THE ROLE OF LOAD IN INITIATION AND PROGRESSION OF CARTILAGE PATHOLOGY

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ABSTRACT

Articular cartilage is primarily responsible for dissipation of load in diarthrodial joints. Load plays a critical role in maintaining cartilage health, but can also be a primary contributing factor in cartilage disease. Osteoarthritis, initially seen as cartilage degradation, can result from application of abnormal loads to normal tissue or application of normal load to abnormal tissue. IL-1β is known to play a major role in the initiation and progression of osteoarthritis through inciting cascades which cause inflammation and degradation. When cartilage degradation and the associated symptoms occur, corticosteroids are used extensively in the equine industry. Corticosteroids do have beneficial effects with respect to lameness and inflammation, but can exacerbate cartilage degradation and hinder tissue healing . Therefore, it is important to understand the roles and interactions of load, IL-1β, and corticosteroids with respect to cartilage health and disease.

To better understand the effects of corticosteroids and IL-1β on articular cartilage *in vivo*, relevant gene expression, extracellular matrix composition, and biomarker production of cartilage subjected to various combinations of load, corticosteroids, and IL-1 β were analyzed using *in vitro* tissue explant and 3-D chondrocyte culture systems. The results from this study have begun to disclose

some of the effects of various loads on articular cartilage. Higher frequencies and durations of compressive loading seemed to have more pronounced deleterious effects on cartilage, even within physiological loading ranges. In combination with corticosteroids, compressive loads at 2 and 6 MPa delivered at a frequency of 1 Hz for 20 minutes three times a day resulted in changes similar to those reported in corticosteroid-induced arthropathy. However similar compressive load delivered at lower frequencies at 2 and 6 MPa delivered at a frequency of 0.1 Hz for 20 minutes three times a day seemed to mitigate some of the deleterious effects of IL-1β as evidenced by decreased expression of matrix metalloproteinases when compared to unloaded samples and samples loaded at higher frequencies.

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OC	Osteochondrosis
OCD	Osteochondrosis Dissecans
OA	Osteoarthritis
IL-1 β	Interleukin 1
TGF β	Transforming Growth Factor
MPA	Methylprednisolone Acetate
MPa	MegaPascals
kPa	KiloPascals

Introduction

Articular cartilage is an aneural, avascular, alymphatic hyaline cartilage and is characterized by an abundant extracellular matrix (ECM) which makes up 95 % of the tissue volume and consists mainly of aggrecan and collagen type II. Chondrocytes fill most of the remaining tissue volume and though, are sparse are the sole producers of the ECM. The chondrocytes play a central role in the production, organization and maintenance of the ECM and are in turn protected by the ECM from the biomechanical forces exerted on the cartilage.

Articular cartilage is a very dynamic organ, the main components of which are the collagens, proteoglycans and chondrocytes which act together to determine the mechanical behavior of cartilage. Any change in the composition of these components either due to increased degradation or decreased production will change the mechanical properties of cartilage. Chondrocyte metabolism is regulated by a variety of factors such as cytokines, growth factors, matrix adhesion, and mechanical loading. In an *in vivo* situation, the coordinated influence of these factors regulate the biologic behavior of chondrocytes and, thus, the state of cartilage health.

Normal mechanical loading is needed for chondrocytes to be able to produce and maintain the ECM. The effect of loading also depends on the magnitude and frequency of applied load. Static compression of tissue to physiological magnitudes causes breakdown of proteoglycan. Moderate exercise has a protective effect on the joint and increases matrix synthesis and content. Moderate exercise has been shown to reduce the severity of OA lesions in rats.

Application of supraphysiologic (excessive frequency/ magnitude) load elicits a net ECM catabolic response by chondrocytes characterized by increased expression of matrix metalloproteinase (MMP)-1, MMP-3, MMP-9 interleukin 1β (IL- 1 β), tumor necrosis factor α, cycloxygenase- 2, increased nitric oxide, prostaglandin E_2 synthesis reduced expression of type II collagen and aggrecan, inhibited the synthesis of DNA, proteoglycan, collagen and protein, and increased chondrocyte cell death.

