The effect of concurrent and non-concurrent (delayed) administration of mepivacaine hydrochloride and triamcinolone acetonide on inflamed equine osteochondral and synovial explants in co-culture

By

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Abstract

During equine lameness investigation, intra-articular administration of a local anesthetic followed by a corticosteroid is common for diagnosis and treatment of osteoarthritis (OA). Anecdotally, veterinarians are advised to separate diagnosis and treatment to allow recovery of articular tissues from the inflammatory effect of the local anesthetic; however, no scientific evidence supports this need for rest. 5 geldings and 1 mare (aged 3-18 years) were euthanized for reasons unrelated to the study. From each horse, 48 synovial explants and 12 osteochondral explants were harvested from the stifles and transferred to a 12-well plate (2 wells per group, 2 synovial explants and 1 osteochondral explant per well). Explants were stimulated with 10 μg/ml recombinant equine interleukin-1β (IL-1β) and 10 μg/ml tumour necrosis factor-α (TNF-α). They were treated as 6 groups: unstimulated control, stimulated control, triamcinolone acetonide (TA, 10^{-6} M), mepivacaine hydrochloride (MH, 4.4 mg/ml), MH + TA (concurrent) and MH + TA (delayed). The delayed group was treated with MH and, six days later, treated with TA. Media were replenished and analysed every 3 days for 9 days total. ELISA analyses for markers of tissue cytotoxicity (lactate dehydrogenase, LDH), inflammatory eicosanoids (prostaglandin E_2, PGE_2), and markers of matrix degradation (matrix metalloproteinase 13, MMP-13 and dimethylmethylene blue assay, DMMB, for glycosaminoglycan, GAG) were performed. Data were analysed with 2-way ANOVA or mixed-effects models with Tukey's multiple comparisons. There were no differences in LDH, PGE_2, MMP-13 and GAG between delayed and concurrent groups at any time point; no differences were found in culture medium levels of cytotoxicity, inflammation or matrix degradation between explants treated concurrently with MH and TA and those that had a 6-day delay between MH and TA treatments. In this model, no benefit of a 6-day delay between local anaesthetic and corticosteroid treatments was found.

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List of Abbreviations

AAEP	American Association of Equine Practitioners
ACS	Autologous conditioned serum
ADAM	A Disintegrin and Metalloproteinase
ADAMTS	A Disintegrin and Metalloproteinase with Thrombospondin Motif
APS	Autologous protein serum
BAP	Betamethasone sodium phosphate
cGCR	Cytosolic glucocorticoid receptor
CNS	Central nervous system
COMP	Cartilage oligomeric matrix protein
COX	Cycloxygenase enzyme
CP	Central protein
CPII	Type 2 pro-collagen
CS	Chondroitin sulfate
CT	Computed tomography
CTX-II	C-terminal crosslinked telopeptide type 2
DMEM	Dulbecco's Modified Eagle's Medium
DMMB	Dimethylmethylene blue assay
d-ROM	Diacron-reactive oxygen metabolites
ECM	Extra-cellular matrix
ELISA	Enzyme-linked immunosorbent assay
FDA	US Food and Drug administration

FEI	Fédération equestre internationale
GAG	Glycosaminoglycan
HA	Hyaluronan
IA	Intra-articular
IGF	Insulin-like growth factor
IL-1β	Interleukin 1 beta
IL-ra	Interleukin receptor antagonist protein
IM	Intra-muscular
IV	Intravenous
KS	Keratin sulfate
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MH	Mepivacaine hydrochloride
MPA	Methylprednisolone acetate
MMP	Matrix metalloproteinases
MRI	Magnetic resonance imaging
NSAID	Non-steroidal anti-inflammatory
OA	Osteoarthritis
PET	Positron emission tomography
PGE_2	Prostaglandin E_2
PRP	Platelet rich plasma
PSGAG	Polysulfated glycosaminoglycan
RMTC	Racing medication and testing consortium

SF	Synovial fluid
SMOAD	Symptom-modifiying osteoarthritis drug
TA	Triamcinolone acetonide
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
TNF-α	Tumor necrosis factor alpha
USDA	United States Department of Agriculture

Chapter 1: Introduction

Osteoarthritis (OA) is defined by the Diagnostic and Therapeutic Criteria Committee of the American Rheumatism Association as "a group of conditions that lead to joint symptoms and signs which are associated with defective integrity of articular cartilage, in addition to related changes in the underlying bone and at the joint margins".[1] This definition encompasses idiopathic OA, in which OA occurs spontaneously, and secondary OA, which develops as a result of joint trauma or congenital or developmental disease. Regardless of the etiology, the end result of OA is usually severe pain, leading to functional disability and limb deformity.[2]

Understanding the problem of OA cannot be achieved without an appreciation for the scale and impact of the disease. In human medicine, OA is recognized as one of the most prevalent chronic diseases in the world. Though OA does not significantly reduce life expectancy, the condition results in pain, disability and reduction in quality of life.[3] Around 54 million adults in the United States (23% of the adult population) have doctor-diagnosed OA.[4] The economic impact of human OA is multi-factorial. Direct costs are those paid directly to the medical system for medications, procedures, home adaptations as well as transportation to medical centers. Indirect costs include lost workdays by the affected persons as well as informal care provided by unpaid caregivers. The annual cost of OA, encompassing direct and indirect costs, is estimated to be $486.4 billion.[5] The lifetime cost of knee OA is estimated to be $104,300 per person.[6] Despite several epidemiological surveys, the true cost of OA is unlikely to be ascertained due to failure to value indirect costs to affected patients and the people that support them.[7] Additionally, over 58% of people with OA have mental health disorders: the resultant chronic pain results in poor sleep, anxiety and depression.[8] This compromise to mental wellness is further exacerbated by

financial pressure from loss of work and costs of medical care. The impact of OA cannot be overstated, especially when considering that there is no known cure.

The impact of OA in the equine industry is similarly profound due to its prevalence and associated economic impact. In 2015 the USDA performed an equine health and welfare survey encompassing approximately 72% of equids in the United States. This survey determined that among equids aged 5 to over 20 years-of-age, lameness that prevented use for intended purpose was the most prevalent health issue reported.[9] The single most common cause of lameness in horses is OA.[10,11] While the true prevalence of equine OA is unknown, it is estimated that 60% of lameness problems in horses are related to OA.[12] Considering that there are currently around 7 million horses in the US, this means that millions of horses are affected by this debilitating condition.[13] The direct costs of equine OA include that of lameness examinations, radiographs, intra-articular medications and systemic medications and are estimated to be $3,000 per horse per year.[13] The indirect costs are harder to appreciate and include those from loss of winnings, lost income due to increased time spent treating the horse and lost leisure time. Nevertheless, it is clear that the cost of equine OA extends into billions of dollars per annum and represents a significant burden on the US equine industry. The progressive, painful nature of the disease, coupled with the high costs associated with management, make OA a frequent cause for equine euthanasia.[14] Osteoarthritis is a major cause of morbidity and mortality in horses and research optimizing diagnosis and treatment is warranted.

In horses, most cases of OA are diagnosed using a combination of subjective lameness assessment, diagnostic analgesia and imaging.[15] The improvement of lameness following intra-

synovial local anesthetic remains one of the most commonly utilized techniques employed.[16] Currently, equine OA is primarily managed medically. The most common treatment is intra-articular (IA) administration of a corticosteroid.[17] The timing of IA corticosteroid treatment in relation to IA local anesthetic is currently controversial. Those in favor of a delay between diagnosis and treatment may speculate that the synovitis caused by IA local anesthetic would reduce the potency of the corticosteroid if administered on the same day, however *in vivo* research has somewhat refuted this.[18] Those that argue in favor of same-day treatment may be alarmed to find that *in vitro* evidence suggests that the steroid-anesthetic combination may be harmful to articular tissues, even more so than either agent alone.[19,20] For optimal timing of diagnosis and treatment of horses with OA, research examining comparing delay and same-day diagnosis-treatment protocols is warranted. In the absence of clinical research, extrapolation from well-designed, relevant *in vitro* studies is justified. This thesis will provide one such study and provide the rationale for and state the clinical conclusions to be made from the described work.

Chapter 2 describes the anatomy of the synovial joint and its component tissues, before describing the known pathophysiology of OA with respect to said tissues. A review of *in vivo* and *in vitro* models of OA research is presented. The principles of diagnosis of equine OA are outlined, including an appraisal on modern imaging techniques. The pharmacology of local anesthetics is discussed, and their effects on articular tissues is reviewed. A brief summary of surgical and medical treatments for equine OA follows, including IA corticosteroid therapy. The rationale for IA corticosteroid treatment, including the regulatory rules concerning their use, incidence, diagnosis and treatment of possible complications relating to IA corticosteroid therapy

are summarized. A summary of the *in vitro* research on local anesthetic/corticosteroid combination treatment effect on articular tissues concludes the literature review.

The study described in this thesis aims to improve the diagnosis and treatment of OA by providing evidence for a clinical rationale that thus far has been based on anecdotal reasoning: the need for a rest period between diagnosis (IA local anesthetic) and treatment (IA corticosteroid) of inflammatory joint pain in the horse. Chapters 3 through 6 describe the aims and hypothesis of the study, materials and methods, results and discussion of the results. Chapter 7 presents conclusions from the work and discusses future directions.

Chapter 2: Literature Review

Joint anatomy and function

A basic understanding of joint anatomy and physiology is required for evidence-based decision making in treatment of joint disease. Joints can be considered according to function and categorized according to range of motion: synarthritic (immovable), amphiarthritic (somewhat moveable) and diarthritic (moveable). Diarthritic joints, found mostly in the appendicular skeleton, enable movement and load transfer of the extremities.[21] More commonly, joints are considered by their structural components and are classified as fibrous, cartilaginous and synovial; fibrous and cartilaginous joints are synarthritic, synovial joints are diarthritic.[22] The synovial joint is encased by a joint capsule, which is lined with synovium and filled with synovial fluid. This fluid nourishes the articular cartilage that overlines the subchondral bone of two opposing bony surfaces (**Figure 1**).[23] When functioning optimally, the synovial joint facilitates the painless movement of the two bones by the peri-articular soft tissue structures.

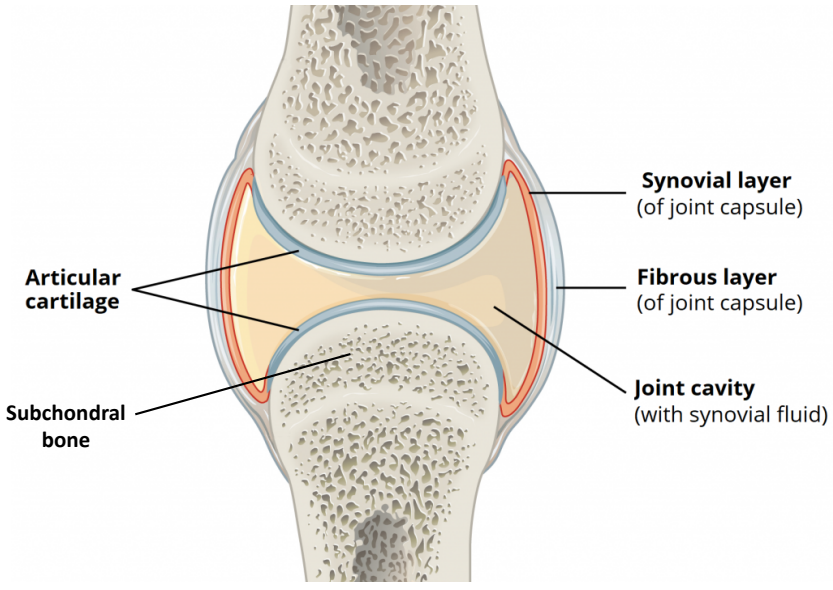


Figure 1. Basic anatomy of the synovial joint. (Adapted from https://teachmeanatomy.info/the-basics/joints-basic/synovial-joint/)

Synovium

The synovium is composed of two layers, the continuous surface intimal layer and the underlying subintimal layer; the subintimal layer consists of dense fibrous connective tissue, areolar tissue and fatty tissue, whereas the intimal layer is predominantly macrophages and fibroblasts (**Figure 2**).[24] The macrophagic cells (type A synoviocytes) phagocytose cell debris and waste produced by the joint cavity.[25] The fibroblast cells (type B synoviocytes) produce specialized synovial matrix constituents such as hyaluronan (HA), lubricin and collagens.[25] A third synovial cell, type C synoviocytes, has been described and is thought to be a transitional cell capable of becoming either type A or type B.[25] Beneath the intimal layer is a fenestrated capillary plexus and lymph network, essential for synovial fluid synthesis through the exchange of nutrients and metabolic wastes.[23]

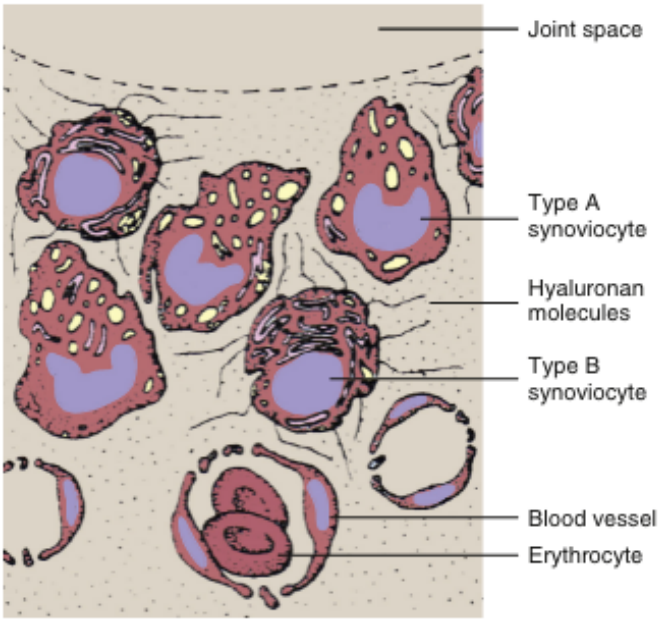


Figure 2. The synovial membrane (schematic). The subintimal layer contains macrophagic type cells (type A synoviocyte) that phagocytose debris and secretory type cells (type B synoviocyte), as well as a transitional cell (type C synoviocyte, not pictured). The predominant secretory molecule is hyaluronan. (From van Weeren R. P. (2016) General anatomy and physiology of joints. In: McIlwraith C. W., Frisbie D. D., Kawack C. E., van Weeren R. P. (Eds). Joint Disease in the Horse (2nd ed.) (p. 11). Philadelphia, PA: W. B. Saunders.)

The synovial membrane acts as a semipermeable membrane: it produces nutrients essential for maintaining the articular cartilage and also removes products of chondrocyte metabolism. The membrane controls molecular traffic in and out of the articular cavity, maintaining the composition of the synovial fluid.

Synovial Fluid

Synovial fluid is essentially an ultrafiltrate of plasma and as such is continually absorbed and replenished by the synovial intima.[21] Ultrafiltration across the fenestrated synovial capillary membrane is driven by a net Starling pressure imbalance: the pressure gradient from capillary plasma to synovial interstitium, minus the difference in colloid osmotic pressure.[26] The most abundant macromolecule of synovial fluid, albumin, maintains a relatively high colloid osmotic pressure within the joint.[26] The net effect is inward movement of plasma components that are less than 10 kDa in size, including oxygen, carbon dioxide, glucose and proteins. Larger molecules and cells present in plasma are excluded through a process known as steric exclusion.[27] Type B synoviocytes actively secrete the glycosaminoglycan HA and the glycoprotein lubricin into the synovial fluid, both of which maintain fluid viscosity, are chondroprotective and play key roles as boundary lubricants.[28] Additionally, articular cartilage is avascular and has no lymphatic or neural supply; nutritional and metabolic support for articular cartilage is derived from the synovial fluid.[29]

Joint capsule and periarticular soft tissues

The fibrous joint capsule, peri-articular ligaments and tendons, as well as the surrounding nerves and musculature, provide mechanical stability to synovial joints.[21] Joint stability is essential for

normal joint function: an unstable joint creates an abnormal loading scenario that damages the articular surface. The joint capsule functions as part of the seal that keeps synovial fluid within the articular space, limits the movement of the joint and facilitates proprioception via mechanoreceptors.[30] Additionally, sensory free nerve endings and corpuscular nerve endings are present in abundance in capsular tissue.[31] This rich innervation explains the severity of lameness seen in the horse following joint injury. Ligaments and the fibrous joint capsule are composed of type 1 collagen, elastin and proteoglycan.[30] The attachment of both structures to bone is considered in 4 layers: the ligament/capsule, the uncalcified fibrocartilage, the calcified fibrocartilage and the bone. The ligamentous/capsular collagen fibres form a grid, embedded within the fibrocartilage, firmly attached to the bone below and inferring high pull-out strength.[32]

Within the temporomandibular and the femorotibial joints are menisci, fibrocartilaginous discs that separate the opposing bony surfaces of the mandibular condyle and temporal bone or the proximal tibia and the distal femoral condyles respectively. They are composed of type 1 collagen, meniscal cells and an extracellular matrix (ECM) of water, collagen and proteoglycans.[33] This structure is similar to articular cartilage and has similar function: dissipation and redirection of compressive forces via the movement of water (see *Articular cartilage*). Their shape provides congruency to the joint and additional stability.[34]

Articular cartilage

Articular cartilage is a specialized connective tissue that has the principal functions of maintaining a smooth, lubricated surface for frictionless movement and conducting load to the underlying subchondral bone.[35–37] Articular cartilage is comprised of chondrocytes and an ECM

consisting of a collagen fibril network in a concentrated solution of proteoglycans, water, inorganic salts, glycoproteins and lipids (**Figure 3**).[21,38] Chondrocytes and the ECM function interdependently; chondrocytes synthesize and degrade the ECM, while the ECM creates a homeostatic environment for chondrocytes to function.

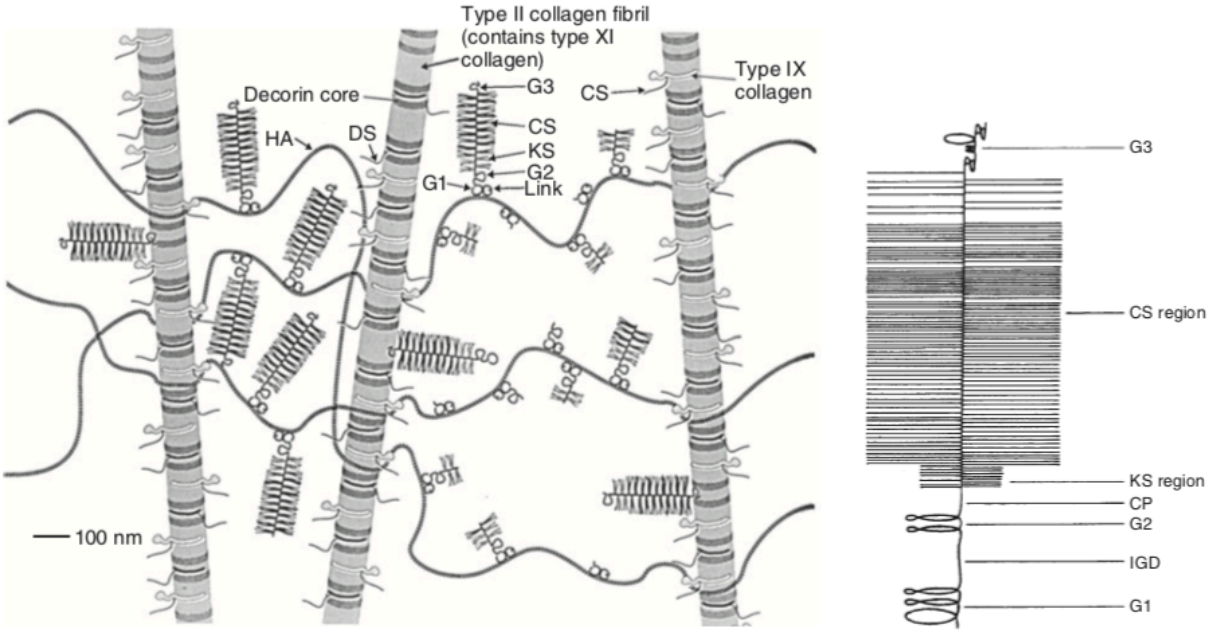


Figure 3. Cartilage extracellular matrix (ECM, schematic). Cartilage ECM is composed of aligned collagen fibrils with hyaluronan (HA) molecules. Each HA is surrounded by aggrecan monomers (right inset): a central protein (CP) with three globular domains (G1 to G3), which are each attached to negatively charged glycosaminoglycans (GAGS; chondroitin sulphate, CS and keratin sulfate, KS). The aggrecan monomers are stabilized to HA via G1 and a link protein. The negative charge of aggrecan GAGs (CS, KS) attracts water and infers compressive stiffness. (From Frisbie D. and Johnson S. (2018) Synovial joint biology and pathobiology. In: Auer J. and Stick J. (Eds). Equine Surgery (Fifth ed.) (p. 1332, 1333). St. Louis, MO: Elsevier.)

