ELISA Kit, Catalog# ELH-MMP13, RayBiotech, Inc., Norcross, GA.

k. Sigma-Aldrich, St. Louis, MO, USA

l. Sigma-Aldrich, St. Louis, MO, USA

m. Quidel, San Diego, CA, USA

n. Roche, Basel, Switzerland

o. SAS Version 9.4, Cary, NC.

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Chapter 4: Effects of Three Corticosteroids on Equine Synovium, Cartilage and Subchondral Bone In Vitro

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Accepted for publication in *American Journal of Veterinary Research* and included with kind permission.

Introduction

Lameness is a common problem for equine athletes with an estimated prevalence up to 5%.[1] Among the most common manifestations of lameness is OA of the appendicular skeleton. OA is a complex disease process characterized by articular tissue catabolism with progressive cartilage deterioration. Regardless of the inciting etiology, pro-inflammatory cytokines (including interleukin-1β) are released by multiple intra-articular tissues.[2] Reduction of inflammatory mediators is one aim of therapeutic intervention. Corticosteroids have been a common intra-articular medication for this aim in horses.[3] These medications act by blockade of the arachidonic acid cascade, reduction

of capillary dilation, and interruption of the congregation of inflammatory cells capable of releasing cytokines contributing to pro-inflammatory and catabolic states.[4]

The in vitro effects of common clinically utilized corticosteroids have been previously investigated for equine articular tissues in inflammatory environments.[5-7] Some controversy exists regarding the metabolic effects of corticosteroids on articular cartilage, with potentially positive (eg, increased GAG synthesis and abrogation of histopathologic changes)[8] and negative (eg, erosion and morphologic lesions)[9] sequelae reported. However, the disparate results regarding the effects of corticosteroids may be attributable to dose differences rather than biological properties of the drugs. Results of one study suggest that low doses of corticosteroids downregulate gene expression of degradative enzymes with no or minimal detrimental effects on equine articular cartilage[6] and that MPA and TA have similar effects on gene expression at equivalent molarities.[7]

The corticosteroids used most commonly by equine practitioners for treatment of OA are TA and MPA.[3] However, a substantial percentage of veterinarians (>15%) also use IPA intra-articularly in horses.[3] Although data have been published regarding effects of other corticosteroids on cartilage, to our knowledge there have been no studies documenting the in vitro effects of IPA.

Typically, in vitro models used to evaluate the effects of corticosteroids have included only cartilage explants or chondrocytes.[5-7,10-12] Although cartilage is susceptible to irreversible loss of function and damage as a result of OA progression[2], crosstalk between cartilage, subchondral bone, and synovium is important.[13,14] Results of several studies indicate culture of other articular tissues, such as synovium[15,16], subchondral bone[17], or synovium and subchondral bone[18], with cartilage has important effects on

biological responses to inflammatory stimuli and treatments. Synovial tissue may be the largest contributor of catabolic cytokines within the articular environment.[19-21] Inclusion of subchondral bone with articular cartilage as an osteochondral explant significantly reduces in vitro chondrocyte death.[17] These coculture models highlight cellular interplay that may contribute to, or mitigate, articular degeneration.

The objective of this study was to compare the effects of 3 corticosteroids (MPA, TA, and IPA) at various concentrations on equine articular cocultures in an inflammatory environment in vitro. Various biomarkers of articular metabolism associated with inflammation (PGE_2), degradation of extracellular matrix (MMP-13), cytotoxicity (LDH), and articular catabolism (GAG) were analyzed. The hypothesis was that all corticosteroids tested (MPA, TA, and IPA) would counteract the negative effects (upregulation of inflammatory biomarkers and dysregulation of cartilage metabolism) of interleukin-1β (IL-1β) on equine articular cocultures (including synovium, cartilage, and subchondral bone), and that low concentrations (10^{-7} and 10^{-10} M) would have more beneficial and fewer detrimental effects than high concentrations (10^{-4} M).

Materials and Methods

Samples

Collection of tissue samples immediately followed euthanasia via barbiturate[a] overdose for reasons unrelated to this study. Collection of samples was performed in accordance with an approved Institutional Animal Care and Use Committee protocol (Number 14-259). Horses included 3 mares, 2 geldings and one intact colt. Ages ranged from 2 to 11 years. Breeds included Quarter Horse (2), Thoroughbred (2), Belgian (1), and Warmblood (1). Horses did not have a history or clinical signs of musculoskeletal disease, and no gross evidence of degenerative joint disease (cartilage fibrillation, erosion, scoring, or discoloration) was detected during sample collection. Tissue samples (osteochondral and synovial explants) were aseptically collected from the femoropatellar joints. Collection of samples from right or left limbs was determined using a randomization procedure (coin flip). Osteochondral explants (diameter, 7.9 mm; cartilage depth, 2 mm; subchondral bone depth, 4 mm) were collected from the axial surface of the lateral trochlear ridge by use of a hollow punch[b] and orthopedic mallet. Synovium (without fibrous articular capsule tissue) was collected and 6-mm-diameter explants prepared. Sufficient explants for all treatments groups for each horse were collected from a single femoropatellar joint; approximately 25 explants (more than the number sufficient for all treatment groups) were collected per joint. Only grossly undamaged and uniform explants were used in experiments. Explants were kept in phosphate buffered physiologic saline solution containing 1% penicillin and streptomycin at room temperature (25°C) for approximately 1 hour prior to tissue culture.