The exact mechanism from mechanical load stimulation to signal transduction and change in gene expression is not yet fully understood. Studies suggest that multiple regulatory pathways could be involved including upstream signaling pathways and mechanisms that may lead to direct changes at the level of transcription, translation, and post-translational modifications and cell-mediated extracellular assembly and degradation of matrix. Correspondingly, the stimulus could be due to changes in cell shape and volume, fluid flow, hydrostatic and osmotic pressure, shear and electrical potential which are brought about by loading. By these pathways, physical stimuli can not only alter the rate of matrix production, but the quality and functionality of newly synthesized proteoglycans, collagens, and other molecules, resulting in diseases like osteoarthritis and osteochondrosis.

Various *in vitro* models have been developed to study cartilage metabolism in response to various stimuli and have their own advantages and disadvantages. The biggest disadvantage of these studies is that none of them have incorporated load as a factor. Since the major aim of an *in vitro* model is to

mimic an *in vivo* situation and since joints are always experiencing some degree of load, it is essential that load be included as a factor in cartilage studies as load has both beneficial and deleterious affects and may influence how the cartilage reacts to a particular stimulus.

Osteoarthritis and osteochondrosis are the biggest problems affecting the equine industry and cost millions of dollars in terms of treatment costs, loss of use and prolonged disability. The main objectives to the studies undertaken were to try and elucidate if load plays a role in the development of these diseases either alone or in combination with other stimuli. Since horses are born athletes and extensively used for sport and pleasure as an athlete, these studies would enable us to better understand if a horse should be exercised in a particular situation and if so to what levels of joint loading as the amount of load experienced by the cartilage would be directly proportional to the activity.

Review of Literature

There are three major types of cartilage in the body: 1) elastic cartilage, 2) fibrocartilage, and 3) hyaline cartilage. Elastic cartilage exists in the epiglottis and the eustachian tube. Fibrocartilage, often exists temporarily at fracture sites, but is permanently present in three major locations in the body: the intervertebral disks of the spine, as a covering of the mandibular condyle in the temporomandibular joint, and in the meniscus of the knee. The third type of cartilage, hyaline cartilage also called the articular cartilage, is most prominently found in diarthroidal joints covering long bones.

Articular cartilage is an aneural, avascular, alymphatic hyaline cartilage and is characterized by an abundant extracellular matrix (ECM) which makes up 95 % of the tissue volume and consists mainly of aggrecan and collagen type II [1,2]. Chondrocytes fill most of the remaining tissue volume and though, are sparse are the sole producers of the ECM. The chondrocytes play a central role in the production, organization and maintenance of the ECM and are in turn protected by the ECM from the biomechanical forces exerted on the cartilage.[3,1]

Articular cartilage can be divided in two major phases: 1) a fluid phase (68-85%) consisting of water and electrolytes, and 2) a solid phase made up of collagen type II (10-20%),trapped proteoglycans (5-10 %), glycoproteins and chondrocytes (2 -5%). [4,3,5,1,6]

Articular cartilage is a very dynamic organ and these three major components act together to determine the mechanical behavior of cartilage, and any change in the composition of these components either due to increased degradation or decreased production will change the mechanical properties of cartilage.[6] In vitro studies have shown that chondrocyte metabolism is regulated by cytokines,[7] growth factors,[8] matrix adhesion,[9,10] and mechanical loading[11,12]. In an in vivo, situation the coordinated influence of these all these factors regulate the biologic behavior of chondrocytes and, thus, the state of cartilage health.

Of the three major components, the most prevalent is water[13-16]. About 30% of the total water exists within the intrafibrillar space of collagen [17-19]. The collagen fibril diameter and the amount of water within the collagen is determined by the swelling pressure due to the fixed charge density (FCD) of the proteoglycans [18,20,19]. So what this means is that the proteoglycans have strong negative electric charges. The proteoglycans are constrained within the collagen matrix. Because the proteoglycans are bound closely, the closeness of the negative charges creates a repulsion force that must be neutralized by positive ions in the surrounding fluid. The higher concentration of ions in the tissue compared to outside the tissue leads to swelling pressures. The exclusion of water raises the density of fixed charge, which in turn raises the swelling pressure and charge-charge repulsion[6]. The amount of water present in cartilage depends on 1) the concentration of proteoglycans which determines FCD and swelling pressure, 2) the organization of the collagen network, and 3) the

stiffness and strength of the collagen network. The FCD also determines the collagen fibril diameter. The collagen network resists the swelling of the articular cartilage. If the collagen network is degraded, as in the case of OA, the amount of water in the cartilage increases[21-23], because more negative ions are exposed to draw in fluid. This increase in fluid can significantly alter the mechanical behavior of the cartilage.