A major component of the ECM are proteoglycans, these include aggrecan, decorin, bigylcan and fibromodulin.[39] Proteoglycans consist of a large, central protein core to which multiple glycosaminoglycans (GAGs) are attached.[21] Aggrecan, the predominant proteoglycan, has three different GAGs: chondroitin-4-sulfate, chondroitin-6-sulfate and keratin sulfate.[21,40] The GAGs molecules have high negative charges which repel each other and create a large osmotic swelling

pressure.[41] Interstitial fluid (mostly water) moves in, the GAG molecules become hydrated in an attempt to increase separation from each other, the aggregates consequently occupy a large volume. The collagen fibril network acts as a framework, containing the aggrecan and providing a limit to its expansion, thus providing integrity to the tissue. As cartilage is loaded, the interstitial fluid pressurizes against the collagen network and absorbs the bulk of the force, protecting the ECM.[42] As the fluid moves out of the tissue the GAG chains are forced closer together: the mutual repulsive force of the negatively-charged chains contributes to the anti-compressive function of cartilage.[43] This in and outward movement of fluid is known as the biphasic lubrication theory of articular cartilage.[44] The solid phase of the cartilage is the collagen-proteoglycan network, while the fluid phase is the interstitial fluid and ions. The ability of the cartilage to resist deformation under load (stress-strain relationship) is hypothesized to depend on the interaction of these two phases, i.e. the fluid movement through the cartilage ECM dictates the biomechanics of cartilage. The triphasic theory considers both the biphasic relationship and the ionic charge of the proteoglycans (and hence the osmotic potential of cartilage).[45]

The structure of cartilage and the properties of the ECM constituents dictate the function and properties of cartilage. Articular cartilage is organized into four zones (from articular surface to bone) based on chondrocyte and collagen composition: the superficial zone, the intermediate zone, the deep zone and the calcified zone.[21] The spatial organization of the collagen network is known as the 'Arcades of Benninghoff'.[46] In the superficial zone, chondrocytes are most abundant and collagen is densely packed and arranged parallel to the joint surface.[47] The parallel orientation of the collagen fibers in this zone infer the cartilage with anti-shear properties. Within

the intermediate zone, the collagen fibril arrangement is randomly arranged. In the deep zone the fibrils are perpendicular to the articular surface. In this zone there is the largest concentration of aggrecan, resulting in resistance to compression (see earlier discussion). Mature articular cartilage is separated from the subchondral bone by a thin layer of calcified cartilage, in which the collagen fibers are encased with osteoid secreted from osteoblasts.[48] These fibers anchor the cartilage to the bone. A 5 μm thick tidemark separates the calcified and non-calcified cartilage portions. This structure-function relationship is important; during osteoarthritis (OA) the structure of cartilage is dismantled and thus the function of cartilage is compromised (see *Pathophysiology of osteoarthritis*). In many cell-culture models of OA, the structure of cartilage is not captured and thus this important component is lost. In explant-based models, structure of tissues can be maintained and this effect can be captured in the data (see *Models of OA*).

Subchondral bone

Deep to the calcified cartilage is the subchondral bone plate, a compact layer of bone around 10 μm to 3 mm thick that separates the cartilage from the trabecular bone adjacent to the medullary cavity.[49] The inorganic component of subchondral bone is similar to cortical bone, in that it is predominantly comprised of hydroxyapatite crystals, however the inorganic components more closely resemble that of cartilage: collagen, proteoglycan, GAGs and water.[50] This unique composition affords the subchondral bone plate with more elasticity and pliancy than cortical bone; subchondral bone is 10 times more deformable than the cortical shaft of long bones, making this tissue more optimized to dissipate the forces of locomotion.[51] Wolff's Law states that bone adapts in response to load. The rich vascular and neural supply of subchondral bone

facilitates an extensive morphological response to any physiological or pathological process within the adjacent cartilage and bone, resulting in a highly adaptable articular component.[50]

Pathophysiology of osteoarthritis

Osteoarthritis (OA) has long been established as a significant pathology causing compromise to welfare and loss of use of the horse.[52] In human medicine, there is a broad consensus that OA can be defined as "a group of overlapping distinct diseases which may have different etiologies, but with similar biologic, morphologic and clinical outcomes. Disease processes not only affect the articular cartilage but involve the entire joint, including the subchondral bone, ligaments, capsule, synovial membrane and peri-articular tissues (**Figure 4**). Ultimately the articular cartilage degenerates with fibrillation, fissure formation, ulceration and full thickness loss of the joint surface".[53] A similar definition can be applied to the equine patient; OA cannot simply be thought-of as a disease of cartilage, but rather a group of differing pathologies that may affect any of the peri-articular tissues and manifests as progressive disruption of articular cartilage.[54]

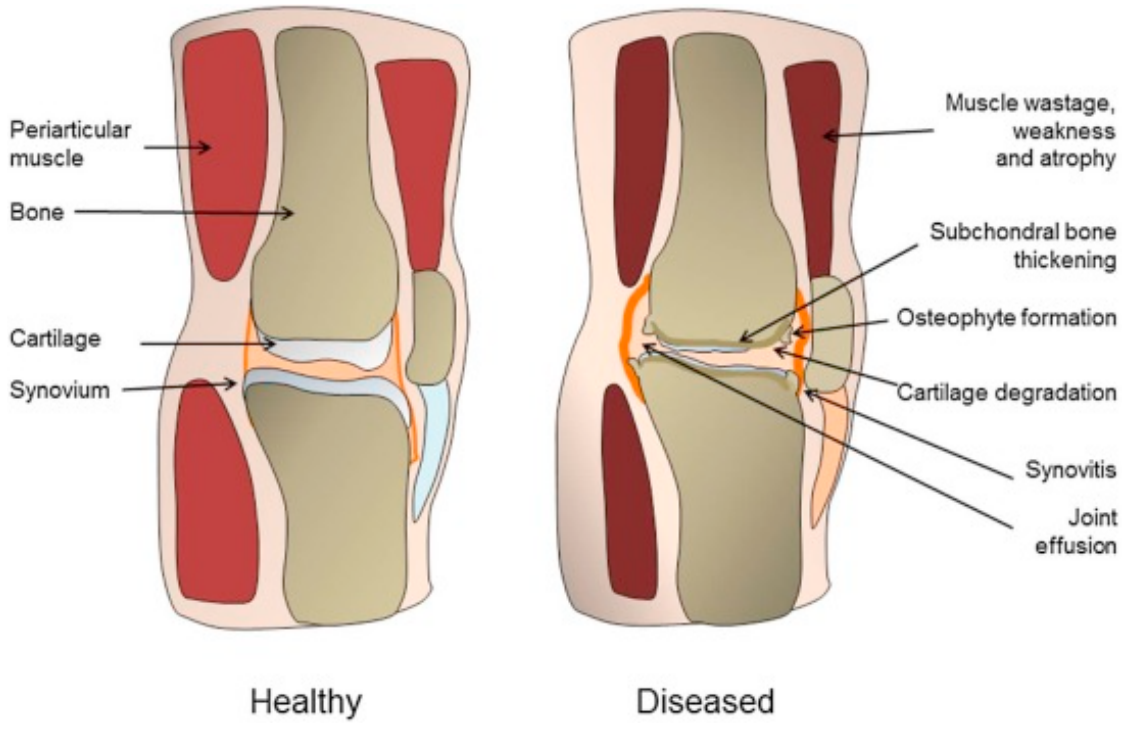


Figure 4. Healthy versus osteoarthritic synovial joints. (From Johnson C.I., Argyle D.J., Clements D.N. In vitro models for the study of osteoarthritis. Vet J. 2016 Mar;209:40-9.)

The synovium and synovial fluid in osteoarthritis

The role of the synovium in OA is likely related to biomechanical and inflammatory factors.[55] Usually, trauma to the synovial membrane establishes a synovitis that is resolved through the action of synovial macrophages.[56] However, in osteoarthritic joints, macrophages further perpetuate inflammation and their activation is directly correlated to disease severity and pain.[57] Synovitis may be established by primary trauma to the synovial membrane or joint capsule, or secondary to pathology in one or more of the other joint tissues. Histologically, the synovial membrane becomes edematous, there is cellular infiltration of inflammatory cells and hypervascularity. Chronic inflammation results in hyperplasia of the intimal layer and fibrosis of the subintimal layer, resulting in a synovial membrane that is less compliant.[58] This change in biomechanical property translates to a reduced range of motion and greater chance of trauma, which in turn further perpetuates inflammation. Type B synoviocytes, in addition to the synthesis of key synovial structural components, also synthesize a variety of mediators implicated in the pathogenesis of OA, including pro-matrix metalloproteinases, cytokines and prostaglandins.[59–61] These mediators can act upon the synovial membrane itself, or move through the synovial fluid to act on the articular cartilage. While OA was traditionally thought-of as a disease of cartilage, the importance of the synovium in disease initiation and progression cannot be overstated.[57,59,62]

The changes to the synovium in synovitis directly results in the permeability of the synovial membrane becoming altered, and thus affects the composition of the synovial fluid.[63] Usually, large molecular weight molecules such as lubricin and HA are maintained within the joint. Lubricin is a glycoprotein found in synovial fluid that acts as a boundary lubricant: it reduces the coefficient of friction of the articular cartilage surface.[64] There is controversy in the literature as

to whether lubricin increases or decreases in osteoarthritis; in most large animal models increased lubricin is reported.[65,66] However, in equine OA the glycosylation profile of lubricin is altered, negatively affecting the boundary lubricating properties and potentially increasing the friction at the articular cartilage surface.[67] In contrast, in synovitic and osteoarthritic joints, there is a net loss of HA from synovial fluid.[68,69] Hyaluronan, similar to lubricin, is an important boundary lubricant for articular cartilage. It is also provides the synovial fluid with its viscoelastic properties and therefore significantly contributes to the mechanical function of the joint.[70]

The joint capsule and periarticular soft tissues in osteoarthritis

The importance of the peri-articular supportive structures should not be overlooked. For example, suprascapular nerve damage results in loss of the stabilizing function of the supraspinatus and infraspinatus muscles, resulting in lateral instability of the scapulohumeral joint.[71] Chronic joint instability accelerates OA development.[72]

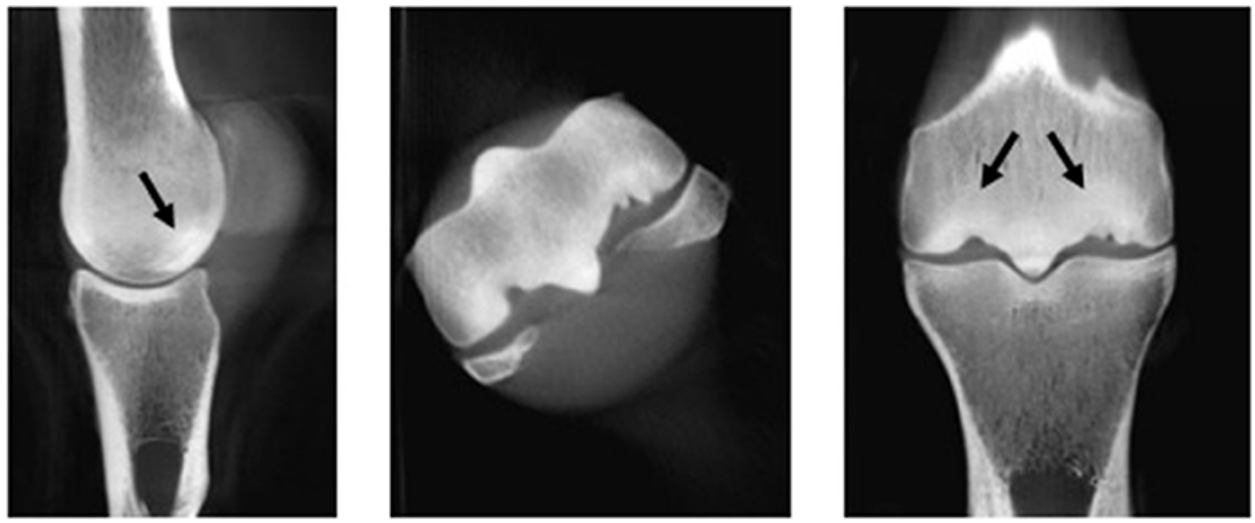
Damage to the joint capsule, either directly or secondary to sustained synovitis, results in capsulitis and hyperplasia, reduction in capsular compliance and subsequently reduced range of motion of the joint. The richly innervated joint capsule contains nociceptors which transmit the pain signal via the ascending pathway and a number of neurotransmitters (including bradykinin and substance P).[73] These same receptors are found to a lesser extent in the synovial membrane, periarticular ligaments, periosteum and subchondral bone. The catabolic cytokines released into the synovial fluid by synoviocytes and chondrocytes stimulate inflammation and cartilage matrix degeneration (see *Catabolic pathways in osteoarthritis*). The inflammatory mediators cause

peripheral sensitization of the joint nociceptors, creating hyperalgesia and further perpetuating pain.[74] Nociceptive pathways that are chronically stimulated are upregulated, enhancing pain transmission and resulting in central sensitization. The development of central sensitization can make pain from OA difficult to control.[75]

As mentioned previously, certain joints contain articular menisci; cartilaginous-like discs that support the structure and function of the joint. Disfunction of these menisci, for example due to tearing or age-related degeneration, disrupts the congruency of the joint and results in OA.[76]

The subchondral bone in osteoarthritis

A common cause of OA in the young horse is that which results from inappropriate cyclic loading to the subchondral bone, for example from overuse or conformational inadequacies. Bone responds to repetitive trauma by remodeling; excessive loading can overwhelm the capacity of the bone to remodel and causes significant changes to bone structure that negatively affect its elasticity and capacity for shock absorption.[77] The responsibility for this function is shifted towards the articular cartilage, a tissue which is unable to remodel in response to the increased stresses placed upon it, and disruption of the articular surface ensues. Sclerosis (loss of elasticity of the bone), osteophytes and clefting within the deep zone of the cartilage ensues.[50] Additionally, repetitive trauma to the subchondral bone results in a pro-inflammatory bone microenvironment that results in a similar inflammatory state within the articular space.[78] Pathology of the subchondral bone following repetitive high-speed loading is a pre-cursor to OA and pathologic fracture, and is an area of intensive research efforts in the horse racing industry (**Figure 5**).[79–81]



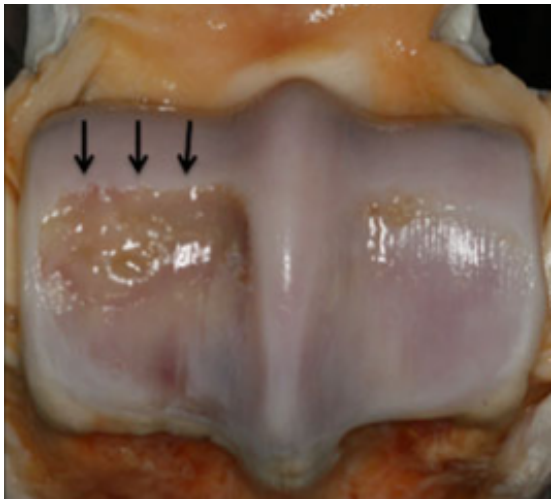


Figure 5. Subchondral bone disease in the equine fetlock. The fetlock is a common site for subchondral bone disease and subsequent cartilage erosion in the racehorse, known as palmar osteochondral disease. Radiographically (CT images above), sclerosis of the subchondral bone is seen (arrows), with loss of congruency at the articular surface. On post-mortem (left), degeneration of the articular cartilage can be profound, with exposure of the underling bone (arrows).

(From Stewart H. L. and Kawcak C. E. The importance of subchondral bone in the pathophysiology of osteoarthritis. Front. Vet. Sci., 28 August 2018;5:178; From Davis, A.M., Fan, X., Shen, L., Robinson, P. and Riggs, C.M. Improved radiological diagnosis of palmar osteochondral disease in the Thoroughbred racehorse. Equine Vet J, 2017 49: 454-460.)

Articular cartilage in osteoarthritis

As stated previously, articular cartilage is comprised of ECM and chondrocytes (see *Articular cartilage*). The function of the chondrocytes is to regulate the homeostasis of the ECM via a balance of anabolic and catabolic pathways, both dismantling and rebuilding collagen and aggrecan.[21] In OA, the catabolic pathway is upregulated, resulting in a net loss of collagen, aggrecan and other proteoglycans. The loss of proteoglycans from cartilage results disrupts the

lubricating function of the cartilage resulting in an increase in the coefficient of friction at the articular surface.[82] Specifically, loss of proteoglycans decreases the osmotic potential of the ECM (triphasic model of lubrication) and depletes the solid and fluid phases (biphasic model of lubrication), resulting in a net loss of viscoelasticity of the tissue.[83] Increased friction at the articular surface is well-correlated with the progression of OA.[84] Cartilage has a limited capacity to repair, and progression of the ECM disruption can progress to full thickness erosions with exposure of the subchondral bone plate. These erosions are filled with fibrocartilage, a biomechanically-inferior tissue to hyaline cartilage, and the function of the joint is compromised.[85]

Catabolic pathways in osteoarthritis

Regardless of the specific etiopathogenesis, the precursor to an arthritic joint is a pro-inflammatory articular environment. Synoviocytes and chondrocytes upregulate several catabolic cytokines, primarily interleukin 1 (Il-1β) and tumor necrosis factor (TNFα).[74] In clinical cases of equine OA, TNFα was abundantly expressed in both cartilage and synovium, whereas Il-1β was primarily produced by the cartilage.[86] Other pro-inflammatory cytokines include Il-6, Il-15, Il-17 and Il-18. These cytokines directly stimulate the chondrocytes and synoviocytes to release matrix metalloproteinases (MMPs), enzymes that degrade different components of the ECM: collagenases degrade collagen, stromelysins degrade proteoglycans and gelatinases further degrade denatured collagens, aggrecan and elastin.[21] In particular, collagenase-3 (MMP-13) has been shown to aggressively degrade type 2 collagen and plays an important role in the induction and progression of OA.[87] Structurally-similar to MMPs, A Disintegrin and Metalloproteinase (ADAM) and A Disintegrin and Metalloproteinase with Thrombospondin Motif (ADAMTS) are

enzymes that cleave aggrecan (aggrecanases) and are also produced by articular cells in response to Il-1β and TNFα.[88] ADAMTS-4 may be the primary aggrecanase in equine OA.[86] Reactive oxygen species such as nitric oxide are produced by inflamed synoviocytes and act to upregulate the production of Il-1β and TNFα, as well as degrading hyaluronan and collagen and activating MMPs, further progressing the destructive cycle.[89] Additionally, pro-inflammatory cytokines induce prostaglandin E_2 (PGE_2) and the neuropeptide substance P, both of which mediate inflammation and contribute to the transmission of pain.[55,90,91]

Anabolic pathways in osteoarthritis

Several modulatory cytokines are also produced by articular cells, in an attempt to off-set the pro-inflammatory action of Il-1β and TNFα, as well as promoting anabolic cartilage metabolism. These include Il-4, Il-10 and Il-13.[92] These cytokines inhibit the secretion of MMPs, downregulating the degradation of the ECM. They also promote the synthesis of MMP inhibitors (tissue inhibitor of matrix metalloproteinase, TIMP); these molecules directly bind to MMP enzymes to form TIMP-MMP complexes with no enzymatic activity.[93] Similarly, natural inhibitors of Il-1β and TNFα (e.g. Il-1 receptor antagonist), bind and neutralize these cytokines. A large family of cytokines, the transforming growth factor (TGF) family, stimulate chondrogenesis, induce chondrogenic differentiation of mesenchymal stem cells in the synovium and increase the production of cartilage ECM.[94] Insulin-like growth factor (IGF) promotes chondrocyte proliferation and ECM production.[95] In healthy joints, there is a balance between anabolic and catabolic pathways, in OA catabolic processes dominate (**Figure 6**).