Tissue Culture

Tissue samples were transferred to 12-well polystyrene transwell plates[c] with inserts lined with polyester porous membranes (12 mm diameter; 3 μm pore size). Synovial tissue samples were placed in the bottom of plate wells and osteochondral explants suspended in well inserts. One 7.9-mm-diameter osteochondral sample and two 6-mm-diameter synovium explants were included in each well (Figure 4.1). This yielded a synovium to articular cartilage surface area ratio (1.2:1) similar to that in human and mouse joints.[22] Articular tissue samples were incubated at 37°C; 95% relative humidity; 5% carbon dioxide in Dulbecco's Modified Eagle Medium (2.8 mL/well) containing 1 g/L glucose, 584 mg/L L-glutamine, and 110 mg/L sodium pyruvate[d]. All articular tissue explants were fully submerged in media. Experiments were performed in duplicate. Tissues were cultured for 48 hours prior to initiation of experimental treatments. Treatment groups included explants with no rhIL-1β[e] (negative control), rhIL-1β alone (10 ng/mL; positive control), and rhIL-1β plus 10^{-4}, 10^{-7}, or 10^{-10} mg/mL of MPA[f], TA[g] or IPA[h]. Doses were chosen on the basis of results of other work[6] and preliminary data within our laboratory that indicated marked cell death at corticosteroid concentrations greater than 10^{-4} M. Media samples were collected at 48 and 96 hours after initiation of treatments and stored at -80°C until analysis; media was replaced at 48 hours with freshly prepared media including treatments.

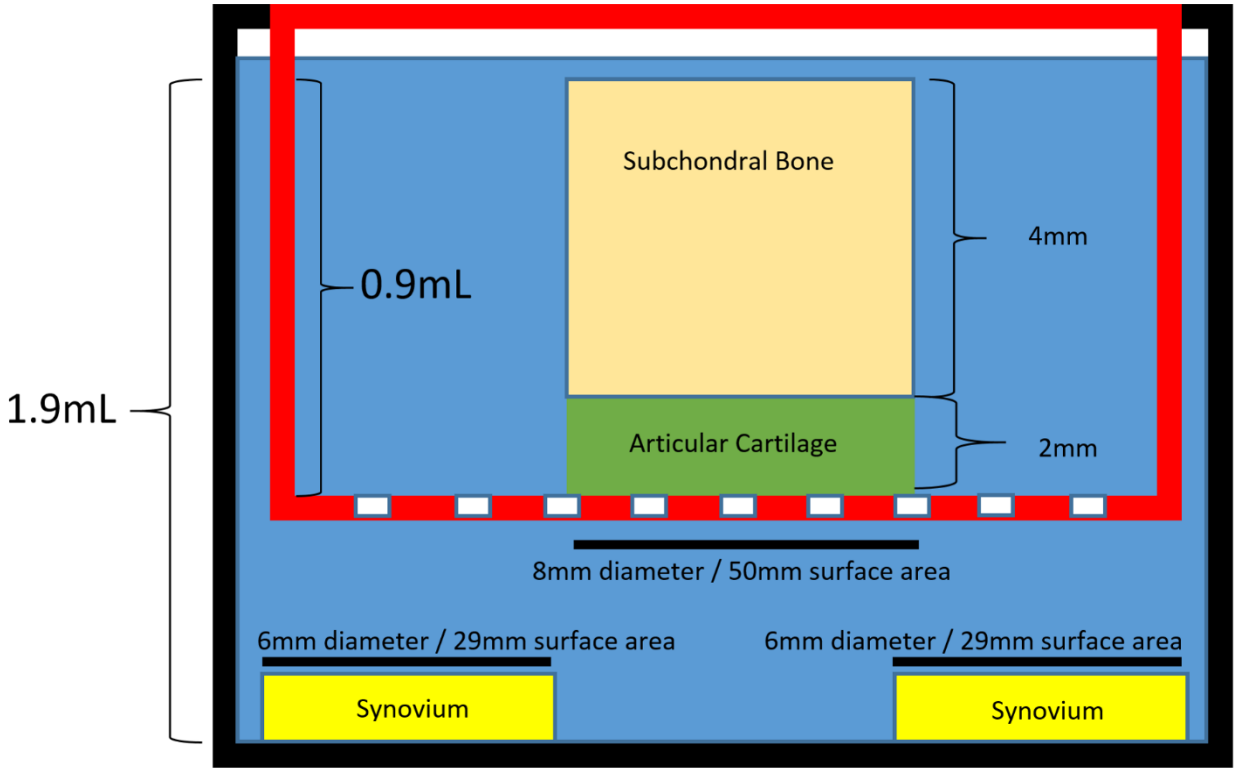

Figure 4.1: Representative individual well + instert. Black rectangle represents main well. Red rectangle represents suspended insert.