In addition, with a pressure gradient or compression, fluid is squeezed out of the cartilage. When the fluid is being squeezed out, there are drag forces between the fluid and the solid matrix that increases with increasing compression and make it more difficult to exude water. This behavior increases the stiffness of the cartilage as the rate of loading is increased.[5,22,6]

Collagen accounts for about two-thirds of the dry weight of adult articular cartilage. Collagen is the component of cartilage that is believed to contribute most to tensile behavior of the tissue. The tissue's material strength depends on the extensive cross-linking of the collagen and the apparent zonal changes in fibrillar architecture with tissue depth. The predominant collagen in articular cartilage is type II, and contains three α 1(II) polypeptide chains. [6]. The collagen network traps proteoglycans and other cartilage molecules. [1] Once this collagen network is laid down during development, the chondrocytes seem to have little capacity to recapitulate the overall collagen architecture if the mature tissue is injured or undergoes advanced degenerative changes. The ability of chondrocytes to remodel the collagen at ultrastructural and molecular levels is

poorly understood, but it may be more significant than previously thought and possible molecular mechanisms are a topic of growing interest.[24] Collagenases are mainly thought to be be responsible for degradation of collagens by an initial cleavage of the collagen molecule into three-quarter and one-quarter length fragments. Chondrocytes in culture with IL-1 stimulation and in tissue removed form arthritic joints have been shown to express s collagenases, including collagenase-3 (MMP13) which is the most active in cleaving type II collagen.[25]

The third major component of cartilage is aggrecan, a large aggregating proteoglycan. The presence of aggrecan is considered the hallmark of chondrogenesis and accounts for 80-90% of the total proteoglycan and has been studied extensively due to its role in skeletal growth, joint function and the development of arthritis. These molecules normally occupy much larger space when not compacted by a collagen network. The compaction of the proteoglycans affects swelling pressure as well as fluid motion under compression. [26-30]

Proteoglycans are formed from GAGs covalently attached to core proteins. GAGs are the most abundant heteropolysaccharides in the body. These molecules are long unbranched polysaccharides containing a repeating disaccharide unit. GAGs are highly negatively charged with extended conformation that imparts high viscosity to the solution. GAGs are located primarily on the surface of cells or in the ECM. Along with the high viscosity of GAGs comes low compressibility, which makes these molecules ideal for a

lubricating fluid in the joints. At the same time, their rigidity provides structural integrity to cells and provides passageways between cells, allowing for cell migration. Proteoglycans are able to bind calcium and hence their changes are thought to play an important role in endochondral ossification.[31]

Articular cartilage can be divided into four zones. The superficial or tangential layer, which is characterized by low proteoglycan content and the collagen fibrils are arranged parallel to the surface. This layer makes up 5-10% of the ECM volume.[32,33,2] The transitional of intermediate layer is characterized by an increase in proteoglycan content and the collagen fibrils are arranged perpendicular to the surface. This layer occupies 40-45 % of the ECM volume. [32,33,2] Another 40-45 % of the matrix volume is occupied by the deep or radial layer and is characterized by high proteoglycan content. The fourth zone is the calcified cartilage layer which occupies 5-10% of the matrix volume. This layer lies between the tidemark and the subchondral plate of the bone and is characterized by a high concentration of calcium salts, the absence of proteoglycans, radial collagen fibers and rounded chondrocytes. [32,33,2]