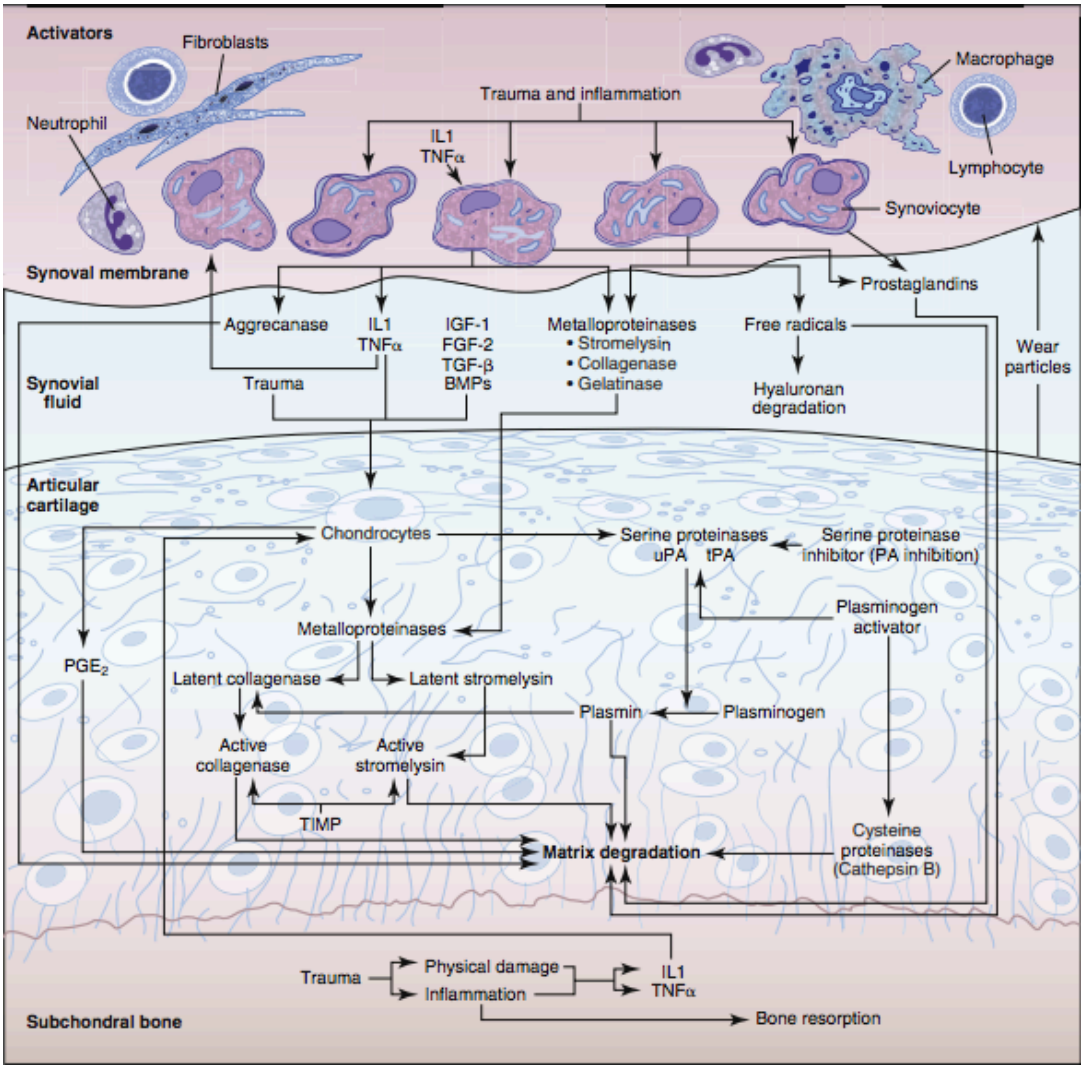


Figure 6. Summary of factors in equine osteoarthritis. It is worth noting that chondrocytes also produce inflammatory cytokines after insult. Key: BMPs, bone morphogenetic proteins; FGF-2, fibroblast growth factor-2; IGF-1, insulin-like growth factor-1; IL1, interleukin-1; TGF-β, transforming growth factor-β; PA, plasminogen activator; PG, prostaglandin; PGE_2, prostaglandin E_2; TIMP, tissue inhibitor of metalloproteinases; TNFα, tumor necrosis factor-α; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator. (From McIlwraith C. W. (2016) Traumatic arthritis and posttraumatic osteoarthritis in the horse. In: McIlwraith C. W., Frisbie D. D., Kawack C. E., van Weeren R. P. (Eds). Joint Disease in the Horse (2nd ed.) (p. 11). Philadelphia, PA: W. B. Saunders.)

In summary, OA is initiated by an articular disturbance, whether this is by mechanical disruption or biochemical deficiency, and progresses to a catabolic imbalance within the joint. This creates a pro-inflammatory environment, resulting in progressive dismantling of the cartilage ECM,

maladaptation of the subchondral bone, inflammation and fibrosis of the synovium with restriction of the joint capsule. The dismantling of the cartilage ECM results in loss of the lubrication function of the cartilage as proteoglycans are depleted, increasing friction at the cartilage surface. Nociceptive pathways are up-regulated and biomechanical function is decreased, resulting in painful joints with limited range of motion, compromising the welfare of the horse.

Models of OA

The widescale impact of OA (see Introduction) in man and in horses has directly resulted in an intense academic interest in the understanding of its pathogenesis and the development of potential therapeutics. This has resulted in the development of a wide range of models, both *in vitro* and *in vivo*, which aim to replicate the arthritic scenario.[96] Each model has advantages and disadvantages; currently no perfect model exists for OA.

In vitro – within the glass

Effective animal models are essential for testing therapeutics prior to human clinical trials. The associated costs, longer timelines and ethical concerns necessitate *in vitro* hypothesis testing prior to animal trial initiation. These models can be categorized as 2D culture (monolayer or co-culture), 3D culture (scaffold-free or scaffold-based), *ex vivo*/explant-based culture, dynamic culture, 3D biofabrication and organotypic models.[97] A summary is provided in **Table 1**.

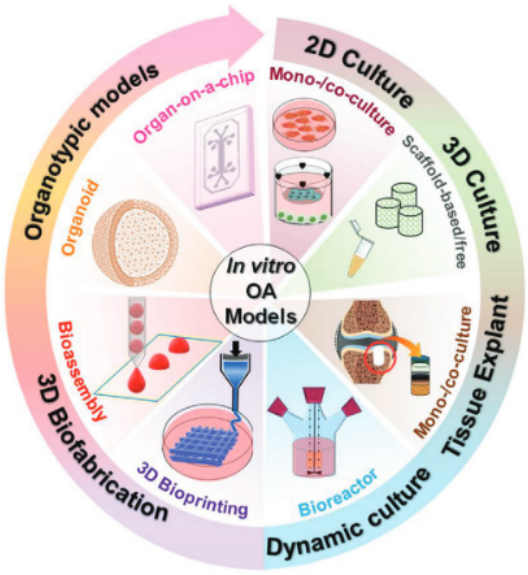


Figure 7. *In vitro* models of osteoarthritis (schematic). (From Singh Y.P., Moses J.C., Bhardwaj N., Mandal B.B. Overcoming the Dependence on Animal Models for Osteoarthritis Therapeutics - The Promises and Prospects of In Vitro Models. Adv Healthc Mater. 2021 Oct;10(20):e2100961.)

Table 1. Summary of *in vitro* models of osteoarthritis.

Model type	Subtype	Summary	Advantages	Disadvantages
2D culture	Monolayer	The culture of immortalized cell lines or primary cells on a flat surface in a culture flask, within nourishing cell media.[98]	Fast, inexpensive, reproducible. Allows expansion of cells from a single sample. Can investigate distinct cellular pathways in isolation.	Limited potential for investigating cell-cell or cell-ECM crosstalk. Cells can de-differentiate and change phenotype.[98] Variable impact of cell media.
	Co-culture	As for monolayer but with multiple cell types in a 2D environment.	Captures the effect of different tissue cell-cell crosstalk.	Can be more challenging and expensive than monolayer (different cells may require different culture conditions; chondrocytes less stable than synoviocytes).[99]
3D culture	Scaffold-free	Cultured mesenchymal stem cells (MSC) that undergo chondrogenesis. Micromass or pellet cultures are the most common. Micromass is a high-density culture system formed by seeding cells in droplets.[100] Pellet cultures/chondrospheres are formed by centrifugation.	Higher levels of chondrogenesis, production of ECM. More robust than monolayer cultures.[101] Can use in a co-culture system so captures different cell type crosstalk.[102]	Cannot be subjected to mechanical forces so unlikely to capture the true pathophysiology of OA. Reduced proliferative capacity of cells. Poor long-term survival.[103] Requires 2D culture first (slow).
	Scaffold-based	Natural or synthetic materials are used to provide a scaffold for MSCs, for example alginate hydrogel, fibrin alginate	Provides structural strength to cultured cells and can be subjected to mechanical forces. Greater proliferative	Expensive. Mechanical forces that can be applied are variable and may not by physiologic. Requires 2D culture first (slow).

		hydrogel, agarose or collagen microspheres.[104,105]	capacity than scaffold-free culture.	
Explant-based culture	Mono-tissue	Culturing tissue harvested from donors either at surgery or post mortem.	Maintains ECM and more closely replicates *in vivo* conditions than monolayer. Can subject explants to mechanical stimulation.[106]	Does not capture different cell-type cross-talk. Expensive, can be difficult to maintain for longer periods. Depends on availability of donors. High donor variability.[107]
	Co-culture	As for mono-tissue but includes multiple tissue types e.g. synovium and cartilage.	As for mono-tissue but is closer to *in vivo* conditions due to capture of different cell-type cross-talk.[108,109]	Expensive, can be difficult to maintain, develops on availability of donors and donor variability.
Dynamic culture	Bioreactor	Culturing cells or larger tissues in vessels that enable mechanical stimulation.	Extended culturing time and can involve multiple tissues.[110] Captures effect of mechanical stimulation on cartilage.[111]	Requires more instrumentation and expertise than traditional culture. Expensive.
Biofabrication	Bioprinting	Cells are cultured within 3D printed scaffolds made from natural or synthetic polymers or bioceramics.[112] Allows direct spatial arrangement of cells.	Material extrusion printing is widely available, low cost and can use a variety of materials. Potential for use in clinical cases to heal cartilage defects.	Technique is not standardized, difficult to incorporate multiple tissue types. Lengthy printing time.
	Bioassembly	Assembly of a 3D structure through cell-driven self-organization of microtissues,	Fast, high cellular density, autonomous/more life-like assembly.	Hard to influence the outcome/shape during assembly process.[107]

		achieved by applying molds or microfluidics.[113]		
Organotypic	Organoid	3D multicellular tissue construct that mimics the corresponding organ, grown from pluripotent stem cells.[114]	Can generate a large tissue mass, somewhat more complete mimicking of *in vivo*.	Lacks vascular/immune supply of a true organ. Gene expression from organoids matches fetal tissue. Large amount of variability. Greater expertise required.
	Organ-on-a-chip	A microfluidic cell culture device created with microchip-manufacturing methods, which contains continuously perfused chamber(s) inhabited by living cells arranged to simulate tissue-level or organ-level physiology.[115]	More precise evaluation of different tissue crosstalk, increased capture of biological processes. More controlled than organoids.[116]	Expensive, complex, require expertise and specialized instrumentation.

In vitro models require induction of OA via mechanical stimulation or cytokine/chemical induction. Mechanical stimulation of articular tissues is required for optimal joint homeostasis and therefore loading is recommended in culture models, with supra-physiological loads required for OA induction.[117] Cytokine stimulation of tissues, usually with 10 μg/ml IL-1β and/or 10 μg/ml TNF-α, is a straightforward method of replicating the pro-inflammatory articular environment. However, it is important to note that these concentrations are much higher than in naturally occurring OA in order to facilitate rapid progression of the *in vitro* model.[97] One study that assayed the synovial fluid of human patients with OA for IL-1β and TNF-α found average concentrations of 21 pg/mL and 80 pg/mL respectively.[118] Though these models cannot fully replicate the true scenario, they are useful for screening therapeutics prior to progression to animals models.

In vivo – within the living

Despite the considerable progress of *in vitro* OA models, they cannot replicate the complexity of the living organism. Animal models are essential for the development of disease-modifying OA drugs, and for gaining further understanding as to the pathogenesis of OA. Ideally, the animal should be biomechanically, histologically and anatomically similar to humans and the model result in disease that is similar to the human condition. Similar to *in vitro* models, no animal model perfectly replicates the human clinical scenario, and as such many different species and techniques for OA induction are utilized. Animal models in at least 18 different species have been developed.[119] These include mice, rats, guinea pigs, cats, rabbits, dogs, goats, sheep, horses, zebrafish, pigs, cattle and non-human primates. While smaller animals are easier to handle and cheaper to manage, their osteochondral unit is anatomically distinct to that of humans, whereas

larger mammalian species are more similar. The ethical and emotional impact of the selected species should also be considered when deciding upon an animal model as well as the natural life-span of the animal involved.[120]

The horse represents a useful model for post-traumatic OA.[54] Their size means that biological fluids such as blood or synovial fluid are more readily available than smaller animals. Additionally, joints such as a stifle are large enough to facilitate arthroscopic evaluation of the articular space or detailed diagnostic imaging examination, facilitating data-gathering at multiple time frames rather than just post euthanasia. A controlled exercise program is easy to implement in horses. The prevalence of OA in horses has directly resulted in concentrated research effort and experience in clinical equine OA, and as such a full genome is available.[119] There are several important anatomical and histological similarities between the stifle and the human knee, despite the obvious bipedal versus quadrupedal difference. One report determined that the average cartilage thickness in the femorotibial joint is 1.5-2 mm in horses, which is similar to the 2.2-2.5 mm seen in man.[121] The distribution of GAGs, collagen and chondrocytes throughout cartilage is similar in horses as it is in man.[122] Horses have femorotibial menisci, also seen in man, and similar loading patterns through their stifle joint. These factors, and the general availability of horses, have resulted in the popularity of horses in pre-clinical OA research.

Nevertheless, there are several important disadvantages to the equine model. Horses require special facilities, experienced personnel and are expensive. Surgery and many advanced imaging modalities require general anesthesia, which carries a risk of injury or death that is increased compared to other species.[123,124] The horse loads the stifle for significantly more hours per day

than the human loads the knee; the potential impact of this difference is not well defined. Horses are herbivores and humans are largely omnivores; this different diet has proteonomic implications, for example horses have a different phospholipid profile to the synovial fluid.[125] Lastly, most experimental models require induction of OA in one joint per horse, this results in the individual response to injury influencing the dataset. More horses are therefore required for statistically sound results, which has ethical and financial implications.

There are several different methods for inducing OA in the horse, including IA injection of chemicals, instability, osteochondral fragmentation with exercise, trauma, spontaneous and disuse.[126] A summary of the different equine *in vivo* OA models described is provided in **Table 2**.

Probably the most commonly utilized model is the carpal osteochondral fragment-exercise model, developed at Colorado State University in the early 90's.[127] In this model an 8 mm osteochondral fragment is created arthroscopically in the distal dorsal aspect of one of the radial carpal bones (**Figure 8**), creating progressive OA without causing severe lameness.[54] The opposite intercarpal joint serves as a control. The horses then undergo a controlled exercise program, with intermittent lameness evaluation, diagnostic imaging and synovial fluid analysis, until 70 days when they are euthanized. This typically results in changes in increased synovial fluid total protein concentrations, synovial membrane hyperplasia and fibrosis and articular cartilage erosions, similar to those seen in post-traumatic OA.[128,129] This model has been utilized to test multiple therapeutics, including several different corticosteroids (see *Intra-articular corticosteroid therapy*), and as such has likely influenced decision making for many equine

practicioners.[127,128,130–136] This model is not without disadvantages: it is expensive, requires specialist facilities and personnel, only utilizes one joint per horse and results in rapid progression of post-traumatic OA that likely does not capture the true pathogenesis of spontaneous OA.

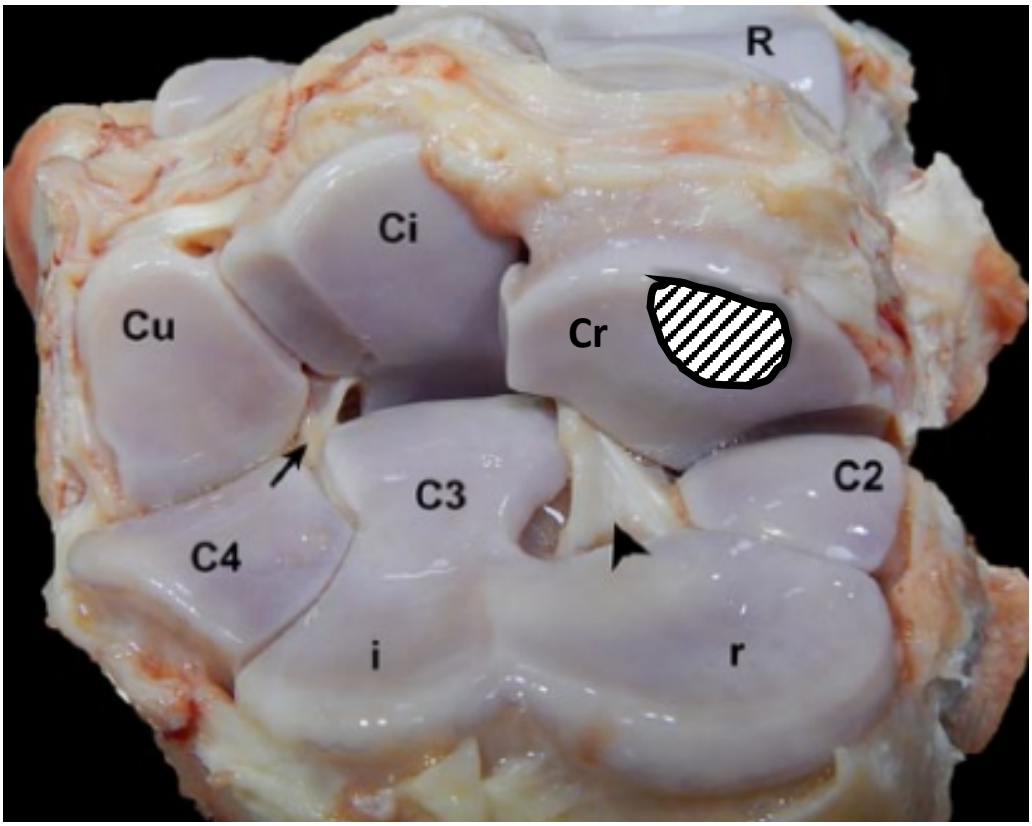


Figure 8. Location of the 8 mm radial carpal bone osteochondral defect (striped zone) as utilized in the carpal osteochondral fragment-exercise model. Key: R, radius; Cu, ulnar carpal bone; Ci, intermediate carpal bone; Cr, radial carpal bone, C2, second carpal bone, C3, third carpal bone, C4, fourth carpal bone; i, intermediate facet of C3; r, radial facet of C3; arrow, lateral palmar intercarpal ligament; arrowhead, medial palmar intercarpal ligament. (Adapted from Engiles J.B., Stewart H., Janes J., Kennedy L.A. A diagnostic pathologist's guide to carpal disease in racehorses. J Vet Diagn Invest. 2017 Jul;29(4):414-430.)

As stated previously, the lack of complete understanding as to the pathogenesis of OA means that there is no perfect, validated model. Advances in tissue engineering and continued refinement of animal models remains necessary for the development of effective therapeutics.

Table 2. Summary of equine *in vivo* models of osteoarthritis. (Adapted from McIlwraith C.W., Frisbie D.D., Kawcak C.E., Fuller C.J., Hurtig M., Cruz A. The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the horse. Osteoarthritis Cartilage. 2010 Oct;18 Suppl 3:S93-105.**)**

Model type	**Subtype**	**Summary**	**Advantages**	**Disadvantages**
Intra-articular chemicals	Filipin[137]	Injecting a chemical to cause synovitis (and capsulitis), results in increased articular inflammatory cytokine concentration (e.g. MMPs) and cartilage degradation without destabilizing the joint.	- Usually inexpensive and reversible - Rapid response seen clinically and histologically, so allows rapid progression to treatment with therapeutics - Easy to perform and requires minimal equipment	- Can cause profound lameness/pain - Does not capture the true pathogenesis of spontaneous OA - The individual response can differ - Usually just one joint at a time - The response to chemicals may also be different between joints[138]
	Sodium monoiodoacetate[139–141]			
	Amphotericin[142–146]			
	E. coli lipopolysaccharide[18,147–153]			
	IL-1β[69,138,154–158]			
	Polyvinyl alcohol foam particles[159]			
	Carrageenan[160–163]			
Instability	Transection of the collateral and collateral sesamoidean ligaments of the fetlock[164]	Desmotomy of supportive ligaments of the fetlock, destabilizing the joint.	- Rapid progression to OA - Produces radiographically apparent lesions - Potential to perform without general anesthesia	- Lesions can be severe and painful - Usually just one joint at a time - Requires surgical expertise

Surgically-created articular trauma and exercise	Osteochondral fragmentation [127–136,165,166]	Surgically created (arthroscopy, arthrotomy) osteochondral defects, grooves or full-thickness chondral defects. Blunt trauma models use a motorized instrument to apply a pre-determined amount of force to the cartilage.	- Lots of experience with these models - Reliably causes post-traumatic OA - Potential to include more than one joint - Lameness is usually mild	- Requires expertise and specialized equipment - Expensive - Wide variation in lesions produced - Lesions can be severe - Irreversible - Does not replicate spontaneous OA
	Cartilage groove[167–169]			
	Single impact blunt trauma[170–172]			
	Full-thickness chondral defects [173,174]			
Disuse	Cast immobilization[175–177]	Induction of osteoporosis via casting. Causes osteochondral fragmentation, joint effusion and lameness.	- Inexpensive - Easy to do - Results in spontaneous OA	- Can be lengthy - Less experience with this model
Spontaneous	Naturally occurring OA[178]	Long term research herds with OA that develops naturally,	- Natural OA is ideal - Ideal for pathogenesis studies	- Very long term and therefore expensive - Requires horses to have a painful disease (OA) for a long period of time - Unable to standardize between individuals

Principals of diagnosis of equine OA

Horses with a diagnosis of OA typically present for lameness or decreased performance.[15] In the horse, spontaneous, post-traumatic and septic arthritis are described; the etiology and stage of the disease dictates the severity of clinical signs, clinicopathological parameters and diagnostic imaging findings.