PGE_2 Assay

Quantification of PGE_2 within culture media from each time point was determined with a commercially available colorimetric assay[i] in accordance with manufacturer's instructions. Media were diluted 25-fold in supplied buffer and incubated within goat anti-mouse antibody-precoated wells at room temperature on a horizontal plate shaker set to 400 rpm for 1 hour. A further 2-hour incubation under the same conditions followed addition of horseradish peroxidase-labeled PGE_2. After washing wells with supplied wash buffer, tetramethylbenzidine was added for luminescence of bound substrate and incubated covered at room temperature for 30 minutes. Sulfuric acid

(1 mol/L) was added to each well as a stop solution. Absorbance was determined at 450 nm using a commercial microplate reader[j]. All samples/standards were run in tandem wells and the average value multiplied by the dilution factor used for statistical evaluation. Sample concentration was determined using a standard curve.

MMP-13 Assay

A commercially available kit[k] was used to determine MMP-13 concentration in media samples in accordance with the manufacturer's instructions. Media were incubated for 2.5 hours at room temperature in assay wells precoated with anti-MMP-13 antibody. Wells were washed and addition of biotinylated anti-human MMP-13 antibody added, followed by a 1-hour incubation at room temperature. Wells were washed to remove unbound biotinylated antibody and horseradish peroxidase-conjugated streptavidin, followed by a 45-minute incubation at room temperature. After a final wash, 3,3,5,5'-tetramethylbenzidine was added for colorimetric change of bound substrate and plates were incubated covered on the benchtop for 30 minutes. Sulfuric acid was added to stop the reaction and optical density was immediately determined at 450 nm. Average of paired sample concentrations was determined utilizing a standard curve.

LDH Assay

A colorimetric assay[l] was used for the quantification of LDH in spent culture media in accordance with manufacturer's instructions. Media and standard curve samples were incubated with substrate solution including a tetrazolium salt for 30 minutes at room

temperature in a flat-bottom clear 96-well polystyrene plate. The coupled enzymatic reaction then produced a proportional amount of red formazan. The reaction was halted with stop solution and absorbance was measured at 490 nm. Paired wells of each standard/sample were used for determining an average. A standard curve was used for calculation of LDH concentrations in media samples.

GAG Assay

As previously published[23], in a flat-bottom clear 96-well polystyrene plate, media samples (diluted 1:3 in formate buffer) and serial dilutions of chondroitin-6-sulfate standards were combined with 1,9-dimethyl-methylene blue dye and ethyl alcohol substrate. After gentle mixing, absorbance was determined at 525 nm. Samples were assayed in duplicate wells and average concentrations determined by use of the standard curve.

Statistical Analysis

Data were tested for normality. Data that were not normally distributed were log transformed. Data for GAG and LDH were summarized as means (± standard deviation). Data for MMP-13 and PGE_2 were summarized as medians (± inter quartile range). Effects of steroid, time and dose on each of the outcomes were assessed using mixed model analysis of variance[m]. The linear model specified steroid, time, dose, and all 2-way interactions and the only 3-way interaction 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 3-way interaction, the following comparisons were extracted: 1) Time point 48 vs time point 96 for each concentration within each steroid. 2) Effect of doses for each combination of steroid and time point. 3) Effect of steroids at each combination of time point and dose. Where appropriate, p-values were adjusted for multiple comparisons using Tukey's procedure. For each outcome, an extra model (mixed model analysis of variance) was implemented to compare each of positive and negative controls to measurements defined by a combination of time, steroid, and dose. P-values for the extra models were adjusted for multiple comparisons using Dunnett's procedure. For all analysis of variance models, residuals were inspected to verify that the errors followed a normal distribution with constant variance. Statistical significance was set to $p < 0.05$.

Results

PGE_2

At 48 hours, significantly less PGE_2 was released into culture media of all 3 tested corticosteroids at the 10^{-10} M dose compared to positive control (IL-1β-stimulated) samples (Figure 4.2). Additional treatment groups with significantly less PGE_2 compared to positive control samples at 48 hours included MPA and TA at 10^{-7} M. At 96 hours, MPA at 10^{-7} and 10^{-10} M and TA at 10^{-10} M had significantly less PGE_2 than positive control samples. At 48 hours, samples with low concentrations (10^{-7} and 10^{-10} M) of MPA and TA contained significantly less PGE_2 than samples with the highest concentration (10^{-4} M) of those corticosteroids. At 96 hours, samples with MPA at 10^{-7} M had significantly less PGE_2 than those with MPA at 10^{-4} M and samples with TA at 10^{-10} M had significantly less than those with TA at 10^{-4} M.

MMP-13

Compared with positive control samples, significantly less MMP-13 was detected for the following treatment groups at 48 hours: MPA at 10^{-7} and 10^{-10} M, TA at 10^{-4} and 10^{-7} M, and IPA at 10^{-10} M (Figure 4.3). At 96 hours, significantly less MMP-13 was detected in media of all tested corticosteroids at all 3 concentrations compared to positive control samples. At 48 hours, only one significant difference in MMP-13 concentration was detected within each corticosteroid; TA at 10^{-7} M had significantly less MMP-13 than TA at 10^{-4} M. At 96 hours, MPA at 10^{-7} M had significantly less MMP-13 than MPA at 10^{-10} M and TA at 10^{-10} M had significantly less MMP-13 than TA at 10^{-4} M.