The articular cartilage is a very dynamic organ and is constantly being remodeled and this requires a balance between degradation and synthesis of matrix molecules and imbalance in this cycle could lead to chemical and biomechanical failure of the cartilage. Chondrocytes through their interactions with the matrix maintain this balance and integration of newly synthesized molecules into the matrix. The degradation part of the process is

controlled by the production and activation of matrix proteinases and their inhibitors. Two proteinases are primarily responsible for collagen and proteoglycan degradation in cartilage tissue 1)matrix metalloproteinases (MMPs) and 2)aggrecanases.[34] Collagen degradation is regulated by MMPs where as both aggrecanases and MMPs can degrade aggrecan. Though the exact mechanism is not understood, in normal articular cartilage the levels of MMPs and aggrecanases is tightly regulated to basal levels for matrix turnover and remodeling, [35] and is accomplished by tissue inhibitors of metalloproteinases (TIMPs).[36,37] In OA this balance is lost resulting in cartilage degradation and failure. [38]

Articular cartilage has an incredibly low coefficient of friction which, along with its ability to bear very large compressive loads, makes it ideally suited for placement in joints which experience varying degrees of load during movement and rest. The articular cartilage thus 1) protects the bone from abrasion and other damage, 2) transmits and distributes compressive loads and shearing forces to the subchondral bone, 3) provides for joint congruity and thus low contact stress between apposing joints 4) provides a smooth, lubricated surface which facilitates movement with little friction between articulating surfaces. [39] The ECM in the cartilage along with the synovial fluid provides a low friction surface. [40] The articular cartilage lacks blood supply and all nutrition is diffused. The articular cartilage has limited healing capacity and any injury can lead to the

development of various orthopedic diseases such as Osteochondrosis (OC)[41-44] and Osteoarthritis (OA) [45-47].

The exact mechanism of the initiation and progression of OA and OC are not understood and various factors ranging from excessive load due to over exercise or abnormal joints [48-53,44]to trauma[54-58] to nutrition[59-66] to genetics[67-72] to metabolic and endocrine disorders [73,64,74,75] have been thought to play a role in the initiation and progression of both these conditions.

The mechanical environment of the chondrocytes is an important factor and influences the health and function of the joint.[76,77] The chondrocytes use mechanical signals in conjunction with other environmental and genetic factors to regulate their metabolic activity. However, the specific sequence of events through which this occurs is not understood.[78] This capability provides a means by which articular cartilage can alter its structure and composition. [11,76,79,12]

Articular cartilage serves as a system for load support, load transfer and motion between bones of the diarthrodial joint and any alterations can compromise the ability of the cartilage to function and survive the strenuous mechanical environment normally found in the load bearing joints.[80] As cartilage develops it responds to load and remodels. Not only is cartilage thickness increased in regions of highest physiological load, [81-83] in young adult animals it is smooth, glistening, pearly white where as in older animals the articular cartilage often acquires a yellowish tint and India ink staining reveals an increasing degree of fibrillation and erosion with advancing age. [81] The applied

forces probably modify cellular behavior by affecting metabolism, gene expression, cytokine and growth factor secretion.

The pressure exerted on cartilage varies between 0 and 20 MPa during movements, [84-88] with the average daily contact stress on a human joint ranging from approximately 3 to 10 MPa.[22] Normal mechanical loading is needed for chondrocytes to be able to produce and maintain the ECM. [89-91] The effect of loading also depends on the magnitude and frequency of load applied. Static compression of tissue to physiological magnitudes causes the breakdown of proteoglycan.[92-94] Moderate exercise has a protective effect on the joint and increases matrix synthesis and content. [95-100] Moderate exercise has been shown to reduce severity of OA lesions in rats.[101] Whereas application of supraphysiologic (excessive frequency/ magnitude) load elicits a net ECM catabolic response by chondrocytes characterized by increased expression of matrix metalloproteinase (MMP)-1, MMP-3, MMP-9 interleukin 1β (IL- 1 β), tumor necrosis factor α, cycloxygenase- 2, increased NO, PGE_2 synthesis reduced expression of type II collagen and aggrecan, inhibited the synthesis of DNA, proteoglycan, collagen and protein, and increased chondrocyte cell death.[102,103,93,104-106]

The exact mechanism from mechanical load stimulation to signal transduction and change in gene expression is not yet fully understood. Studies suggest that multiple regulatory pathways could be involved including upstream signaling pathways,[107-109] and mechanisms that may lead to direct changes at the

level of transcription,[110-112] translation, and post-translational modifications[113-115] and cell-mediated extracellular assembly and degradation of matrix.[116-118] Correspondingly, the stimulus could be due to changes in cell shape and volume, [119,78] fluid flow, [120-122] hydrostatic and osmotic pressure, shear and electrical potential which are brought about by loading.[123] By these pathways physical stimuli can not only alter the rate of matrix production, but the quality and functionality of newly synthesized proteoglycans, collagens, and other molecules.