The lameness examination starts with a detailed history and musculoskeletal examination. Any history of trauma or use for strenuous activity such as flat racing or barrel racing increases suspicion for post-traumatic OA. If the horse has a history of a penetrating wound into a synovial structure, degenerative joint disease associated with sepsis may be more likely.[179] Joint disease can often be detected by judicious palpation of synovial structures: horses with synovitis subsequent to OA often have effusion of the respective joint.[21] In chronic cases, synovitis results in fibrosis and reduced compliance of the joint capsule, with resultant decreased range of motion and subjective hardening of the capsule. Boney proliferation can sometimes be appreciated at articular surfaces, for example at the abaxial aspects of the pastern, colloquially known as 'high ringbone' (**Figure 9**).

Figure 9. Photograph of a horse with proximal interphalangeal joint osteoarthritis 'high ringbone'. (From http://www.horsedvm.com/disease/ringbone/)

Once the static examination is completed, the clinician can progress to dynamic evaluation of the horse. Successful subjective evaluation of the horse's gait is dependent on the skill and experience of the diagnostician. Use of objective measures, such as computerized sensor-based lameness evaluation systems, should be considered to aid and provide unbiased documentation of the examination.[180–182] Ideally, the complete lameness examination should involve perineal or intra-articular analgesia (see *Local anesthetic use in horses*).

Arthrocentesis, for example prior to IA administration of local anesthetic or OA therapeutic, provides the additional benefit of allowing sampling of the synovial fluid. Macroscopic inspection of arthritic fluid typically reveals a decrease in viscosity, suspected to be due to decreased HA concentration.[183] Standard synovial fluid analysis parameters include nucleated cell count, total protein and cytological determination of cell morphology; these parameters are relative to the degree of inflammation and can be influenced by repeated needle sticks as well as by various medications and systemic health status.[184,185] Evaluation of these conventional parameters is less useful in the early stages of disease when intervention has a higher chance of success, therefore more sensitive markers of OA have been sought.

Biomarkers of OA are molecules that are products or by-products of metabolic processes that occur during OA.[186] Ideally, they should detect early-stage joint damage or provide information that can stage the disease and predict the cause or prognosis. Several biomarkers have been identified in equine synovial fluid, plasma and urine, though translation of their use from the laboratory to the clinical setting has been lacking. A summary of selected biomarkers identified in horses is provided in **Table 3**.

Table 3. Brief description of selected biomarkers identified in equine OA. Note – this is not an exhaustive list and this is an ever-changing field. Key: SF, synovial fluid; OA, osteoarthritis

Biomarker type	Example	Sample	Notes
Catabolic cytokines	Interleukin-1β, IL-1β[187–189]	SF Serum	Major catabolic cytokine implicated in OA. - Concentrations rose prior to the onset of symptoms in an *in vivo* model.[187] - Levels were increased in racehorses with OA compared to controls, however differences not seen at early timepoints.[188] - Not more effective than SF cell count at predicting clinical joint disease.[189]
	Interleukin-6, Il-6[187–190]	SF Serum	Catabolic cytokine. - Concentrations rose prior to the onset of symptoms in an *in vivo* model.[187] - Dramatically elevated in horses with natural osteochondral chip fractures/traumatic OA.[190] - Elevated in racehorses without OA, affected by exercise.[188] - Very well correlated with clinical joint disease.[189]
	Tumour necrosis factor-α, TNF-α[187,189–191]	SF Serum	Major catabolic cytokine implicated in OA. - Concentrations rose after the onset of symptoms in an *in vivo* model.[187] - Activity was low in clinical cases with chip fractures/traumatic OA.[190] - Levels were increased in racehorses with OA compared to controls, however differences not seen at early timepoints.[188] - Not more effective than SF cell count at predicting clinical joint disease.[189] - Concentrations were not correlated with cartilage damage in a post mortem study.[191]

Degradative enzymes	Matrix metalloproteinase 9 (Gelatinase B), MMP-9[187,191]	SF	Expressed by chondrocytes, denatures aggrecan, fibronectin, collagen, procollagens, link protein, decorin and elastin.[21] - Concentrations rose prior to the onset of symptoms in an *in vivo* model.[187] - SF concentrations were highly correlated with cartilage damage in a post mortem study.[191]
	Collagenase 3, MMP-13[187,192]	SF	Expressed by chondrocytes, denatures collagen, aggrecan, fibronectin.[21] - Concentrations rose prior to the onset of symptoms in an *in vivo* model.[187] - Concentrations rose dramatically in an LPS-synovitis model of OA.[192]
	A disintegrin and metalloproteinase with thrombospondin motifs 5 (Aggrecanase), ADAMTS-5[187]	SF	Denatures aggrecan. - Concentrations rose prior to the onset of symptoms in an *in vivo* model.[187]
	Gelatinase A, MMP-2[187]	SF	Expressed by chondrocytes, denatures collagen and elastin.[21] - Concentrations rose after the onset of symptoms in an *in vivo* model.[187]
	Stromelysin 1,	SF	Expressed by chondrocytes, denatures aggrecan, fibronectin and further dismantles denatured type 2 collagen. - Concentrations rose after the onset of symptoms in an *in vivo* model.[187]

	MMP-3[187]		
Markers of oxidative stress	Nitric Oxide, NO[193]	SF	Non-specific for joint trauma and levels affected by systemic disease. - Levels were increased following repeated arthrocentesis.[193]
	Diacron-reactive oxygen metabolites, d-ROMs[194]	SF	Used to calculate oxidative stress index. - Levels were increased in joints with carpal bone fracture, indicating high oxidative stress in this joint.[194]
Markers of inflammation	Prostaglandin E_2 PGE_2[129,189,192,193,195]	SF	Major eicosanoid in OA inflammation. - Significantly elevated in OA-affected joints compared to controls in a carpal osteochondral fragment-exercise model.[129] Was not increased by exercise in this model. - Concentrations rose dramatically in an LPS-synovitis model of OA.[192] - Very well correlated with clinical joint disease.[189] Significantly elevated in clinical cases compared to normal controls.[90,195] - Levels were increased following repeated arthrocentesis.[193]
	Substance P[192]	SF	Neurotransmitter, concentrations are increased in inflammation. - Concentrations rose dramatically in an LPS-synovitis model of OA.[192] - Concentrations increased in osteoarthritic horses, but not correlated with radiographic appearance of the joint.[90]
	Bradykinin[192]	SF	Potent inflammatory peptide. - Concentrations rose dramatically in an LPS-synovitis model of OA.[192]
Components of cartilage ECM/	Hyaluronan, HA[187,196]	SF	Component of cartilage ECM. - Concentrations rose prior to the onset of symptoms in an *in vivo* model.[187] - Levels were not different between horses with tarsal OA compared to controls.[196]

products of cartilage degradation			
	Glycosaminoglycan, GAG[129,187,192,193,197]	SF Serum	Concentrations are speculated to be increased in SF following cartilage proteoglycan degradation. - Concentrations rose prior to the onset of symptoms in an *in vivo* model.[187] - Significantly elevated in OA-affected joints compared to controls in a carpal osteochondral fragment-exercise model, however differences were small.[129] Also increased with exercise in this model. - SF concentrations rose dramatically in an LPS-synovitis model of OA.[192] - No correlation between SF GAG levels and severity of cartilage degeneration at post mortem.[197] - SF Levels were increased following repeated arthrocentesis.[193]
	Cartilage oligomeric matrix protein, COMP[187,188,196]	SF Serum	Cartilage-specific protein bound to type 2 collagen, released into SF after cartilage damage. - Concentrations rose prior to the onset of symptoms in an *in vivo* model.[187] - Levels were increased in racehorses with OA compared to controls, however differences not seen at early timepoints.[188] - COMP concentrations were lower in horses with tarsal OA compared to controls and levels were not correlated with radiographic changes.[196]
	Chondroitin sulphate, CS846[129,187,192,198,199]	SF Serum	Concentrations are increased in SF following cartilage proteoglycan degradation. - Concentrations rose prior to the onset of symptoms in an *in vivo* model.[187] - Significantly elevated in OA-affected joints compared to controls in a carpal osteochondral fragment-exercise model.[129] Also increased with exercise in this model. - No differences in concentration between horses with radiographic tarsal OA and controls.[198] - Significantly elevated in clinical horses with osteochondral fragments compared to healthy controls.[199] - Concentrations rose dramatically in an LPS-synovitis model of OA.[192]

	C-terminal crosslinked telopeptide type 2 collagen, CTX-II[187,188]	SF Serum	One of the primary products of type 2 collagen degeneration. - Concentrations rose prior to the onset of symptoms in an *in vivo* model.[187] - Levels were increased in racehorses with OA compared to controls, differences were seen at early timepoints and were more pronounced in later timepoints.[188]
	Type 2 pro-collagen, CPII[129,192,198–200]	SF Serum	- Significantly elevated in OA-affected joints compared to controls in a carpal osteochondral fragment-exercise model.[129] Also increased with exercise in this model. - Concentrations were well-correlated with radiographic signs of tarsal OA in clinical cases.[198] - Significantly elevated in clinical horses with osteochondral fragments compared to healthy controls.[199] - Concentrations rose dramatically in an LPS-synovitis model of OA.[192] - Levels were influenced by age in an LPS-synovitis model.[200]

Following localization of the source of pain in equine lameness, diagnostic imaging is performed. Modalities to assess equine joints include radiography, ultrasound, magnetic resonance imaging (MRI), computed tomography (CT), nuclear scintigraphy and positron emission tomography (PET).

Radiography and ultrasound are the most readily available in general practice. The maladaptation of the subchondral bone results in increased density of the bone (sclerosis) which is apparent on radiographs as areas of increased radiodensity. Other radiographic findings include presence of osteophytes, joint space thinning, subchondral lysis, osteochondral fragments, periarticular soft tissue mineralization, synovial effusion and eventual ankylosis of the affected joint.[21] It is important to note that radiographic changes underestimate cartilage pathology and often changes indicate more advanced disease.[201,202] Ultrasound is useful for examining peri-articular tissues such as the synovium and joint capsule, ligaments, menisci (when present) and in certain joints can estimate cartilage depth.[203] Additionally, osteophytes can be identified with ultrasonography.[204]

Advanced imaging techniques, such as CT and MRI, are becoming more readily available and provide a more complete assessment of articular health than radiography or ultrasound.[205] The soft tissue detail provided by MRI allows a thorough assessment of the peri-articular tissues such as supportive ligaments. In humans, MRI is considered the standard cartilage imaging modality and can provide detail concerning cartilage morphology and volume.[206] In the equine carpus, excellent correlation between MRI assessment and histologic measurements of cartilage and subchondral bone has been demonstrated.[207] Conversely, in the

metacarpophalangeal/metatarsophalangeal (fetlock) joint, MRI measurements of cartilage thickness are less accurate.[208,209] Additionally, several studies have demonstrated that MRI underestimates cartilage damage in equine OA.[210–212] The use of CT is postulated to provide more information as to the health of the subchondral bone, and correlation between CT-identified subchondral bone pathology and histologic cartilage damage has been demonstrated.[213] However, both modalities have been shown to have poor sensitivity for identifying defects in the cartilage, with one study demonstrating sensitivity of 33% and 18% for MRI and CT respectively.[214] Additionally, the shape of current units generally limits their use to the distal appendages and cranial cervical region.

The main limitation of these advanced modalities is the need for general anesthesia. Standing MRI units have been developed; however, standing units are low-field which lack the detail of high-field units. Studies have demonstrated the reduced sensitivity of low-field units for cartilage damage in the horse, limiting the use of this modality in early-stage OA.[215,216] Moreover, when the horse is weight-bearing the resulting compression of the articular cartilage can limit the reader's ability to accurately identify abnormalities.[217] Standing cone-beam CT has recently been advocated for use in the horse.[218] Robotics-controlled units have enabled evaluation of the subchondral bone in the fetlock, which may eventually allow this modality to become a screening tool.[219] Correlation between standing caudal cervical CT and several biomarkers of OA has been demonstrated.[220] However, research comparing this imaging modality to post mortem and histological findings is lacking.

Nuclear scintigraphy (bone scan) involves the intravenous injection of radio-isotypes such as technetium-99m (^{99m}Tc). These give off gamma rays which are detected using a scintillation camera. This isotope binds to exposed sites on inorganic hydroxyapatite crystals, these sites are exposed in areas of remodeling bone or mineralizing soft tissues.[221,222] As such, scintigraphy provides a functional evaluation of bone and is very sensitive for detection of active pathology.[223] Increased scintigraphic uptake is demonstrated in joints with experimentally-created OA.[224,225] The location of uptake intensity corresponds with the location of pathologic changes, however this modality cannot provide anatomic detail and therefore is more useful as a localizing tool rather than for focused joint assessment.[226]

Similar to nuclear scintigraphy, PET imaging uses a radioisotope, ^{18} F-sodium Fluoride, which has a high affinity for the hydroxyapatite complex (the mineral component of bone).[227] This can be combined with CT or MRI imaging to create 3D, functional images which can identify bone metabolic activity and synovitis in clinical cases of OA.[228] Recently, PET has been investigated for use in the horse.[229] A study that compared PET imaging with CT and scintigraphy of the racing fetlock found that PET identified areas of histologically-abnormal bone that were not detected with other modalities.[230] The authors speculated that this modality, particularly if combined with CT or MRI, may help identify horses suffering from early joint disease prior to progression to OA, however this modality is still in its infancy in the horse.

Despite the advances in diagnostic imaging, the gold standard for diagnosis of OA in the horse remains diagnostic arthroscopy (see *Surgical treatment of OA*).[231] Surgery also permits treatment and prognostication of lesions and therefore is of outstanding value in equine joint disease.

Local anesthetic use in horses

As stated previously, diagnosis of joint disease in the horse involves localization of lameness to the articular unit with perineal or intra-articular analgesia. Painful stimuli are transmitted to the central nervous system (CNS) via myelinated type A delta fibers and nonmyelinated C fibers.[232] Local anesthetics disrupt this transmission by blocking the trans-membranous movement of sodium ions in a dose-dependent manner.[233] Typically pain sensation is lost first, followed by cold, warm, touch and deep pressure sensation, and finally by loss of motor function.[234]

The molecular structure of all local anesthetics consists of three components: a lipophilic aromatic ring, an intermediate linkage (either an ester or an amide) and a terminal amine (**Figure 10**).[235] The structure of the intermediate linkage dictates the chemical stability and metabolism of the drug: esters are less stable and rapidly metabolized by plasma cholinesterase whereas amides are more stable and undergo hepatic metabolism. Examples of esters include cocaine, benzocaine, procaine and tetracaine. Examples of amides include lidocaine, mepivacaine, bupivacaine and ropivacaine.

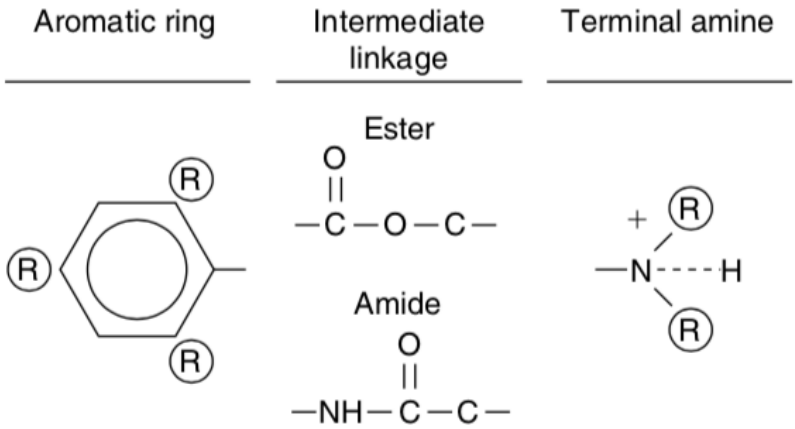


Figure 10. Basic structure of local anesthetics. (From Skarda R., Muir W., Hubbell J. (2009) Local anesthetic drugs and techniques. In: Equine anesthesia (2nd ed.) (p. 212). St. Louis, MO: W. B. Saunders.)

The properties of the different local anesthetics are dictated by their chemical structure and dictate how they behave in biological tissues. The properties of concern with regards to lameness examinations in horses include onset of action, duration, potency and safety to local tissues.[16,236] These properties are influenced by the dose administrated: increasing the dose shortens the onset, increases potency and duration of action and increases the risk for adverse events.[237] The properties of commonly used local anesthetic drugs are presented in **Table 4**.

The onset of action is dictated by lipid solubility, which in turn is determined by the dissociation constant (pKa).[238] The closer the pKa of the drug to the pH of the tissue, the more rapidly the drug can cross the neuronal lipid bilayer. For example, lidocaine has a pKa of 7.8 and mepivacaine has a pKa of 7.7, the closeness of these to the physiological pH of 7.4 results in both having a rapid onset of action.[239]

Duration of action is primarily determined by the ability of the drug to bind to the voltage-gated sodium channels in the neuronal membrane, i.e. their protein-binding capacity and affinity for the target receptor. For example, lidocaine has a protein-binding potential of 64% and mepivacaine has a protein-binding potential of 77%, thereby increasing the duration of mepivacaine relative to lidocaine.[240] Additionally, mepivacaine has less vasodilatory activity than lidocaine and is therefore cleared less readily from the neurovascualture.[241] The vasodilatory effect of local anesthetic can be offset by the addition of a vasoconstrictor, for example epinephrine. Addition of 0.5 ml of 1:1000 epinephrine to lidocaine to create a solution containing 5 μg/ml of epinephrine increases the duration of action and potency of palmar digital nerve blocks.[242] This effect also extends to more proximal nerves: this solution was found to be

equivalent to mepivacaine in alleviating lameness when performed as median and ulnar nerve blocks.[243]

Potency is defined, in this instance, as efficiency in resolving lameness and is dictated by lipid solubility, volume of local anesthetic used and characteristics of the tissue. When volume is controlled and the target tissue is the same, 2% lidocaine is less potent than 2% mepivacaine when administered as palmar digital nerve blocks as it is less efficacious at resolving foot pain in horses.[244] When the local tissues are inflamed, the local pH is lowered and the amount of local anesthetic present in the ionized form is increased, decreasing the lipid solubility of the drug and thus decreasing efficacy.[236]

Lastly, safety to local tissues is dictated by the drugs biocompatibility and local inflammatory response. Anesthetics have the potential to produce dose-dependent CNS toxicity proportional to their inherent potency, as well as cardiovascular and respiratory effects.[245] The volumes utilized for lameness examination are unlikely to produce systemic effects, but the clinician should be mindful in smaller patients when multiple blocks are performed. Lidocaine is considered to be more irritating to soft tissues than mepivacaine, however definitive evidence is lacking.[232] The same is not true for synovial structures, where local anesthetics differ vastly in toxicity to articular tissues, for example lidocaine is considered more toxic to cartilage than mepivacaine (see later discussion).

Table 4. Properties of commonly used local anesthetics. (Adapted from Skarda R., Muir W., Hubbell J. (2009) Local anesthetic drugs and techniques. In: Equine anesthesia (2nd ed.) (p. 212). St. Louis, MO: W. B. Saunders.)

Drug type	**Drug name**	**Lipid solubility**	**Relative potency to procaine**	**pKa**	**Onset**	**Plasma protein binding (%)**	**Duration of action (minutes)**
Ester	Procaine	0.5	NA	8.9	Slow	6	60-90
	Chloroprocaine	1	1	9.1	Fast	?	30-60
	Tetracaine	8	8.6	8.6	Slow	80	180-360
Amide	Lidocaine	3	2	7.8	Fast	64	90-180
	Mepivacaine	2	2	7.7	Fast	77	120-180
	Prilocaine	1	2	7.7	Fast	55	120-240
	Ropivacaine	15	6	8.1	Fast	95	180-360
	Bupivacaine	28	8	8.1	Intermediate	95	180-500

The lameness examination is complicated by several factors. The differing size of nerve fiber size and degree of myelination can result in blockade of pain fibers without abolition of skin sensation.[246] The region of skin desensitization can vary between horses after more proximal nerve blocks.[247] Additionally, skin desensitization can occur without the resolution of lameness, this occurs more often after blocks are performed with lidocaine than with mepivacaine, further confusing the interpretation of the lameness examination.[244] During the standard lameness examination, the clinician starts with the most distal nerves and works 'from the ground up', sequentially progressing to more proximal blocks when no improvement in lameness is seen.[16,232] However, numerous studies have indicated that proximal diffusion of the anesthetic occurs, increasing the chance for false positive results.[248–250] The process can be time consuming and the horse may not tolerate multiple needle sticks.