LDH

At 48 and 96 hours, samples with IPA at 10^{-4} M had significantly increased LDH compared with samples in all other treatment groups (IPA at 10^{-7} and 10^{-10} M; MPA and TA at all concentration) or positive control samples (Figure 4.4). At neither 48 or 96 hours were there any significant differences among samples within MPA or TA groups at any concentration or between MPA or TA groups and control groups.

GAG

Significantly less GAG was detected for the TA 10^{-7} M group at 48 hours and MPA 10^{-10} M group at 96 hours compared to the positive control group (Figure 4.5). At 48 hours, significantly less GAG was detected in media for groups with lower concentrations of each corticosteroid (MPA at 10^{-10} M, TA at 10^{-7} M, and IPA at 10^{-10} M) compared with the highest concentration (10^{-4} M). At 96 hours, no significant differences in GAG concentration were detectable among tested concentrations of each corticosteroid.

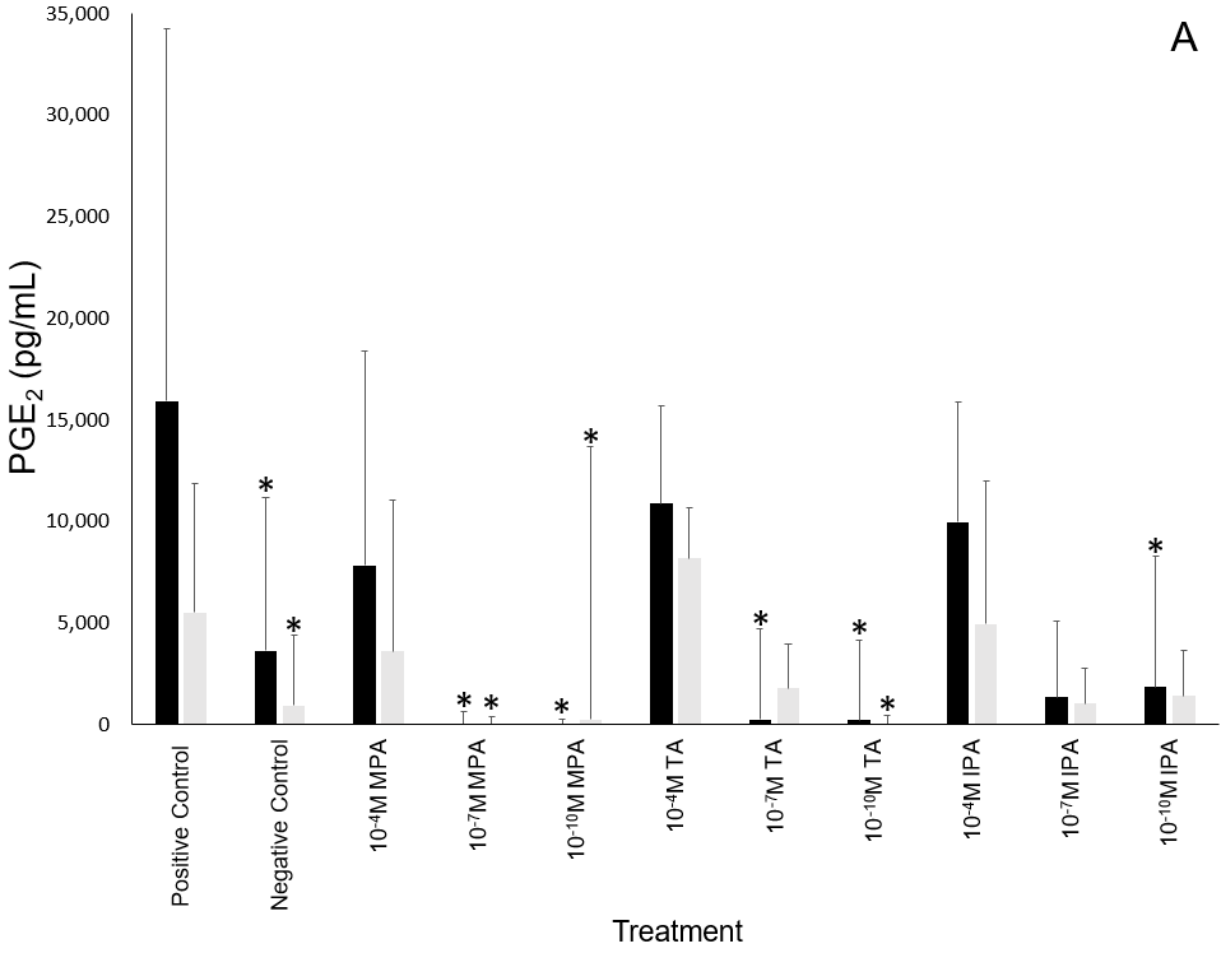

Figure 4.2: Log_e median ± IQR PGE_2 concentrations in media samples collected from co-cultures of equine synovial and osteochondral explants (n = 6) in basal media (Neg Cont), IL-1β (10 ng/mL; Pos Cont), or IL-1β and MPA, TA, and IPA at concentrations of 10^{-4}, 10^{-7}, and 10^{-10} M. Samples were collected for analysis at 48 hours (black bars) and 96 hours (gray bars). *Significantly ($P < 0.05$) different than positive control values.