Intra-synovial local anesthesia is generally considered more specific than perineural anesthesia for a diagnosis of joint pain. The synovium and joint capsule contain numerous nociceptive nerve endings. Several articular pathologies that cause lameness are typically responsive to IA local anesthetic, these include synovitis, capsulitis, osteochondral fragmentation with cartilage erosion, peri-articular ligament or meniscus tears and osteoarthritis.[251] The need for life-long therapy for equine OA generally justifies the use of IA anesthetic to obtain a fast, accurate diagnosis, however there are numerous issues associated with their use. Several studies have demonstrated that the local anesthetic will diffuse out of the synovial structure to desensitize surrounding structures in a dose-dependent manner.[252–255] Complex synovial structures such as the stifle and tarsus, which contain multiple distinct compartments, demonstrate variable communication.[256,257] Diffusion of anesthetic from joints to peripheral nerves can result in

desensitization of distant structures, for example anesthesia of all three compartments of the stifle can reduce foot lameness.[258]

Another important consideration is the effect of local anesthetics on synovial tissues. Following some reports of chondrolysis associated with the use of pain pumps (continuous infusions of local anesthetic into the target joint, usually administered in man following arthroscopy), the compatibility of these drugs with synovial tissues has been questioned.[259] *In vitro*, bupivacaine, lidocaine, ropivacaine and mepivacaine all demonstrate dose-dependent cytotoxicity to human chondrocytes by increasing cell death, necrosis and apoptosis.[260–262] Lidocaine and bupivacaine are considered to be the more chondrotoxic of the anesthetic agents.[263] In an *in vivo* rat model, there were no differences between 0.5% bupivacaine-treated joints and saline controls in the short term, but at the six month time point a 50% reduction in chondrocyte density was observed, indicating long-term toxicity.[264] A similar reduction in live chondrocytes was see after IA injection of 2% lidocaine in rabbits.[265] These two drugs were also evaluated in horses, with a small *in vivo* model demonstrating that both drugs resulted in an increase in two OA biomarkers, CS846 and CPII (see *Principles of diagnosis*).[266] This was a non-euthanasia study, and as such cartilage histology was not performed and the effect on chondrocyte viability could not be determined.

The two more cartilage-friendly drugs, mepivacaine and ropivacaine, are lacking *in vivo* trials. Interestingly, in a rat OA monosodium iodoacetate model, IA administration of ropivacaine reduced lameness and suppressed the expression of TNF-α, Il-6, MMP-1 and MMP-13, suggesting that this product may actually slow synovitis-driven degradation of cartilage.[267] *In*

vitro exposure of equine chondrocytes to ropivacaine resulted in less chondrotoxicity than mepivacaine, but decreased cell viability was seen with both agents.[268] In a similar study, mepivacaine was less cytotoxic than lidocaine or bupivacaine to equine chondrocytes.[20] These studies were both limited by their inclusion of only one cell type. Recently, the effect of local anesthetics on both equine chondrocytes and fibroblast-like synoviocytes was examined.[269] This study found that bupivacaine was more chondrotoxic than lidocaine, mepivacaine and ropivacaine, however the inverse was true when considering synoviocytes, with lidocaine being the most synoviocyte-friendly.

Though the long-term impact of local anesthetics on the equine articular unit is not known at this time, in the short-term IA administration of local anesthetics causes synovitis.[270,271] An *in vivo* equine study compared middle carpal joint inflammation and catabolism following IA 2% lidocaine and 2% mepivacaine.[271] Synovial fluid white blood cell count, neutrophil percentage, and total protein, neutrophil enzymes (myeloperoxidase and elastase, enzymes released from neutrophils in response to inflammation), and Coll2-1 (released from articular cartilage ECM during degradation) concentrations were determined at set time points after injection in 17 horses. Both local anesthetics increased joint total protein, neutrophil myeloperoxidase, neutrophil elastase and Coll2-1, indicating articular inflammation and subsequent damage to the articular cartilage. Lidocaine-injected joints also increased white blood cell count and neutrophil percentage compared to controls. These changes were seen as long as 14 days after treatment.

Knowing that local anesthetics create a pro-inflammatory environment, the clinician may therefore elect to delay progression to IA treatment with expensive therapeutics (see *Medical*

treatment of OA) for fear that the temporarily-increased synovitis may reduce the efficacy of the drug. Alternatively, administration of a potent anti-inflammatory, for example a corticosteroid, may be performed with the aim of treating the original joint pain and the anesthetic-induced synovitis. Guidelines for concurrent or delayed administration of corticosteroid following IA local anesthetic are currently lacking (see *Local anesthetic and corticosteroid combination*).

Treatment of OA in horses

Medical treatment of OA

Once a diagnosis of equine OA is established, appropriate medical and/or surgical therapy is initiated. As stated previously, there is no known cure for OA. Therefore, therapy is aimed at reducing pain and slowing joint degeneration. In most cases, medical management and exercise protocols are utilized unless intra-articular pathology amenable to surgical correction is suspected, for example if an osteochondral fragment is present.[272]

Currently, medications used to treat OA in horses include non-steroidal anti-inflammatory drugs, intra-articular (IA) corticosteroids, hyaluronan, polysulfated polysaccharides, HA-sodium chondroitin sulfate and N-acetyl-D-glucosamine combination (Polyglycan), oral joint supplements, bisphosphonates and orthobiologics.[40] This section of the literature review will summarize these treatments, which the exception of IA corticosteroid therapy, which is discussed in more detail later (*see Intra-articular corticosteroid therapy*).

Non-steroidal anti-inflammatory drugs (NSAIDs) are therapeutic agents that disrupt the enzymatic conversion of arachidonic acid to prostaglandins.[273] All cells, including articular cells, contain arachidonic acid as a component of the phospholipid membrane. Following the release of arachidonic acid from the membrane by phospholipase, cyclooxygenase (COX) enzymes and 5-lipoxygenase convert it to prostaglandins/thromboxane and leukotrienes respectively.[274] While prostaglandins, in particular PGE_2, are important inflammatory mediators, they are also critical for the optimal function of several organs. For example, prostaglandins synthesized in the renal medulla are essential to regulating salt and water excretion by increasing renal blood flow, inhibiting sodium transport in the ascending limb of the loop of Henle, antagonizing the action of

vasopressin and inhibiting urea and sodium reabsorption in the collecting duct.[275] Prostaglandins inhibit parietal cell acid secretion, stimulate production of gastric mucus and bicarbonate secretion and increase mucosal blood flow.[275] Traditionally, the isoenzyme COX-1 is thought of as the producer of these protective prostaglandins, whereas COX-2 is associated with inflammatory events. Therefore, the ideal NSAID preferentially inhibits COX-2, with minimal COX-1 disruption. Unfortunately, the majority of NSAIDs for use in the horse are non-selective and as such prolonged use is associated with considerable pathology such as renal insufficiency and gastric ulceration.[276–278] They are, however, highly effective in alleviating lameness associated with synovitis.[160] The ease of administration, wide availability and cost-effectiveness make them a popular choice for equine joint disease despite their obvious drawbacks.

Hyaluronan is an essential component of articular cartilage ECM and synovial fluid (see *Joint anatomy and function*). The rationale of treatment with HA is that exogenously administered HA supplements or re-establishes the depleted HA in the arthritic joint, thereby restoring the viscosity of the synovial fluid and lubrication of articular tissues.[279] It is also antioxidative, anti-inflammatory and analgesic.[280] These effects are determined by the molecular weight of the HA formulation, total dose and the route of administration.[281,282] The effectiveness of IA HA as an OA treatment is controversial. In an osteochondral fragment model, 20 mg of IA HA administered at 7 day intervals for a total of 3 doses did not reduce lameness, response to joint flexion and decreased joint effusion compared to saline controls.[283] However, histologic fibrillation of the articular cartilage was reduced in treated horses. A similar model concluded that HA had no significant effect on several OA biomarkers, but did cause clinical improvement.[284] In a clinical trial of horses with moderate to severe lameness, HA significantly

reduced lameness compared to a placebo control.[285] Conversely, in an amphotericin B model of synovitis, treatment with either 8 mg, 16 mg or 32 mg of IA HA did not decrease the severity of lameness, perhaps due to the severity of lameness in this model.[143] A 2021 meta-analysis of the effectiveness of IA HA determined that HA reduced lameness in the short-term but was ineffective in improving long-term comfort in horses with OA.[286] The combination of HA with a corticosteroid has been proposed, with HA postulated to offset the harmful effects of steroids on chondrocytes while preserving the anti-inflammatory effect of the steroid.[287] However, in an equine *in vitro* cartilage explant study, the addition of HA to methylprednisolone acetate had no discernible effect.[288] Furthermore, in a multi-center clinical trial, the rate of successful treatment of lameness with intra-articular triamcinolone acetonide and HA was reduced as compared to treatment with triamcinolone alone.[289] Despite this, two recent surveys of equine practitioners found that HA was the most common IA medication administered concurrently with IA corticosteroids.[17,290]

Polysulfated polysaccharides include polysulfated glycosaminoglycan (PSGAG; Adequan) and pentosan polysulfate (Cartrophen).[291] PSGAG contains bovine lung and trachea chondroitin sulfate, a structural component of cartilage ECM; similar to HA, exogenous administration is aimed at re-establishing degraded ECM. *In vitro*, PSGAG reduced the synthesis of PGE_2 by LPS-stimulated synoviocytes.[292] PSGAG treatment also decreased collagenase and proteoglycanase production by stimulated chondrocytes.[293] In an osteochondral model, 250 mg of IA PSGAG administered at 7 day intervals reduced synovial membrane vascularity and fibrosis, as well as reducing synovial effusion.[283] Conversely, in a similar model that examined the effect of six doses delivered at weekly intervals, PSGAG decreased the quality of the

cartilage repair.[294] It is important to note that PSGAGs inhibit inflammation and impede articular bacterial clearance by inhibiting the complement pathway.[295] This results in a significantly increased risk of synovial sepsis as compared to other IA medications such as HA.[296] Perhaps to avoid this potentially life-threatening complication, intramuscular (IM) administration has become common. Radioisotope-labelled PSGAG was detected in synovial fluid, cartilage and subchondral bone following IM treatment, indicating good distribution to the articular environment.[297] However, in an *in vivo* model, 500 mg of IM PSGAG every 4 days for 7 treatments had no effect on healing of articular cartilage lesions or on protecting the articular environment from chemically-induced synovitis.[140] Chemical-induced synovitis is usually severe and rapidly progressive and may not replicate naturally-occurring disease (see discussion of *in vivo* models in OA). Interestingly, the same protocol reduced synovitis and lameness in the same chemical synovitis model in a separate study.[298]

HA-sodium chondroitin sulfate and N-acetyl-D-glucosamine combination (Polyglycan) is currently labelled for IA administration postsurgical lavage. This product contains 25 mg HA, 500 mg chondroitin sulfate and 500 mg glucosamine. In an osteochondral fragment model, IA administration transiently improved lameness and had a modest positive impact on healing of cartilage erosions.[299] A osteochondral fragment study that examined this product looked at the treatment effect following IV administration and determined that treatment improved the macroscopic appearance of the articular cartilage but had no effect on clinical parameters.[300] A similar study contrasted the effect of treatment both beginning the same day as OA initiation (prophylactic group) and beginning 16 days after OA initiation (treatment group).[301] Interestingly, prophylactic treatment resulted in poorer clinical outcomes than placebo. The

treatment group had fewer histologic articular cartilage abnormalities, but increased bone oedema identified on MRI. Overall, the efficacy of this drug in equine OA is yet to be fully elicited.

There are a large number of oral joint supplements available for the horse and they remain popular with horse owners. Most products contain GAGs or HA and attempt to replenish these cartilage ECM components. Quality *in vivo* studies on these products are lacking. One study utilized the osteochondral fragment model to examine the effect of oral avocado and soy unsaponifiables (ASU).[133] They found that ASU did not have an effect on clinical parameters, however the macroscopic and histologic quality of the articular cartilage was superior in the treated horse as compared to controls. This effect may be achieved by decreasing chondrocyte PGE_2 production.[302] Another supplement, extract of green-lipped mussel (Perna canaliculus), decreased lameness and reduced joint pain in clinical cases of fetlock OA.[303]

Bisphosphonates represent a new and evolving area of veterinary medicine. These drugs regulate bone metabolism through inhibition of bone resorption.[304] There are two distinct classes of bisphosphonate, non-nitrogenous and nitrogenous, and these differ in their mechanism of action and effect.[305] The bisphosphonates licensed for veterinary medicine are the non-nitrogenous drugs clodronate and tiludronate. These drugs cause osteoclastic inhibition and apoptosis.[306] Clodronate has mostly been investigated for treatment of navicular disease and results have been promising.[307] Treatment of equine OA has mostly focused on tiludronate. Intraarticular administration of tiludronate is not currently recommended due to some detrimental effects on articular cartilage observed *in vitro*.[308] In a retrospective study, horses with fetlock disease

treated with IV tiludronate had improved radiographic scores and lameness at 6 month follow up, however CTX-II, a biomarker of cartilage damage, was increased.[309] A clinical trial that compared IV tiludronate to placebo in the treatment of distal tarsal OA found improved lameness and radiographic findings in the treatment group.[310] Currently, the clinical use of these drugs is somewhat limited by the concern that interfering with bone metabolism could predispose the patient to catastrophic injury by limiting the physiological capacity of bone to respond to microfracture.[311] This likely stems from a study that detected skeletal microdamage in Beagle dogs, however the dogs were treated daily for a year with very high doses.[312] In an equine bone biopsy model, treatment with either clodronate or tiludronate did not affect bone structure or remodelling.[313] Widespread treatment of equine OA with these agents is unlikely until their long-term effects on bone metabolism and fracture healing are well-established in the horse.

Regenerative Treatments

Regenerative medicine is a fast-expanding area of veterinary medicine. The aim of regenerative medicine in equine OA is ultimately to repair articular cartilage and re-establish the biomechanical function of the joint. The most investigated of these medicines are platelet rich plasma (PRP), autologous conditioned serum (ACS), autologous protein solution (APS) and stem cells.

PRP is a plasma suspension containing a higher concentration of platelets compared to that of whole blood.[314] It contains multiple growth factors with the ability to modulate tissue healing and inflammation.[315] *In vitro*, PRP reduces the expression of MMPs through the secretion of anti-inflammatory factors and chemotaxic effects, as well as stimulating pathways of cartilage

repair such as increasing expression of aggrecan and collagen type 2.[316–318] These *in vitro* findings have translated well to human OA, with multiple meta-analysis indicating superior outcomes with IA PRP treatment versus corticosteroids.[319–323] There has been a limited number of clinical trials in horses, but thus far the results have been promising.[324,325]

ACS and APS are both autologous products that contain increased concentrations of interleukin-1 receptor antagonist protein (Il-1ra) as compared to whole blood. Il-ra is a soluble receptor that binds and neutralizes IL-1β prior to its catabolic action on synoviocytes and chondrocytes.[326] ACS is produced by conditioning monocytes to increase endogenous production of Il-ra by exposure of whole blood to chromium sulfate-treated glass beads, a process that takes 24 hours.[327] APS is produced by 2 step centrifugation and takes 20 minutes.[328] In an equine co-culture model, both treatments decreased PGE_2 production from IL-1β-stimulated explants and increased type 2 collagen and aggrecan expression in cartilage.[329] ACS has been evaluated in an osteochondral fragment model; treatment resulted in significant improvement in lameness and superior cartilage and synovial histology to placebo controls.[134] Although no equine *in vivo* models have evaluated the treatment effect of APS thus far, a small clinical trial has been conducted.[328] In this trial, APS improved lameness scores from baseline and as compared to a saline placebo up to 52 weeks after treatment.

Stem cells used in equine veterinary medicine are largely adult bone marrow-derived or adipose tissue-derived mesenchymal stem cells. Cells obtained from either location can either be cultured and isolated to be injected at a later date, or centrifuged and injected immediately as a point of care treatment.[330] Additionally, some neonatal stem cells, umbilical cord tissue-derived and

placentally-derived stem cells have been evaluated. Mesenchymal stem cells adhere to injured tissues, differentiate, induce differentiation of endogenous progenitor cells and thus stimulate the regeneration of the articular cartilage ECM.[331] Overall, translation to the horse has been disappointing. In an osteochondral-fragment model evaluating both bone marrow-derived mesenchymal stem cells and adipose tissue-derived stem cells, no significant treatment effect was determined with the exception of reduced PGE_2 levels in bone marrow-derived stem cell-treated horses.[332] In a cartilage-defect model, bone marrow-derived stem cells in fibrin did not improve arthroscopic scores at 8 month follow up compared to fibrin alone.[333] However, in this study, the healing defects were also evaluated via biopsy at 30 days, which may been detrimental to the stem cells. A similar model, this time without the 30 day biopsy, found significant improvements in macroscopic and histologic assessments in stem cell treated defects at 3 and 8 months.[334] Conversely, a year-long trial examining the effect of IA bone marrow-derived stem cells with HA for treatment of a full-thickness cartilage defect found only minor histological differences between treated and HA-only controls.[335] No differences in imaging, biochemical or macroscopic cartilage scores were found. A similar, more recent trial, found no differences between cartilage defects treated with bone marrow-derived stem cells and controls, with defects being filled with fibrocartilage at 1 year follow up.[336]

Perhaps the most successful application of this therapeutic has been for treatment of soft tissues with the joint, in particular for femorotibial meniscal injuries.[337,338] In one study, meniscal defects treated with either bone marrow-derived or adipose tissue-derived stem cells were healed with fibrocartilaginous tissue at 12 months, whereas untreated control defects were partially repaired or not repaired.[338] While these results were exciting, a recent retrospective study

examining meniscal injuries in sports horses found that treatment with an orthobiologic (including ACS, PRP and bone marrow-derived stem cells) had no influence on long-term prognosis.[339] However, there were only 6 horses treated with stem cells in this study with the majority being treated with ACS. Long-term follow up was available for 2 of the stem cell-treated horses, both of whom returned to athletic performance.

Surgical treatment of OA

While the majority of research focus has been on medical therapy of OA in the horse, there are several situations in which surgical intervention is indicated.[272] Arthroscopy serves the dual purpose of providing the gold standard antemortem diagnosis of articular cartilage pathology as well as a treatment modality. Removal of osteochondral fragments, articular enthesophytes, defibrillated cartilage, diseased intra-articular ligament fibers or menisci can all be achieved. Additionally, this procedure can assist in the repair of articular fractures, re-establishing the congruency of the disrupted cartilage surface.[340,341]

One technique for stimulating cartilage repair is the arthroscopic liberation of bone marrow stem cells from beneath the subchondral bone plate into the synovial environment. This is facilitated by various techniques, including abrasion arthroplasty (debridement of the cartilage to the level of the subchondral bone), spongialization (debridement past the level of the subchondral bone, into the cancellous bone or 'spongiosa'), osteostixis (focal drilling through the subchondral bone in the cartilage defect) and microfracture (focal penetration of the subchondral bone plate using an awl).[272] As the subchondral bone architecture is completely disrupted during spongialization, it is currently not indicated in equine arthroscopy due to the mechanical detriments that result

from this technique.[342] Currently, debriding defibrillated cartilage to the level of the subchondral bone, combined with microfracture (penetrating the bone into the marrow), is suspected to be most ideal for stimulating cartilage repair. In horses, microfracture increased the collagen content in the tissue that filled cartilage defects as compared to non-treated defects.[343] Microfracture increases the expression of type 2 collagen, though there is less stimulation of aggrecan.[344] Despite these benefits, long-term clinical advantage of microfracture is yet to be demonstrated in the horse.

Arthrodesis is indicated when therapies fail to relieve the pain of OA and destruction of the articular tissues has resulted in irreversible compromise to the function of the joint. In the fetlock, carpal and distal interphalangeal joints, rigid fixation using metallic implants is required. Despite the technical difficulty of these procedures, successful returns to pasture soundness for arthritic horses are reported.[345–347] In low-motion joints such as distal tarsal joints, drilling, laser-based and chemical techniques are often sufficient to return the horse to athletic use.

Intra-articular corticosteroid therapy

Corticosteroids are steroid hormones produced in the adrenal cortex or made synthetically. Endogenous corticosteroids are grouped as mineralocorticoids, involved in water and salt balance, glucocorticoids, involved in cellular metabolism and androgenic steroids, involved in sexual characteristics.[348] In veterinary medicine, the glucocorticoids have the most widespread applications, and for the purpose of this review any reference to 'corticosteroid' refers to exogenous glucocorticoids.

All corticosteroids consist of a 21-carbon, 4-ring steroid skeleton. Slight variations in their chemical structure result in differences in potency, duration of action, affinity for their respective receptors and degree of protein binding.[349] Corticosteroids have two main mechanisms of action: genomic and non-genomic. The genomic mechanism occurs as a result of cellular glucocorticoid-receptor drug interactions; steroids are low molecular weight molecules that are lipophilic and freely cross the phospholipid cell membrane to bind to cytosolic receptors (cGCR, cytosolic glucocorticoid receptor).[350] The glucocorticoid-cGCR complex moves to the nucleus and increases the expression of anti-inflammatory proteins (transactivation) or decreases the production of pro-inflammatory proteins (transpression).[351] The non-genomic mechanisms include non-specific effects caused by interactions with cellular membranes, specific effects caused by interactions with membrane-bound receptors (mGCR) and by non-genomic effects caused by interactions with the cGCR.[352] The non-genomic mechanisms are more rapid than the genomic and can drive both inflammatory and non-inflammatory pathways such as cell calcium homeostasis, muscle tone and reactive oxygen species formation.[352] The non-genomic mechanisms of glucocorticoids are currently an area of research interest, particularly as most of the side-effects of glucocorticoid therapy are thought to be driven through the genomic effects.