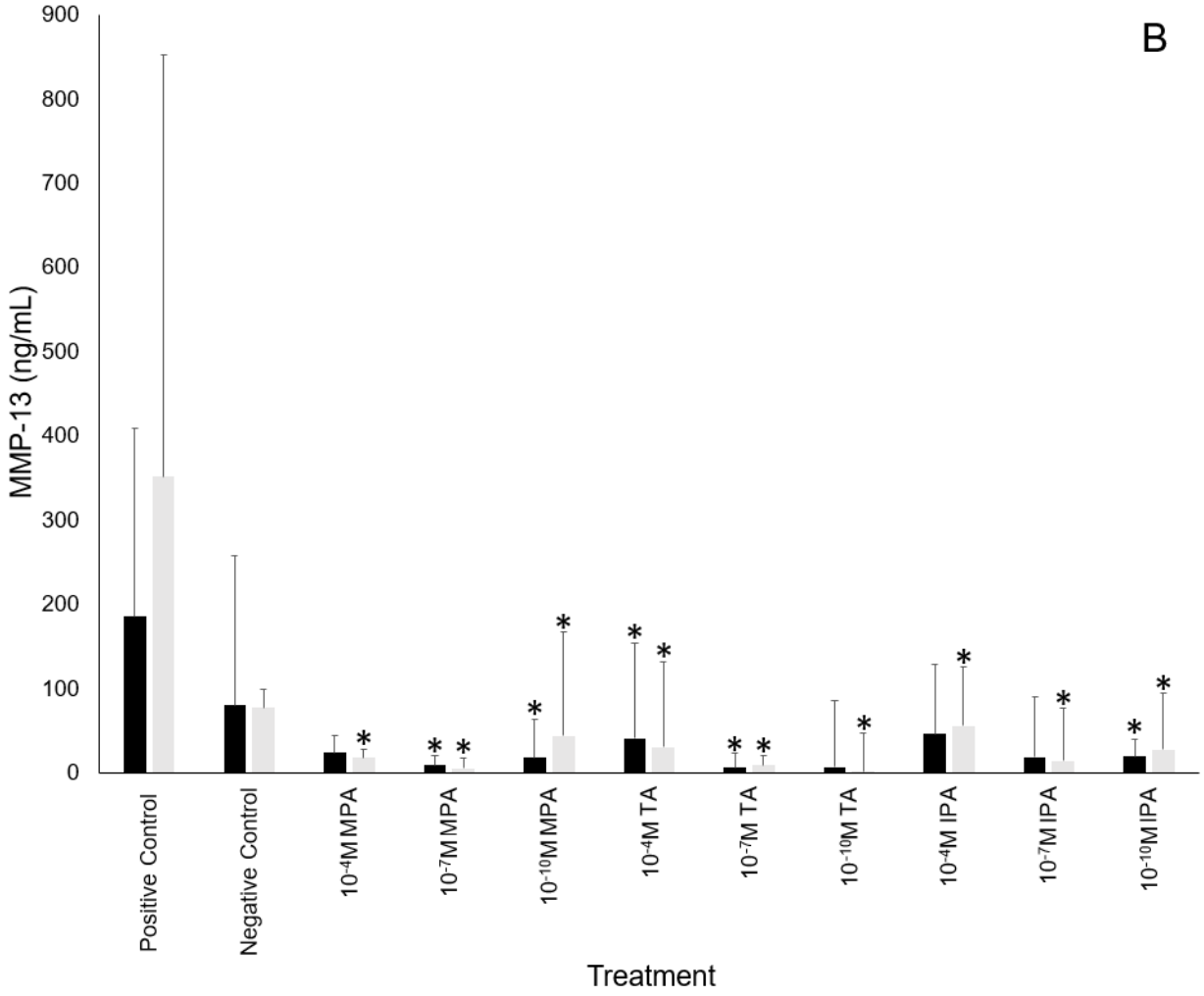

Figure 4.3: Log_e median ± IQR MMP-13 concentrations in media samples collected from co-cultures of equine synovial and osteochondral explants (n = 6) in basal media (Neg Cont), IL-1β (10 ng/mL; Pos Cont), or IL-1β and MPA, TA, and IPA at concentrations of 10^{-4}, 10^{-7}, and 10^{-10} M. Samples were collected for analysis at 48 hours (black bars) and 96 hours (gray bars). *Significantly ($P < 0.05$) different than positive control values.