Corticosteroid action in cells depend on the local concentration of the corticosteroid, the receptor expression and on local corticosteroid metabolism within the target tissue. At a tissue level, exogenous glucocorticoids and endogenous glucocorticoids are both interconverted between inactive and active forms by the 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes.[353] 11β-HSD1, in particular, is responsible for increasing local levels of active glucocorticoids, whereas 11β-HSD2 is the primary inactivation pathway.[354] Expression of 11β-HSD1 varies between cell types and increases with age and in response to Il-1β and TNFα.[355] This indicates that synovial tissue metabolizes glucocorticoids (mostly activation) and this metabolism increases with inflammation.

The effects of corticosteroid treatment are catabolic and anti-anabolic. Corticosteroids modulate pain by reducing inflammation, primarily by suppressing the expression of pro-inflammatory genes and inhibiting the arachidonic acid cascade.[356] They induce the expression of lipocortin-1, which inhibits phospholipase and thereby the arachidonic acid cascade, decreasing inflammation.[357] Intra-articular administration is hypothesized to provide a maximal decrease in inflammation, while limiting the considerable systemic effects of corticosteroids.[357] There is also evidence to suggest that they have the potential to slow the catabolism of the articular cartilage ECM. Part of the regulatory process triggered by corticosteroids within the cell is the decreased release of Il-1β and TNFα.[358,359] Therefore, corticosteroids directly inhibit the collagenolysis that results from cytokine-driven production of MMPs and aggrecanse by articular cells.

The main argumentation against corticosteroid therapy for OA is that OA is not primarily an inflammatory disease. In transgenic mice with reduced expression of cGCR in chondrocytes

(therefore with decreased intra-cellular corticosteroid activity), surgical destabilization of the medial meniscus resulted in reduced macroscopic signs of OA compared to normal controls.[360] In transgenic mice with overexpression of 11β-HSD2 in osteoblasts and osteoclasts (therefore with more local inactivation of corticosteroid), surgical destabilization of the medial meniscus resulted in reduced macroscopic signs of OA compared to normal controls in older but not younger mice.[361] The surgical destabilization model used in these studies produces minimal inflammation, therefore the results imply that the increased local glucocorticoid activity in bone and cartilage as part of the aging process has a role in the pathogenesis of OA. These findings also raise concerns as to the appropriateness of corticosteroid therapy for OA. However, it is important to note that this research primarily involves the disruption to endogenous glucocorticoids in mice, and it is currently unknown if the same situation is present in the horse.

Intra-articular corticosteroid therapy has been employed in the treatment of OA since the 1950's and is widely utilized in both human and equine practice.[362,363] Corticosteroids currently approved for use in the United States by the FDA for intra-synovial use in horses include methylprednisolone acetate, triamcinolone acetonide, betamethasone acetate and betamethasone sodium phosphate, isoflupredone acetate, flumethasone and prednisolone acetate (animaldrugsatfda.fda.gov) (**Table 5**). Of these, the three most common corticosteroid preparations utilized include methylprednisolone acetate, triamcinolone acetonide and betamethasone acetate and betamethasone sodium phosphate.[364]

Table 5. Corticosteroids approved by the FDA for intra-synovial use in horses. (From animaldrugsatfda.fda.gov)

Corticosteroid	**Trade Name**	**Concentration (mg/mL)**	**Dose (mg)**	**Potency Relative to Hydrocortisone**	**Relative Duration of Action**
Methylprednisolone acetate	Depo-Medrol	40	40-120	5	Long
Triamcinolone acetonide	Vetalog	6	6-18	5	Medium
Compounded Betamethasone acetate and betamethasone sodium phosphate	Betavet	6	3-18	30	Medium-long
Isoflupredone acetate	Predef 2X	2	5-20	50	Short-medium
Flumethasone	Flucort	0.5	1.25-2.5	120	Short-medium
Prednisolone acetate	Metricortelone (discontinued)	25	50-100	4	Short

Though the use of articular corticosteroids should theoretically be beneficial for the synovial environment, *in vitro* research indicates that certain corticosteroid formulations may in fact be detrimental. Methylprednisolone acetate (MPA) decreases proteoglycan production in the articular cartilage ECM by equine chondrocytes, depresses collagen production and prevents chondrocyte differentiation.[365,366] Reduction in the viability of chondrocytes and synoviocytes following treatment of MPA at therapeutic doses suggests that they are somewhat cytotoxic to synovial cells.[367] Similar findings were observed in deep digital flexor tendon and navicular bone fibrocartilage explants treated with MPA, suggesting that use in synovial structures other than joints may also be questionable.[368]

These findings are supported by *in vivo* research. Normal horses treated with 60 mg MPA at 2 week intervals for a total of 3 doses had suppression of procollagen II (CPII) synthesis and increased degradation products of aggrecan (keratan sulfate epitope and aggrecan 846 epitope) present in synovial fluid as determined by gel chromatography.[369] In an osteochondral fragment model of OA, horses treated with 100 mg MPA once a week for 4 treatments had inferior healing of cartilage defects when compared to saline-treated controls.[370] A second osteochondral fragment experiment found that joints treated with 100 mg MPA on postsurgical days 14 and 28 had lower PGE_2 concentrations in sampled synovial fluid and less intimal layer hyperplasia and vascularity than control joints.[130] Intimal layer hyperplasia and increased synovial membrane vascularity are signs of synovitis; the intimal layer filters the synovial fluid and produces essential molecules such as HA. However, increased cartilage erosion and other morphologic lesions present (such as cartilage defibrillation) in MPA-treated joints led the authors to conclude that the net effect of MPA on the synovial joint was negative. Furthermore, no clinical

improvement in lameness or joint effusion was noted, which challenges MPA's status as a symptom-relieving drug. It is important to note that the doses and treatment intervals used in these studies exceed those usually recommended in clinical patients. The negative effect of MPA on cartilage has led to its use being generally restricted to 'low-motion' joints, where preservation of cartilage health and joint function is not paramount to the horse's movement.[364]

In contrast to MPA, much of the in vitro research concerning triamcinolone acetonide (TA) suggests a positive treatment effect on synovial tissues. Treatment of IL-1β stimulated chondrocytes with 0.06 mg/ml or 0.6 mg/ml TA mitigated the catabolic effect of IL-1β on cartilage GAG metabolism.[371] In a synovium and cartilage co-culture model, 10^{-10}M TA inhibited the production of catabolic cytokines and MMP activity.[62] Administration of TA decreased PGE_2 production by inflamed synovium and cartilage in co-culture.[372] Importantly, treatment with 40 mg TA did not negatively affect chondrocyte or synoviocyte viability, suggesting that, in contrast to MPA, TA is not cytotoxic at physiologic doses.[367]

These positive results have also translated to *in vivo* research. In a LPS-synovitis model, horses treated with 9 mg TA had reduced lameness, oedema and synovial fluid total protein compared to controls.[18] In an osteochondral fragment model, horses treated with 12 mg TA on days 13 and 27 post-surgery were significantly less lame than saline treated controls.[128] Triamcinolone-treated horses had less synovial membrane hyperplasia and fibrosis, improved cartilage histomorphological parameters and higher synovial fluid GAG concentrations.[128] In a second osteochondral fragment model with the same doses, TA improved lameness without altering bone remodeling or fragility.[131] Interestingly, not all results have been positive. Gene expression

in synovial fluid of exercised, TA-treated horses revealed an increase in anti-inflammatory gene expression and downregulation of pro-inflammatory cytokines, however collagen gene expression was also suppressed after a single 9 mg dose.[373] Recently, a LPS model of synovitis compared a single 12 mg TA treatment with saline control and found higher synovial fluid GAG and PGE_2 levels in the TA group, though there were also lower white blood cell counts and lower MMP activity in TA-treated horses.[374] This suggests that in the presence of inflammation, TA may potentiate matrix destruction and endogenous joint inflammation. The different results between the studies may in part be due to the model utilized – LPS typically produces more profound synovitis and inflammation than the osteochondral fragment model.[119] However, an osteochondral fragment study that examined repeated TA treatment (12 mg every 2 weeks for 3 doses) determined that TA resulted in increased collagen degradation and aggrecan turnover.[375] Typically TA is thought of as a more 'cartilage friendly' corticosteroid and therefore is utilized in more 'high motion' joints in clinical practice.[364] The somewhat conflicting evidence from the scientific literature merits careful consideration, and further *in vivo* trials are required to fully elucidate the beneficial versus deleterious action of TA.

While MPA is considered 'cartilage unfriendly' and TA is 'cartilage friendly', betamethasone acetate and betamethasone sodium phosphate (BAP) appears to be 'cartilage neutral'. Much of the scientific literature is conflicting. One study found that treatment with 6 mg/ml BAP resulted in no significant chondrocyte death after 7 days of treatment.[376] Another saw complete loss of chondrocyte and synoviocyte viability after 1 and 7 days of co-culture after 5 mg BAP.[367] At higher concentrations (0.1-100 µg/ml), BAP suppressed proteoglycan synthesis by cartilage and had no significant effect at lower concentrations (0.001-0.05 µg/ml).[377] These conflicting results

have somewhat translated to the *in vivo* research. Treatment of normal joints with 3 doses of 24 mg BAP resulted in increased levels of synovial fluid hyaluronate and proteoglycan, suggesting increased cartilage ECM turnover.[287] In an osteochondral fragment model, no clinical benefit nor any histological detriment of 16 mg BAP treatment performed at days 14 and 35 post-surgery was determined.[127]

Unfortunately, in comparison to the numerous pre-clinical research trials concerning IA corticosteroids there are few clinical studies. A retrospective study of 51 horses compared the clinical response to IA MPA (median dose per joint was 55 mg) and IA TA (median 9.8 mg per joint) in the tarsometatarsal and/or distal intertarsal joints.[378] This study found no difference between MPA and TA treatment; of the 34 horses that had follow up information available, only 13/34 had a 'positive outcome' as defined as able to return to work without oral non-steroidal anti-inflammatory drugs. Horses were enrolled in that study based on a positive response to IA analgesia and diagnostic imaging findings, however the small number of cases and the non-standardized treatment protocol jeopardize the validity of the results. A large-scale clinical trial examined outcomes for horses treated intra-articularly for 'high motion' joint lameness with 12 mg TA and 12 mg TA with 20 mg high molecular weight hyaluronan (HA).[289] Treated joints included distal interphalangeal, metacarpo/metatarsophalangeal, middle carpal and radiocarpal joints. Concurrent TA-HA treatment resulted in poorer success rates compared to TA alone. Only half of all treated horses returned to their previous level of activity; although most *in vivo* work indicates that TA is chondroprotective, this does not appear to consistently translate to benefiting the clinical patient long-term.

Despite the long-standing use of intra-articular corticosteroids, choice of corticosteroid, dose and frequency of administration remains mostly based on clinical experience.[357,364] Common dosages of corticosteroids approved for IA use are listed in **Table 5**. In a 2011 survey of members of the American Association of Equine Practitioners (AAEP), it was found that 77% respondents to the survey use TA in high-motion joints and 73% of respondents use MPA in low-motion joints.[364] The most common dose ranges per joint reported in this study included 3-5 mg TA (29% respondents), 5-10 mg TA per joint (53%), 20-40 mg MPA (37%) and 20-40 mg MPA (44%). A more recent survey of AAEP members found that corticosteroids remain the treatment of choice for joint pathology, above newer therapies such as autologous conditioned serum or platelet rich plasma.[290] As interest in non-steroidal intra-articular therapies increases, it will be interesting to see if *in vivo* research can demonstrate any advantage of these therapies over traditional corticosteroid therapy, particularly with regards to cartilage metabolism and longevity.

Regulation of intra-articular corticosteroid therapy in horses

The rationale for prohibition of corticosteroids in competition horses, be that racing, eventing, hunting or other, is that they may provide an unfair advantage to the horse by enhancing performance. This does not just pertain to decreased lameness. For example pulmonary function is improved by intra-articular administration of corticosteroid.[379] Additionally, associations between intra-articular corticosteroid administration and musculoskeletal injuries have been identified.[380] This is theorized to be due to the potent 'symptom relieving' effect of these drugs, masking the underlying condition without correction and facilitating its exacerbation into injury via intense exercise.[381] Subsequently, horses that test positive for these drugs can be removed from competition. However, regulatory bodies, such as the Racing Medication and Testing

Consortium (RMTC) and Fédération Équestre Internationale (FEI) among others, recognize that the use of these medications between competitions is justified as they pertain to treatment of the horse.

In order to help veterinarians and owners ensure that the treated horse does not test positive, these regulatory bodies have released various guidelines to help dictate treatment timing. Most define 'detection time' as the approximate time for which a drug or its metabolite remains within a horse's system and is therefore the minimum amount of time that must pass between administration and competition.[382] The 'withdrawal time' is highly variable and is based upon the known detection time plus individual physiological factors that may affect pharmacokinetics such as age, metabolism, sex, disease status etc. Multiple pharmacokinetic studies examining common intra-articular corticosteroids have facilitated the publication of withdrawal times for each medication, based upon detection in plasma, urine and synovial fluid (**Table 6**). The rules pertaining to intra-articular corticosteroid use in competition horses are continuously updated, and judicious double-checking of the current regulations prior use is recommended. At the time of writing, the RMTC recommends a mandatory 14 day stand down period for all intra-articular medications.

Table 6. Detection threshold and recommended withdrawal time of commonly utilized intra-articular corticosteroids, based on recommendations by the Racing Medication and Testing Consortium (RMTC). Note – the RMTC currently recommends a mandatory 14 day stand down period for all intra-articular medications. Key: LOQ, limit of quantification; LOD, limit of detection.

Corticosteroid	Detection threshold	Experimental administration dosage	Time to LOQ /LOD	Detection time (minimum withdrawal time)	Recommended withdrawal time	References
Methylprednisolone acetate	100 pg/mL of plasma or serum	Total of 100 mg in one joint, 200 mg in two joints	240 hours to LOQ (plasma) 21 days to LOQ (urine) 77 days above LOD (synovial fluid)	14 days (100 mg dose) 28 days (200 mg dose)	21 days (100 mg dose)	Lillich *et al.* 1996, Soma *et al.* 2006
Triamcinolone acetonide	100 pg/mL of plasma or serum	Total of 9 mg in one joint	144 hours to LOQ, 168 hours to LOD (plasma) 240 hours to LOD (urine)	7 days	14 days	Soma *et al.* 2011, Knych *et al.* 2013
Betamethasone	10 pg/mL of plasma or serum	Total of 9 mg in one joint	72 hours to LOQ (plasma)	7 days	14 days	Luo *et al.* 2005, Knych *et al.* 2017
Isoflupredone acetate	100 pg/mL of plasma or serum	Total of 20 mg in one joint	144 hours to LOQ, 168 hours to LOD (plasma)	7 days	14 days	Lillich *et al.* 1996, Knych *et al.* 2016

Complications of administration of intra-articular corticosteroids

Complications associated with administration of intra-articular corticosteroids can be considered broadly as complications inherent to the act of intra-articular administration (i.e. septic arthritis, acute aseptic inflammation/joint flare and periarticular cellulitis) and complications specific to corticosteroids (i.e. musculoskeletal injury, steroid arthropathy, osseous metaplasia and laminitis). It is well-established that corticosteroids do not remain within the synovial environment, therefore there is potential for any of the known side-effects of corticosteroid administration to occur.[349]

Septic arthritis

The incidence of septic arthritis following administration of intra-articular medications is low, reports range from 2.1 to 7.8 septic joints per 10,000 joint injections.[390–392] Sepsis results from an inoculation of the equine skin microflora into the synovial environment by the needle, usually Staphylococcus species.[393] Diagnosis is made via a combination of recent history of intra-articular medication, sudden onset of lameness, presence of heat or sensitivity of the afflicted joint and synovial fluid analysis. A positive synovial fluid culture is the gold standard of diagnosis but is obtained in less than 50% of confirmed cases.[394] Differentiation between septic and non-septic inflammatory arthritis is somewhat confused by the overlap between clinicopathological values, however the presence of synovial fluid leucocytosis (>30 $x10^9$ nucleated cells/L), neutrophilia (>80-90% neutrophils), hyperproteinemia (>30 g/L), cytological evidence of sepsis such as neutrophil degeneration, presence of intra-cellular bacteria and increased serum amyloid A concentration all provide convincing evidence for diagnosis.[184,395,396] Treatment strategies include arthroscopic evaluation, through-and-through needle lavage,

arthrotomy and systemic and local antibiotic administration.[397] With appropriate therapy, prognosis for survival and return to use is good.[397]

The significant emotional, financial and clinical consequences to the horse, client and veterinarian following the development of synovial sepsis after IA injection has resulted in justified interest into prevention strategies.[398] Concurrent prophylactic administration of an antibiotic theoretically prevents synovial sepsis by eliminating any iatrogenically inoculated bacteria through locally established MIC. However, prophylactic antibiotic use does not appear to decrease incidence of sepsis and may be detrimental to the synovial tissue.[391,399,400] Risk factors for synovial sepsis following intra-articular injection that have been identified include faulty aseptic technique, the experience of the clinician with injections, use of multi-dose vials, clipping hair from injection sites, larger needle gauge, needle angle of insertion, reusing a needle and not using a stylet in a spinal needle.[390,391,401] Despite a lack of clear scientific evidence for prophylactic antibiotic use with IA injection, concurrent treatment remains common in clinical practice.[17]

Acute aseptic inflammation (joint flare)

A usually self-limiting, aseptic inflammatory response following intra-articular medication administration is referred to as a 'joint flare', and frequency of occurrence is thought to be around 2%.[357] This response can be seen following administration of any intra-articular medication, and may be more common following treatment with certain non-steroidal intra-articular therapies such as orthobiologics.[290] In the case of corticosteroid treatment, the inflammatory response is thought to be secondary to the microcrystalline suspension of the

corticosteroid ester.[402] The horse is typically lame, with pain, swelling and heat in the affected joint. Crucially, the synovial clinicopathological parameters do not indicate sepsis.[348] As initial differentiation between a joint flare and septic arthritis can be difficult and successful therapy of septic synovial structures is time-dependent, treatment for either condition is usually similar. Cases of joint flare will usually improve rapidly even with more conservative therapy (i.e. medical treatment alone.

Periarticular cellulitis

Cellulitis is infection and/or inflammation of the subcutaneous tissues: in cases of periarticular cellulitis an inoculation of skin microflora into the subcutaneous space overlying the joint occurs during medication delivery.[403] While exact incidence is unknown, it is thought to be fairly uncommon.[357] In two retrospective reports of limb cellulitis, a history of recent intra-articular medication administration was identified as the cause of cellulitis in 0-7% cases.[404,405] Both of these studies found Staphylococcus and Streptococcus species to be the most commonly identified. Diagnosis is made based on history of recent intra-articular medication administration, sudden occurrence of lameness, periarticular swelling and heat and the presence of pitting oedema.[403] Treatment strategies include regional and systemic antimicrobial administration, cold hydrotherapy, hand-walking and compressive bandaging.[405] Attempts at locally medicating the joint are actively discouraged as *in vitro* models have shown that needle insertion through cellulitic tissue can inoculate the synovial environment and potentially result in septic arthritis.[406] However, reports of actual clinical instances of synovial sepsis due to cellulitic tissue puncture are lacking.

Musculoskeletal injury

Previously, corticosteroid use in foals was associated with pathologic changes in developing bone.[407] This early research increased concern for the risk of musculoskeletal injury following corticosteroid injection due to osteonecrosis or disruption of the bone's ability to adapt to cyclic loading, though subsequent work found that IA TA and MPA had no effect on subchondral bone.[131,408] Interestingly, epidemiological studies have shown both an increased risk for catastrophic injury following corticosteroid injection and no increased risk.[380,409] It is postulated that IA corticosteroid injection provides analgesia that has a masking effect; mild clinical signs of impending fracture are subsequently alleviated and the horse is pushed closer to injury when instead rest would be beneficial. This is somewhat corroborated by the findings of Smith and colleagues, who reviewed 1488 cases of racing Thoroughbreds treated with intra-synovial medications and determined that 3 or more treatments greatly increased the risk of fracture.[381] However, this study did not have a control group of unmedicated horses and thus the validity of this finding is questionable. While no direct link between these medications and catastrophic injury has been definitively established, the possibility for increased risk of injury should caution the veterinarian from administering them close to intensive exercise. The detection and withdrawal times for different sporting activities should also be considered (see earlier discussion).

Steroid arthropathy

Steroid arthropathy is the accelerated degeneration of the joint following repeated intra-articular corticosteroid administration.[348] Long before *in vitro* and *in vivo* models demonstrated the potential for a negative effect of corticosteroids on articular cartilage health and metabolism, it

was recognized that repeated administration of these drugs had the potential to accelerate joint degeneration.[410–412] To avoid this scenario, low doses of these drugs and appropriate dosing interval is recommended, as well as selection of the most 'cartilage friendly' preparation possible (see earlier discussion).

Osseous metaplasia

Metaplastic bone formation can result following inadvertent deposition of corticosteroids (primarily MPA) into the periarticular tissues.[348] The mechanism and incidence of this is unknown, and few scientific reports are available for reference. Potential clinical implications include the progressive limitation of joint movement and resultant lameness. Diagnosis is by physical examination and diagnostic imaging and no treatments are currently described.