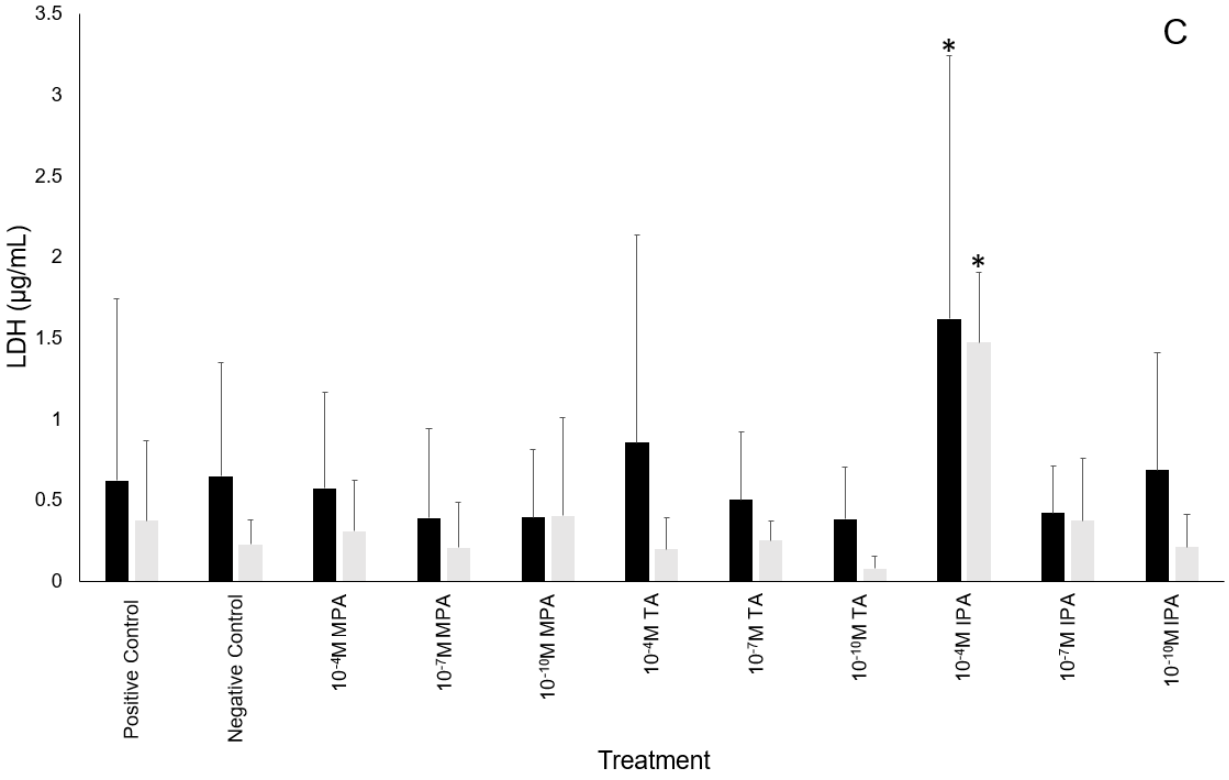

Figure 4.4: Mean ± SD LDH concentrations in media samples collected from co-cultures of equine synovial and osteochondral explants (n = 6) in basal media (Neg Cont), IL-1β (10 ng/mL; Pos Cont), or IL-1β and MPA, TA, and IPA at concentrations of 10^{-4}, 10^{-7}, and 10^{-10} M. Samples were collected for analysis at 48 hours (black bars) and 96 hours (gray bars). *Significantly ($P < 0.05$) different than positive control values.