Laminitis

Currently, there is no scientific proof that corticosteroids cause laminitis, however veterinarians still perceive that some formulations, particularly TA, may contribute to a laminitic event.[364] This is despite several retrospective studies finding no causative link between TA use and laminitis.[413,414] One hypothesis is that corticosteroids cause a rapid increase in plasma insulin and glucose; systemic hyperinsulinemia results in upregulation of lamellar inflammatory pathways (i.e. laminitis) and subsequent clinical disease.[415] While no study has been able to demonstrate a causative link between corticosteroid administration and laminitis, it may be prudent to limit the dosage and frequency of treatment in horses that appear to be at risk for equine metabolic syndrome-associated laminitis.[416] Diagnosis is by presence of typical clinical signs such as 'leaning back' shifting lameness, presence of bounding digital pulses and radiographic changes.

Attempts at treatment are based upon severity and include continuous cold therapy, corrective podiatry, analgesia and supportive care.

Local anesthetic and corticosteroid combination

For maximum efficiency in combating equine OA, the clinician proceeds to IA corticosteroid treatment soon after diagnosing joint pain via response to IA local anesthetic. In human medicine, local anesthetic is added to the same syringe during IA administration of therapeutics to improve patient comfort.[417] The equine practitioner may also elect to do this if a high degree of suspicion for joint pain is present to avoid multiple arthrocenteses, or may proceed to 'same day' treatment to avoid multiple journeys. Perhaps more commonly, an up to 6-day delay between IA local anaesthetic and IA corticosteroid injections is elected to prevent anaesthetic-induced synovitis from reducing the absorption and efficacy of the corticosteroid.[418] The presence of synovitis may also alter the effect of corticosteroid on joint metabolism.[419] The need for this delay was called into question by an *in vivo* LPS study, which demonstrated that concurrent administration of mepivacaine and TA did not alter the potency or duration of action of TA.[18]

The effect of combining corticosteroids and local anesthetics has been examined both *in vitro* and *in vivo*. Exposure of human chondrocytes to combinations of betamethasone and lidocaine, bupivacaine and ropivacaine resulted in greater apoptosis and necrosis rates as compared to the local anesthetic agents alone.[19] This decrease in viability was time-dependent, with longer exposure times causing increased cell death. Bovine chondrocytes treated *in vitro* with different concentrations of MPA and with MPA and 1% lidocaine demonstrated concentration-dependent toxicity, with the MPA-lidocaine combination resulting in almost 100% cell death.[420] The combination of TA and bupivacaine increased chondrotoxicity as compared to bupivacaine.[421] Conversely, a study that examined combinations of MPA, betamethasone and TA and lidocaine or bupivacaine in a bioreactor system determined that the TA-bupivacaine was relatively safe as

compared to other combinations.[422] The addition of the other steroids to either local anesthetic resulted in increased toxicity as compared to the local anesthetic alone, in particular few cells survived after addition of betamethasone. The doses used in this study were titrated by surface area and fluid volume to try to mimic physiologic doses, as well as exposure times limited based on duration of action. Interestingly, while corticosteroid and local anesthetic-treated chondrocytes in cell-culture had decreased viability and metabolic activity compared to those treated with local anesthetics alone, the addition of HA improved all measured outcomes.[423] The authors concluded that HA supported the metabolic effect of corticosteroids on cartilage and reduced the chondrotoxic effect of local anesthetic.

Currently, two *in vivo* studies have examined corticosteroid-local anesthetic combinations. Dogs were injected with MPA-lidocaine (4 ml of 1% lidocaine and 40 mg MPA), TA-lidocaine (4 ml of 1% lidocaine and 40 mg TA) and TA-bupivacaine (4 ml of 0.0625% bupivacaine and 40 mg TA) or a saline control.[424] Doses were based upon recommended doses for the human wrist (as the human wrist is the approximate size of the canine shoulder). Synovium and cartilage explants harvested following euthanasia were assessed for viability, and all treated groups showed significant losses in synoviocyte viability compared to the negative control. Only the MPA-lidocaine group had signs of decreased chondrocyte viability and metabolism versus other groups. In a rat stifle model, TA-ropivacaine combinations of different concentrations were compared to ropivacaine and TA alone.[425] The higher concentrations of TA (4 mg/ml, 0.03 ml total volume) resulted in increased cartilage toxicity. Interestingly, the combination of the lowest concentrations (1 mg/ml, 0.03 ml total volume) of TA and ropivacaine resulted in the least amount of cell death including compared to either agent alone.

Currently, no studies have examined corticosteroid and local anesthetic combination on equine tissues, either *in vivo* or *in vitro*. Additionally, the research thus far has focused on articular cell viability and the effect on ECM turnover and potential implications for OA is unknown.

Chapter 3: Objectives and Hypotheses

The objective of this study was to determine the need or lack thereof for an articular tissue recovery period after exposure to local anesthetic before corticosteroid treatment using an *in vitro* co-culture model of equine OA.

The hypotheses were that a 6-day delay following mepivacaine hydrochloride (MH) administration before triamcinolone acetonide (TA), as compared to concurrent administration, would:

a) Diminish chondrocyte and synoviocyte cell death as indicated by decreased lactate dehydrogenase (LDH)
b) Decrease inflammation as indicated by decreased prostaglandin E_2 (PGE_2)
c) Decrease matrix destruction as indicated by decreased matrix metalloproteinase 13, (MMP-13) and decreased concentration of glycosaminoglycans (GAGs) in culture media as estimated by the dimethylmethylene blue assay (DMMB)

Chapter 4: Materials and Methods

Articular tissue samples

All animal procedures were performed with approval from the Institutional Animal Care and Use Committee (protocol # 2020-3735). Six adult horses (three Quarter Horses, 1 Warmblood, 1Thoroughbred, 1 Paso Fino, aged 3-18 years), 5 geldings and 1 mare, were euthanized for reasons unrelated to the study. Immediately following euthanasia, the hair overlying both stifle joints was clipped and the skin aseptically prepared for arthrotomy. The skin and subcutaneous tissues were removed, exposing the synovium of the femoropatellar and femorotibial joints. The synovium was inspected for macroscopic evidence of synovitis such as hyperemia, hypertrophy or fibrosis and any horses showing any of these changes were excluded from the study.[426] A minimum of 24 synovial membrane explants were harvested from these joints (at least 12 from each joint) using a 6 mm sterile biopsy punch (Integra Militex, Mansfield, MA). The stifles were disarticulated to expose the medial condyle of the distal femur and the articular cartilage was inspected. Any joint with macroscopically evident signs of osteoarthritis, such as cartilage erosions, score lines, discoloration or fibrillation were excluded from the study.[426] A minimum of 12 osteochondral explants were harvested from the medial condyle of the distal femur (6 from each joint). Explants were placed in phosphate buffered saline (PBS) for transport between harvest and culture sites (time interval between harvest and culture did not exceed 1 hour).

Explant co-culture

Explants were co-cultured in duplicate in standard serum-free culture medium, defined as Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine and sodium bicarbonate, free of sodium pyruvate (Lonza, Walkersville, MD), supplemented with 1% streptomycin, 1%

penicillin and 0.0025% amphotericin B (Cytiva, Marlborough, MA), 1% ascorbate-2-phosphate (Sigma-Aldrich, St. Louis, MA), 1% insulin-transferrin-sodium (Discovery Labware, Bedford, MA) and 50 μg/ml L-proline (Sigma-Aldrich). Each well of an unmodified polystyrene 12-well tissue co-culture plate (VWR, Radmor, PA; n=6) contained 3 ml total media, 2 synovium explants in the bottom of the well and 1 osteochondral explant within an overhanging tissue culture plate insert with polyester microporous membrane (pore size 0.4 μm).[427] Each of the six horses were assigned their own co-culture plate with all samples from the same horse cultured together. Co-culture plates were incubated at 37°C with 95% relative humidity and 5% carbon dioxide.

Study design

Each well of each of the 6 co-culture plates (1 plate per horse) was randomly assigned to 1 of 6 treatment groups (2 wells/group – **Figure 5**). Forty-eight hours after initiation of co-culture, with the exception of the 2 unstimulated control group wells, the remaining 10 wells were treated with 10 μg/ml recombinant equine interleukin-1β (IL-1β; R&D Systems, Minneapolis, MN) and 10 μg/ml tumour necrosis factor-α (TNF-α; R&D Systems; **Figure 6**).[109] After 48 hours, 2 wells were treated with TA (10^{-6}M; Kenalog, Bristol-Myers Squibb Company, New York, NY), 2 with MH (4.4 mg/ml; Carbocaine, Zoetis, Kalamazoo, MI) and 2 with TA + MH (concurrent treatment group). Doses were based upon physiologically appropriate data, each dose indicated is the final drug concentration per well.[358,428] An additional 2 wells were treated with MH and, six days later, were then treated with TA (delayed treatment group). Cell co-culture supernatant was harvested and media replenished every 3 days for a total of 9 days in co-culture following treatment.

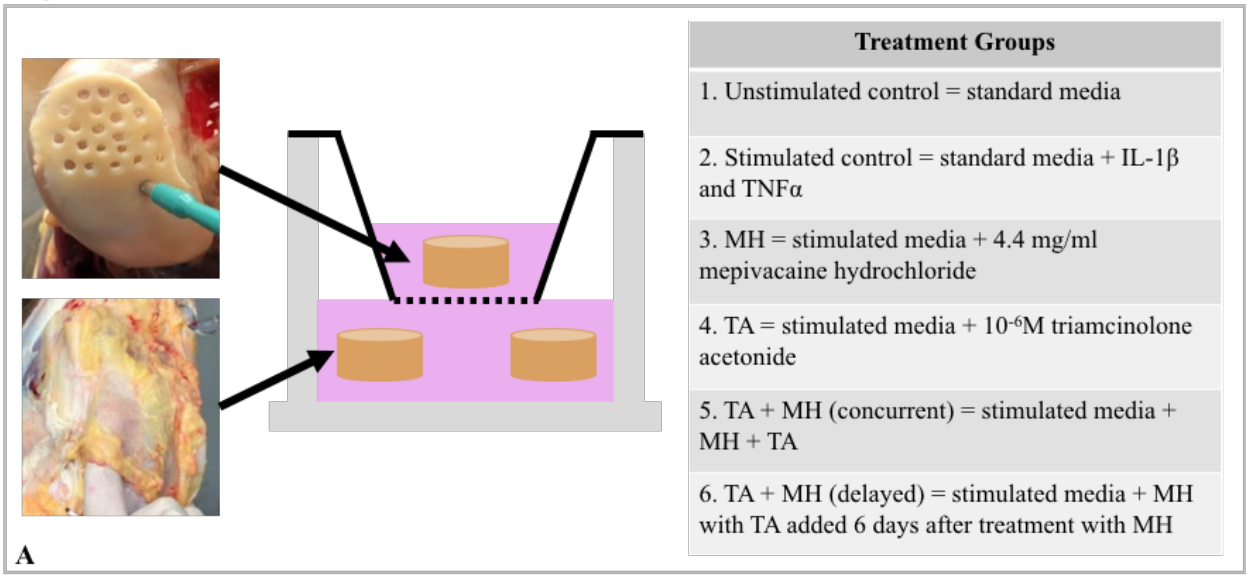


Figure 11. Experimental groups. One osteochondral explant and 2 synovial explants were co-cultured in standard culture medium. Two wells on each 12-well co-culture plate were randomly assigned to each treatment group. Key: IL-1β, equine interleukin-1β; TNF-α, tumour necrosis factor-α; MH, mepivacaine hydrochloride; TA, triamcinolone acetonide.

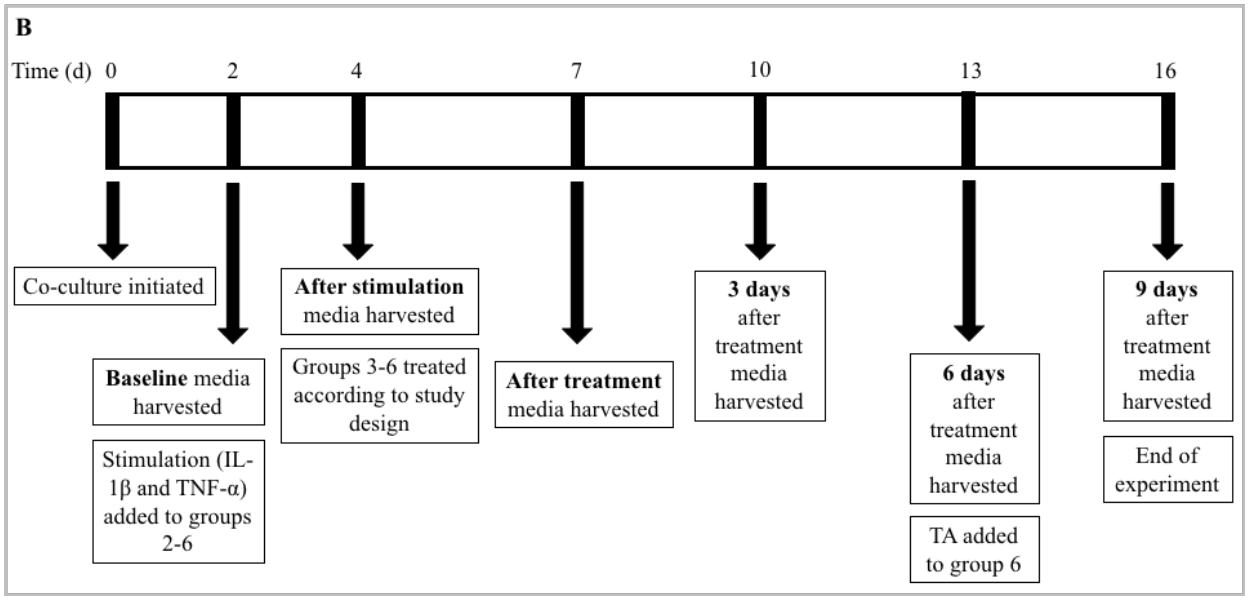


Figure 12. Study timeline. Media were harvested and replenished at day 2 (baseline), day 4 (after stimulation), day 7 (after treatment), day 10 (3 days after treatment), day 13 (6 days after treatment) and day 16 (9 days after treatment). Triamcinolone acetonide was added to group 6 (TA + MH, delayed) on day 13.

Media analysis

At each media change, the media from the 2 wells in each group were pooled, vortexed and stored in 200 μL aliquots. Harvested media was stored at 4°C and analyzed within 72 hours for a marker of cellular toxicity (lactate dehydrogenase, LDH) using a colorimetric assay (Roche Applied Science, Mannheim, Germany) following the manufacturer's instructions.[427] Cells were removed from media via centrifugation at 250 x g for 10 minutes and absorbance measured at 490 nm with a correction wavelength of 620 nm (SpectraMax ID3, Molecular Devices, Sunnyvale, CA). Measurements were performed in triplicate. Additionally, media samples were stored at -80°C for a maximum of 6 months and then thawed, centrifuged at 9000 rpm for 15 minutes and analyzed for analysis of inflammatory eicosanoids (prostaglandin E_2, PGE_2) and markers of matrix degradation (matrix metalloproteinase 13, MMP-13 and dimethylmethylene blue assay, DMMB).

For determination of PGE_2 concentration in co-culture media, a commercially available ELISA assay (R&D Systems, Minneapolis, USA) was used according to the manufacturer's instructions.[429] This assay uses horseradish peroxidase-conjugated PGE_2 binding sites on a mouse monoclonal antibody and is detected by a hydrogen peroxide and tetramethylbenzidine substrate. Samples were diluted with a buffered protein base to 1:30 and measured in duplicate. Absorbance was measured at 450 nm with correction set at 540 nm.

A commercially available sandwich ELISA (R&D Systems) was used to quantify MMP-13 concentrations in media in accordance with the instructions of the manufacturer. In this assay, a mouse anti-human MMP-13 capture antibody and biotinylated goat anti-human MMP-13

detection antibody detect MMP-13 in media using streptavidin conjugated to horseradish peroxidase and a hydrogen peroxide and tetramethylbenzidine substrate. Samples were diluted with 1% bovine serum albumin (BSA) in PBS to 1:1-15 and measured in duplicate. Absorbance was measured at 450 nm with a correction set at 540 nm.

The DMMB assay was performed by combining diluted media (1:4) with papain at 65°C for 4 hours. This assay estmates the glycosaminoglycan (GAG) content of the media, GAGs are released upon damage to cartilage ECM. The estimate was made by measuring optical density at 525 nm.[430]

Statistical analysis

Normality was determined with the Kolmogorov-Smirnov test. Data were analyzed using two-way repeated measures ANOVA for normally-distributed data or mixed-effects models for non-normally distributed data with Tukey's test for post-hoc testing. The two independent variables assessed were treatment group (unstimulated control, stimulated control, MH only, TA only, TA + MH [concurrent] and TA + MH [delayed]) and time point. The outcome variable was LDH, PGE_2, MMP-13 or GAG. All six horses contributed data to the outcome variables, with the exception of MMP-13 (5 horses). All statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, CA) and significance was set at $P < 0.05$.

Chapter 5: Results

Cell toxicity (lactate dehydrogenase)

Stimulation (IL-1β and TNF-α) did not result in an increase in media LDH concentration at any time compared to unstimulated controls (**Figure 7**). All explants treated with MH had increased LDH in culture media immediately after treatment (MH, $P < 0.0005$; TA + MH [concurrent], $P < 0.0005$; TA + MH [delayed], $P < 0.0005$) and 3 days after treatment (MH, $P = 0.006$; TA + MH [concurrent], $P = 0.008$; TA + MH [delayed], $P = 0.020$) as compared to stimulated controls. Immediately after treatment, explants treated with TA alone had decreased LDH as compared to explants treated with MH (MH versus TA, $P = 0.003$; TA + MH [concurrent] versus TA, $P = 0.006$; TA + MH [delayed] versus TA, $P = 0.003$). Treatment with TA alone did not result in an increase in LDH at any time. There was no difference in LDH between TA + MH (concurrent) and TA + MH (delayed) at any time (before or after stimulation).

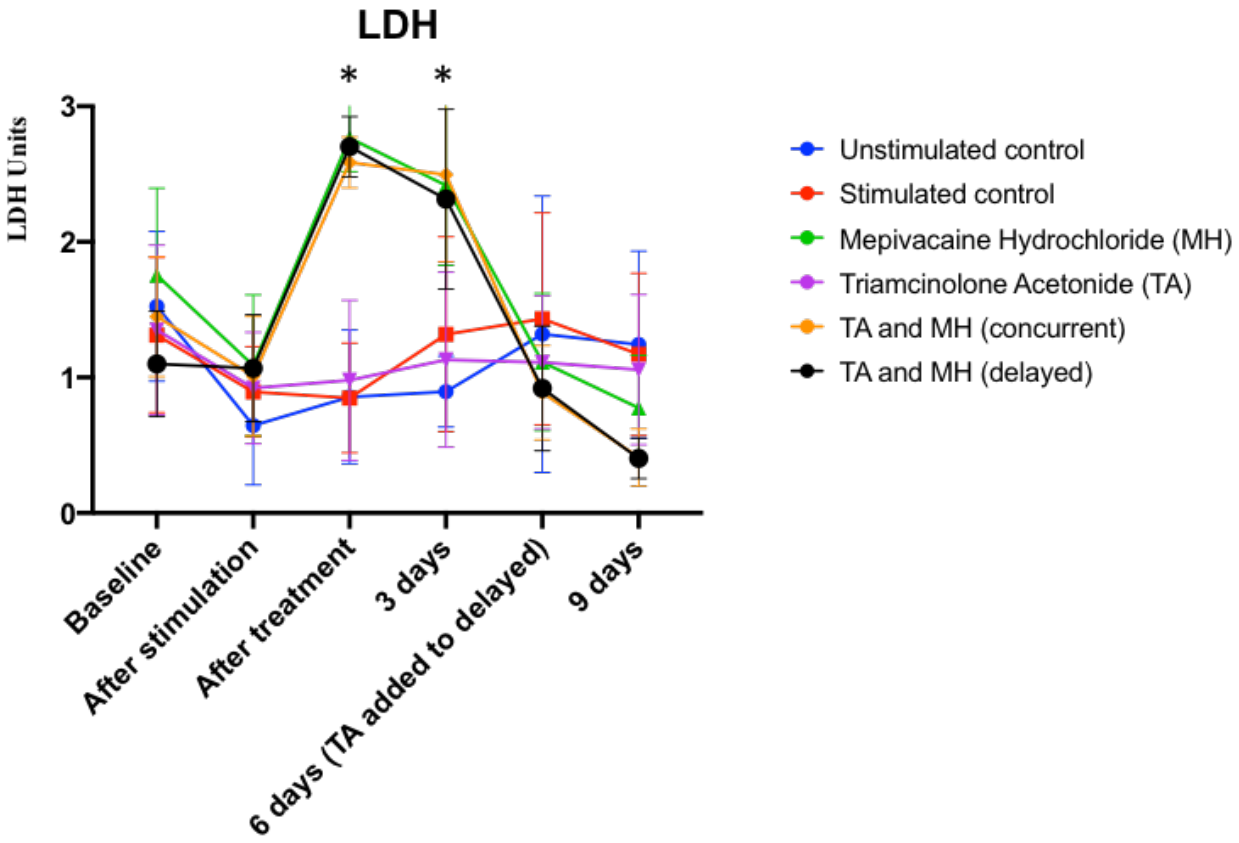


Figure 13. Lactate dehydrogenase (LDH) levels over time for equine explants in co-culture. All explants treated with MH had increased LDH immediately after treatment (MH, $P < 0.0005$; TA + MH [concurrent], $P < 0.0005$; TA + MH [delayed], $P < 0.0005$) and 3 days after treatment (MH, $P = 0.006$; TA + MH [concurrent], $P = 0.008$; TA + MH [delayed], $P = 0.020$) as compared to stimulated controls. Treatment with TA did not result in an increase in LDH at any time. Data points are mean values (n=6); error bars represent SD; * significant differences in LDH between explants treated with MH (MH, TA + MH [concurrent], TA + MH [delayed]) and stimulated controls, $P < 0.05$.