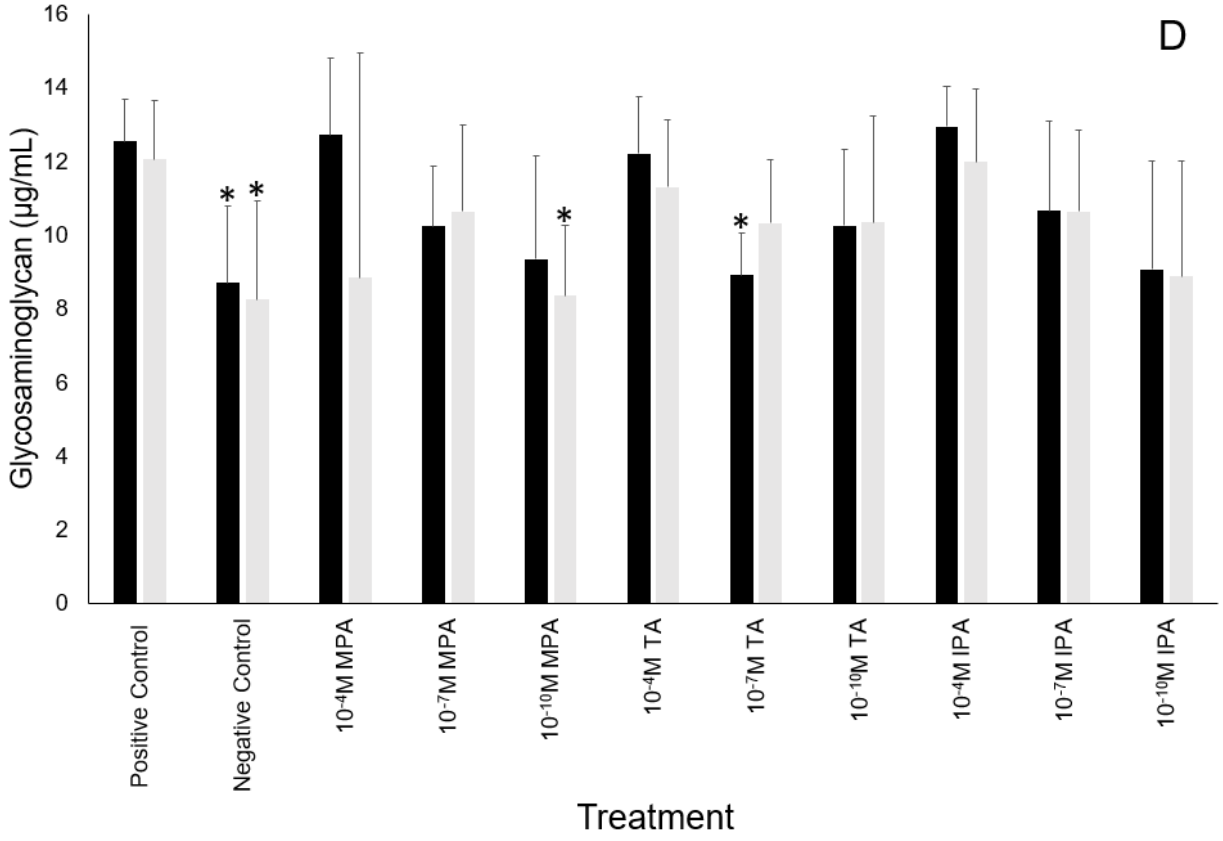

Figure 4.5: Mean ± SD GAG concentrations in media samples collected from co-cultures of equine synovial and osteochondral explants (n = 6) in basal media (Neg Cont), IL-1β (10 ng/mL; Pos Cont), or IL-1β and MPA, TA, and IPA at concentrations of 10^{-4}, 10^{-7}, and 10^{-10} M. Samples were collected for analysis at 48 hours (black bars) and 96 hours (gray bars). *Significantly ($P < 0.05$) different than positive control values.

Discussion

As hypothesized, all tested corticosteroids mitigated the inflammatory and catabolic effects of IL-1β on equine articular tissues, as indicated by results for PGE_2 and MMP-13. Additionally, less GAG was released into media at 48 hours for MPA at 10^{-10} M, TA at 10^{-7} M, and IPA at 10^{-10} M compared with high concentrations (10^{-4} M) of these corticosteroids. Importantly, none of the corticosteroids at low concentrations (10^{-7} and 10^{-10} M) caused cytotoxicity, as determined by LDH quantification.

Increased intra-articular eicosanoid (PGE_2) quantity has been shown to be a relevant marker of joint disease[24,25], and clinical improvements have been associated with reduction of synovial fluid PGE_2 content,[26,27] regardless of therapy. In the present study, stimulation of articular tissues with IL-1β provided a predictable increase in media PGE_2 content and low concentrations of all 3 corticosteroids significantly decreased PGE_2 at 48 hours. This is an expected finding and corroborates findings of prior in vitro studies,[7,12,28] demonstrating various corticosteroids impede upregulation of eicosanoid production. At 96 hours, low concentrations of all 3 corticosteroids decreased PGE_2, although results were only significant for MPA and TA.

Similar to PGE_2 results, low doses of all 3 corticosteroids had significantly reduced media concentrations of MMP-13 compared with positive control samples. Matrix metalloproteinase-13 degrades type II collagen and has been detected in elevated concentrations within joints in naturally occurring disease[19,29] and in experimental short-term[30] and long-term[31] in vitro models of disease. Results of other studies indicate that MMP-13 production is modulated in cartilage by stimulation with IL-1 (upregulation), coculture with synovial tissue (upregulation) and treatment with MPA

(downregulation).[21,32] Interestingly, results from another study within our laboratory[18] suggested that inclusion of subchondral bone within the in vitro model might partially mitigate upregulation of MMP-13 in an inflammatory environment. Results of the present study indicate that low doses of corticosteroids induce equal or greater downregulation of MMP-13 in an inflammatory in vitro model that includes all major articular tissues. This may have important clinical implications, suggesting that low concentrations of corticosteroids are unlikely to cause detrimental effects in cartilage while still achieving clinical efficacy.

Results of this study showed significantly less GAG release into media compared with positive control samples only for TA at 10^{-7} M (48 hours) and MPA at 10^{-10} M (96 hours). Cultures with low concentrations of MPA (10^{-7} M at 96 hours) and TA (10^{-7} M at 48 hours and 10^{-10} M at 96 hours) had less GAG release than cultures with high concentrations. Other authors[5] investigated the effects of MPA and TA on cartilage explants in an interleukin-1 conditioned in vitro environment. Findings indicated low concentrations of each corticosteroid were no less detrimental to cartilage homeostasis than high doses, as measured by GAG metabolism, though in this model no corticosteroid treatment was able to nullify the effects of interleukin stimulation. Interestingly, those authors[5] suggested the in vitro model used in the study (use of articular cartilage explants alone) may not be relevant to in vivo joint physiology. The use of articular cocultures as in the present study may increase physiologic relevance of data and differences between our results and those of the other study could be attributable to this. Although we did have 2 treatment groups negate interleukin stimulation, the effect was short-lived and not

observed at both detection times of each treatment, and as such may not reflect a clinically important difference.