Inflammation (prostaglandin E_2)

Stimulation (IL-1β and TNF-α) resulted in an increase in media PGE_2 concentration immediately after stimulation ($P = 0.013$), after treatment ($P = 0.004$) and 3 days after treatment ($P = 0.022$) compared to unstimulated controls (**Figure 8**). All treated explants had decreased PGE_2 as compared to stimulated controls immediately after treatment: MH ($P = 0.007$), TA ($P = 0.045$), TA + MH (concurrent, $P = 0.008$) and TA + MH (delayed, $P = 0.010$). These differences were not seen at 3, 6 or 9 days after treatment. There were no differences in PGE_2 between the treated groups (TA, MH, TA + MH (concurrent) and TA + MH (delayed) at any time.

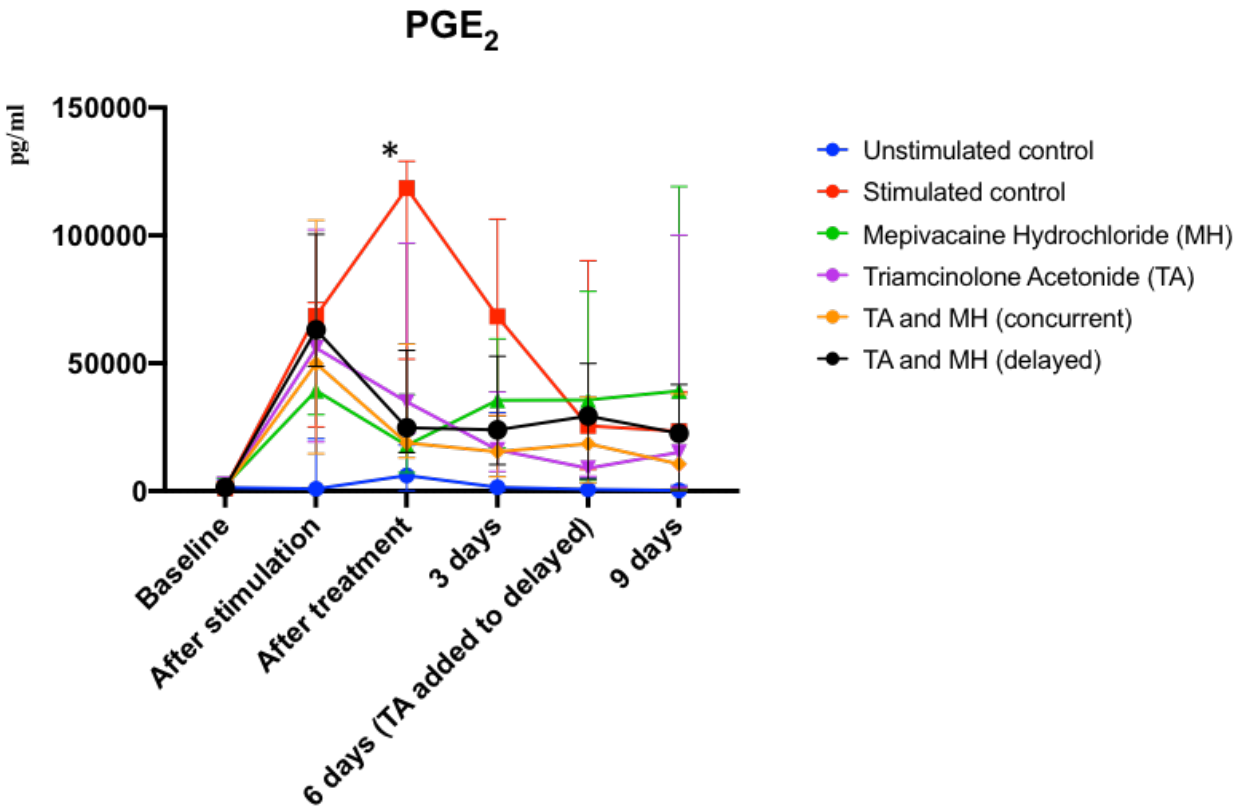


Figure 14. Prostaglandin E2 (PGE2) levels over time for equine explants in co-culture. All treated explants had decreased PGE2 as compared to stimulated controls immediately after treatment: MH (P = 0.007), TA (P = 0.045), TA + MH (concurrent, P = 0.008) and TA + MH (delayed, P = 0.010). Data points are median values (n=6); error bars represent 95% CI; * differences in PGE2 treated (TA, MH, TA + MH [concurrent] and TA + MH [delayed]) explants and stimulated controls, P < 0.05.

Matrix degradation (matrix metalloproteinase 13)

Stimulation (IL-1β and TNF-α) resulted in an increase in media MMP-13 concentration after stimulation ($P = 0.009$), treatment ($P < 0.0005$), 3 days after treatment ($P = 0.029$), 6 days after treatment ($P = 0.022$) and 9 days after treatment ($P = 0.044$) compared to unstimulated controls (**Figure 9**). Treated explants had less MMP-13 than stimulated controls immediately after treatment: MH ($P < 0.0005$), TA ($P = 0.046$), TA + MH (concurrent, $P < 0.0005$) and TA + MH (delayed, $P < 0.0005$). This same difference was seen at 3 days after treatment: MH ($P = 0.041$), TA ($P = 0.047$), TA + MH (concurrent, $P = 0.036$) and TA + MH (delayed, $P = 0.037$) and at 6 days after treatment: MH ($P = 0.027$), TA ($P = 0.047$), TA + MH (concurrent, $P = 0.026$) and TA + MH (delayed, $P = 0.024$). There were no differences in MMP-13 between the treated groups (TA, MH, TA + MH [concurrent] and TA + MH [delayed] at any time.

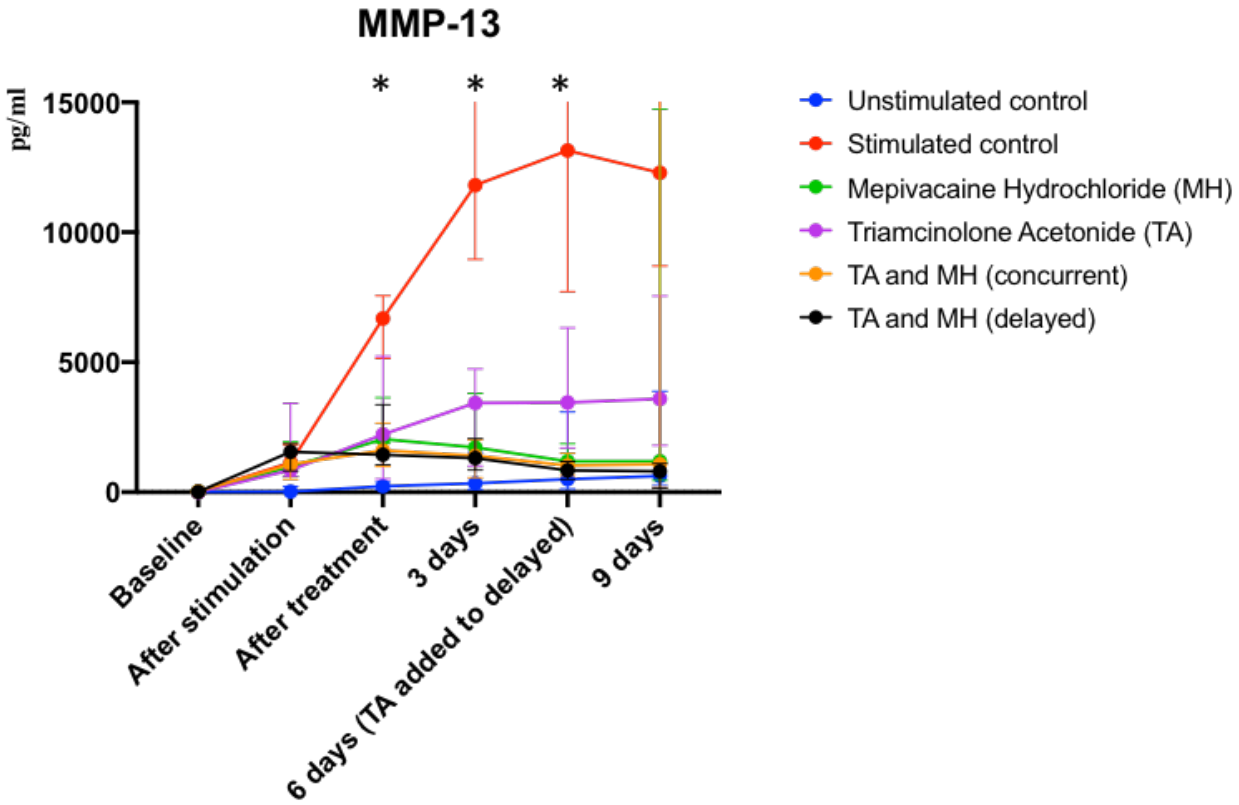


Figure 15. Matrix metalloproteinase 13 (MMP-13) levels over time for equine explants in co-culture. Treated explants had less MMP-13 than stimulated controls immediately after treatment: MH ($P < 0.0005$), TA ($P = 0.046$), TA + MH (concurrent, $P < 0.0005$) and TA + MH (delayed, $P < 0.0005$). This same difference was seen at 3 days after treatment: MH ($P = 0.041$), TA ($P = 0.047$), TA + MH (concurrent, $P = 0.036$) and TA + MH (delayed, $P = 0.037$). It was also observed at 6 days after treatment: MH ($P = 0.0027$), TA ($P = 0.047$), TA + MH (concurrent, $P = 0.026$) and TA + MH (delayed, $P = 0.024$). Data points are mean values (n=5); error bars represent SD; * differences in MMP-13 in treated (TA, MH, TA + MH [concurrent] and TA + MH [delayed]) explants and stimulated controls, $P < 0.05$.

Matrix degradation (dimethylmethylene blue)

Stimulation (IL-1β and TNF-α) did not result in an increase in media GAG concentration at any time compared to unstimulated controls (**Figure 10**). There were no differences in GAG concentration between the treated groups (TA, MH, TA + MH (concurrent) and TA + MH (delayed) at any time.

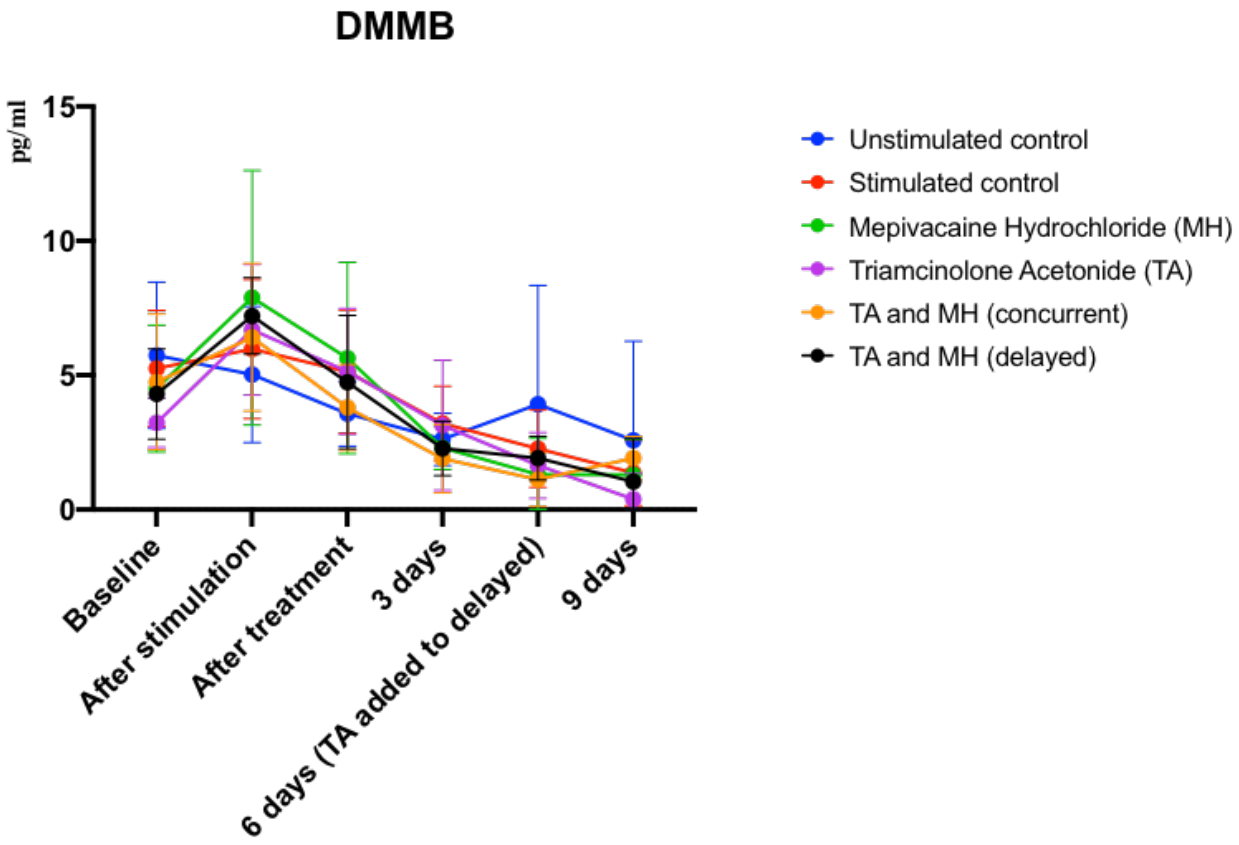


Figure 16. Dimethylmethylene blue (DMMB) levels over time for equine explants in co-culture. There were no differences in GAG concentration in media between treated explants and controls at any time. Data points are mean values (n=6); error bars represent SD.

Chapter 6: Discussion

There was no difference in synovial and osteochondral tissue cytotoxicity (LDH), inflammatory eicosanoid production (PGE_2) or matrix destruction (MMP-13, DMMB) between articular tissue samples treated with TA and MH concurrently or those with delayed TA administration 6 days subsequent to MH. In the described *in vitro* biochemical model of equine inflammatory joint disease, no benefit of a delay between treatment with intra-articular local anaesthetic (pain localisation) and corticosteroid (treatment) could be demonstrated. Therefore, we can reject our hypothesis.

Measuring LDH estimates cell death and lysis as LDH is released from damaged cells into the culture media. Following treatment with MH there were large increases in media LDH concentration in all MH-treated groups (MH, concurrent and delayed). Local anaesthetics demonstrate drug and dose-dependent cytotoxicity to articular tissues, with MH seeming to be more safe than lidocaine or bupivacaine but not as safe as ropivacaine, a drug less commonly used for lameness examination in horses.[20,262,268] The cytotoxic effect of MH in synoviocyte and chondrocyte co-culture has previously been demonstrated.[428] Interestingly, treatment with MH resulted in suppression of PGE_2 and MMP-13 in the current study. Prostaglandins are synthesized from arachidonic acid via the actions of cyclooxygenase (COX) enzymes, i.e. via an active process that requires viable cells.[431] Similarly, MMP-13 is synthesized and secreted by fibroblasts and chondrocytes in response to catabolic cytokines and also requires viable cells.[87] It may be that the apparent anti-inflammatory and anti-collagenase treatment effect of MH seen in the current study is due to the cell cytotoxicity rather than these properties being innate properties of the local anaesthetic.[432]

Treatment with TA suppressed PGE_2 and MMP-13 in the current study. This finding was not unexpected, as corticosteroids decrease inflammation by inhibiting the arachidonic acid cascade and COX-2 enzymes, thereby blocking prostaglandin synthesis.[348] Additionally, corticosteroids inhibit the collagenolysis associated with IL-1β stimulation of chondrocytes and suppress the subsequent matrix metalloproteinase enzyme production by inflamed chondrocytes.[62,433] Currently there is conflicting evidence regarding the effect of treatment with TA on articular cell viability. Previously, a single injection of TA resulted in significant decreases in clonal chondrocyte viability in monoculture.[434] Similarly, TA induced chondrotoxicity when administered to human monolayer chondrocyte culture.[421] However, synoviocyte and chondrocyte viability was not reduced after TA treatment in a canine explant model.[367] Equine *in vivo* studies have similarly mixed results, with both beneficial and detrimental effects of treatment reported.[128,373,374] In the current study, treatment with 10^{-6} M TA did not result in an increase in LDH, suggesting drug safety at this dose to these tissues evaluated *in vitro*. This finding is in agreement with Trahan et al, who found that LDH production was not increased in synovial and osteochondral explants treated with TA at this clinically relevant dose.[372] The different results concerning the safety of TA may be due to the different *in vitro* models utilized, as well as the different TA doses and methodology for determining cell viability.

In the current study, treatment of explants with TA resulted in PGE_2, MMP-13 and GAG levels in media that were not different to explants also treated with MH. However, explants treated with TA alone had less LDH in media than those also treated with MH. This finding is in agreement with two previous *in vitro* studies (one examining bovine and the other examining human tissues) which determined that corticosteroid and local anaesthetic combination treatment results

in more dramatic decreases in chondrocyte viability (either due to increased apoptosis, necrosis or both) than treatment with either agent alone.[19,420] However, in the one previous equine study examining the combination of TA and MH, a protective effect of TA was determined in unstimulated explants, as determined by histomorphometric evaluation.[435] That study also found no difference in GAG content between explants treated with TA alone and those treated with TA and MH. The protective effect of TA was lost when explants were stimulated with lipopolysaccharide. In the current study, no protective effect of TA was determined in synovial and osteochondral explants treated with IL-1β and TNF-α, suggesting that in a stimulated environment (either by LPS or by catabolic cytokines) the cytotoxic effect of MH overwhelms the protective capacity of TA.

The *in vitro* co-culture model utilized in this study was modified from those originally described by Haltmayer et al, Byron and Trahan.[109,427] We elected not to create the 2-mm partial-thickness defect within each of our cartilage explants as described by Haltmayer et al in order to avoid excessive handling of the explants. Interestingly, Haltmayer et al did not see significant differences in media MMP-13 concentration between control and OA-model groups as determined by ELISA, whereas we saw increased MMP-13 over time in stimulated control explants, in agreement with Byron and Trahan. This difference is likely due to the different sampling times between studies. We observed that stimulation (10 μg/ml IL-1β and 10 μg/ml TNF-α) did not affect LDH or DMMB concentrations, but resulted in a transient increase in PGE_2, a finding also shared by Byron and Trahan.[427] *In vitro* co-culture models are useful for determining the treatment effect of various compounds without the need for animal models of OA. In addition, the use of a multi-tissue model increases the likelihood of capturing the effect of

articular tissue cross-talk.[62,427,436,437] However, they cannot fully capture the complexities of *in vivo* systems and extrapolation of these results to clinical practice should be done cautiously.

Aside from the *in vitro* nature of the experiment, this study had several limitations. The low number of horses reduced the statistical power of the study and increased the risk for type II error. The number (n=6) chosen was based upon previous experience with this co-culture model and aimed at minimizing the number of horses required.[329] Additionally, while horses with a previous diagnosis of OA were not enrolled in the study, diagnostic investigation such as radiography or synovial fluid analysis was not performed and previously established inflammatory joint disease cannot be definitively ruled out. Macroscopic evaluation of cartilage has been validated using MRI in models of cartilage repair, therefore it was determined that macroscopic evaluation of the cartilage during harvest would be sufficient to determine the presence of clinically relevant joint disease.[438] The variable range in results between individuals may have been due to the wide range in ages of horses included in the study. Use of explants means that outcome correlations cannot be performed for cell numbers, additionally we did not weigh explants and therefore some variation between each 6-mm diameter explant (obtained using a biopsy punch) was likely. Another limitation was the long interval between media changes, which may have resulted in failure to capture shorter-duration treatment effects. While every effort was made to keep experimentation conditions the same during using ELISA analysis, we acknowledge that minor variations beyond our control may have impacted results. Additionally, the MMP-13 ELISA used in this experiment has not been cross-validated for equine species reactivity which may limit the validity of the results. However, similar human MMP-13 assays have been used previously for equine tissue analysis with success.[372]

Chapter 7: Conclusions

This study was conducted to determine if a beneficial effect of a delay period between local anesthetic and corticosteroid treatment of equine articular tissues could be demonstrated *in vitro*. As there were no differences in markers of synoviocyte and chondrocyte cell death, inflammation, and matrix destruction, no such beneficial effect could be demonstrated. This was likely due to the overwhelming cytotoxicity seen after treatment with MH, which was not attenuated by treatment with TA. Translation of this work to an appropriate *in vivo* model would be ideal to more accurately test our hypothesis. Our results do not support the use of MH for joint blocks in horses and alternative methods of diagnosis, such as perineural anesthesia, are preferrable.

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