LDH quantification is utilized as a nonspecific marker of cytotoxicity and cellular death. The only treatment that caused statistically significant cytotoxicity was IPA at a high concentration of 10^{-4} M. This concentration is well in excess of clinically administered doses. We did not determine the cellular or tissue origin of LDH. Potential sources in this in vitro model include synoviocytes, chondrocytes, and osteocytes. Other than this finding, results for IPA regarding the other evaluated biomarkers in this study were similar to results for MPA and TA. Further consideration for the increased LDH measured is the difference in anti-inflammatory potency of the compared corticosteroids. IPA has 5 to 10 times more potent glucocorticoid activity than both MPA and TA[33-35], though commonly used clinical dosing and results of this study do not directly support that. It is possible that the elevated cytotoxicity of IPA at the highest evaluated concentration may be related to this increased potency on an equimolar basis. However, our preliminary work in preparation for this study indicated that all corticosteroids caused marked cell death at a concentration of 10^{-3} M.

Limitations of this study include a lack of inclusion of tissue sample histology to corroborate media biomarker results and to allow for characterization of effects in different anatomic tissues. Also, a 96-hour timeframe is a short-term simulation of inflammatory joint disease and would not account for changes in a chronic process. The inflammatory stimulus (IL-1β) dose was selected on the basis of preliminary investigation and to achieve consistency with prior studies[16,18,36]; however, the use of this concentration of IL-1β and a lack of inclusion of other inflammatory and degradative

stimuli may not accurately mimic natural disease. The tested corticosteroid concentrations were chosen based on pilot data within our laboratory. The 10^{-4} M concentration is well in excess of clinically used doses, but 10^{-7} and10^{-10} M are clinically relevant.[37,38] One advantage of our study design is that the in vitro model used, which included all major articular tissue types, may be more representative of physiologic processes than other models. However, similar to another study[17] in which osteochondral explants were used, this model exposed cut edges of subchondral bone to media. Since these surfaces are not exposed to joints in vivo, this component of our coculture system is not physiologically accurate and future refinement is warranted.

Results of this study suggest that high concentrations of corticosteroids (10^{-4}), IPA in particular, are detrimental to articular tissues. However, low doses (10^{-7} and 10^{-10} M) of MPA, TA, and IPA are more effective than high doses at mitigating the negative effects of IL-1. Effects of these 3 corticosteroids on articular tissues in an inflammatory environment are similar at clinically relevant equimolar concentrations. Therefore, we believe these corticosteroids should be used intra-articularly at doses closer to 10 mg per joint rather than an order of magnitude higher (100 mg per joint). This suggestion is supported by our results and findings of other authors that triamcinolone acetate at a dose of 12 mg in a middle carpal joint has primarily beneficial effects[8] and methylprednisolone acetate at an intra-articular dose of 100 mg has some detrimental effects.[9] These results suggest that it may be best to choose similar dosages for these corticosteroids when injecting them intra-articularly for treatment of joint disease. Comparisons of the effects of such dosages in vivo are warranted.

Footnotes

a. TEKTON Hollow Punch Set Catalog# 6586, Michigan Industrial Tools, Grand Rapids, MI.

b. Corning Transwell Catalog# 3462, Corning Inc., Corning, NY.

c. Corning cellgro, Mediatech Inc., Manassas, VA.

d. rhIL-1β Catalog #201-LB, R&D Systems, Minneapolis, MN.

e. Depo-medrol, Zoetis Inc., Kalamazoo, MI.

f. Kenalog-40, Bristol-Myers Squibb Company, Princeton, NJ.

g. Predef 2x, Zoetis Inc., Kalamazoo, MI.

h. Parameter Prostaglandin E_2 Catalog# KGE004B, R&D Systems, Minneapolis, MN.

i. Molecular Devices SpectraMax M5, Sunnyvale, CA.

j. RayBio Human MMP-13 ELISA Kit, Catalog# ELH-MMP13, RayBiotech, Inc., Norcross, GA.

k. Pierce LDH Cytotoxicity Assay Kit Catalog# 88954, Thermo Scientific, Rockford, IL.

l. SAS Version 9.4, Cary, NC.

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Chapter 5: Final Comments

The compilation of manuscript and thesis is an invaluable process for any scientist. It was our goal to advance in vitro modelling of OA and provide an appropriate model for comparison testing of an increasingly utilized medication against historically and currently relevant products.

The results of the first manuscript provide documentation that the model is appropriate and add to the evidence that OA is not a monocellular process and that tissue types adjacent to articular cartilage (synovium, subchondral bone) should not be forgotten in vitro or in vivo. The latter manuscript provides the first evidence of the response subsequent to isoflupredone acetate treatment on any in vitro arthritis model. Results indicated similar response of all comparative corticosteroids for most measured outcomes. Both manuscripts also document temporal differences in outcome measures when subchondral bone or corticosteroids are included within the model. Further investigation of this association may provide valuable information as to the most appropriate or efficacious timeframe for clinical intervention. Using equimolar concentrations was critical to avoiding clinical dosing biases and provided comparison based on pharmacologic data. One must thus be cautioned against extrapolating controlled in vitro data to an in vivo reality without further investigation on live patients.

Future use of the model may provide valuable comparative responses for a multitude of currently utilized therapies. Therapies amenable to testing within this model include those regenerative and biologic in nature (IRAP, PRP, various stem cell preparations) as well as alternative intra-articular medications (HA, PSGAGs). Reevaluation of systemic supplements with this novel model may provide more evidence for clinical utility. Coculture tissue interaction has not been accounted for in prior models, and this may more appropriately reflect in vivo interaction.