OA and OC not only effect horses but are also seen in humans and several other species. [124-127] Both these are painful conditions and there is currently no cure for either OA or OC and the available treatments are palliative at best. As stated earlier the pathophysiology of OA and OC are not fully understood and further studies to understand the pathogenesis and pathophysiology of these arthropathies are necessary for both human and veterinary medicine. The potential to address both these conditions exists in various animal models,[128-136,127] and further investigation using animal models will give us a better understanding of the arthropathies. Though *in vivo* models are closer to the clinical situation, an inherent defect with *in vivo* models is the lack of ability to look at the effects of a single factor in the initiation and progression of these conditions. Thus the use of *in vitro* models is gaining wider acceptance. The main advantages of these *in vitro* systems over the *in vivo* models are reduced cost, reduction of confounding variables, reduced biological variability,

increased experimental control, the use of methodologies which are not feasible in vivo, their basic simplicity and above all the need for fewer animals.

Various *in vitro* models have been proposed for the study of cartilage health and pathology. Ranging from monolayer cultures to co-cultures. Although it is possible to isolate large numbers of chondrocytes from normal cartilage, growth in an adherent monolayer culture results in dedifferentiation to a more fibroblast- like phenotype characterized by change form round to spindle- shaped cells, increased proliferative capacity, decreased expression of collagen type II and aggrecan and increased expression of collagen type I and III.[137-139]

Various three-dimensional (3-D) cultures systems have been used by suspending chondrocytes in collagen gel [140-142], agraose gel [143-153], in alginate[154-161] and using polyglycolic acid scaffolds[162,163] and these cultures have been shown to maintain cell viability differentiation, and appropriate ECM production, and are probably the best way to culture chondrocytes or study the effects of various substances on chondrocytes, but these models lack cell matrix interaction which is essential for the normal function of the cartilage. A good example to support the importance of cell matrix interactions is that this is that chondrocytes in agarose cultures do not show significant response to load until they have synthesized enough/measurable ECM. [164,165]

In contrast, chondrocytes in cartilage explant culture have low mitotic activity, maintain their differentiated phenotype, and the ECM is similar to the one observed

in vivo.[138] However significant differences in biochemical characteristics [total collagen, Glycosaminoglycan (GAG) and DNA concentration] and chondrocyte ECM metabolism (aggrecan, decorin and biglycan synthesis) have been noted in explants isolated from different locations within a joint, thus limiting the number of explants available per animal for critical evaluation.[166-168] Although explant cultures seem to be the best model[169] as these are closer to the *in vivo* situation the above mentioned factors must be taken into consideration and the samples should be limited to a particular region of the joint, so as to minimize any differences in composition and biomechanical characteristics.

An inherent problem with most of the *in vitro* models proposed for the study of chondrocytes and cartilage, is that they have ignored the role of biomechanical stress in the maintenance of chondrocyte homeostasis and the role it plays in initiation and or progression of disease. The main function of articular cartilage is to serve as a system for load support, load transfer and motion between bones of the diarthrodial joint and a strenuous mechanical environment is normally found in the load bearing joints.[80] Also since load plays an important role in the health and disease states of the cartilage it is important that the effects of load on articular cartilage be studied and be included in any *in vitro* models. Various attempts have been made to apply compressive, tensile, hydrostatic, and sheer stresses. . Mechanical loads have been applied under various conditions, frequencies and durations ranging from single compressions of up to 50% strain,[170-173] to large-amplitude cyclic compression at varying

frequencies for up to 2 hours.[174-177] Injurious mechanical compression of cartilage *in vitro* has been shown to damage the ECM, resulting in increased water content,[174,170,178,179] decreased stiffness,[178,179] increased hydraulic permeability, [176] loss of glycosaminoglycan(GAG)to the culture medium,[180,178,171,179,176,172] loss of collagen to the medium,[176] temporary denaturation of collagen in the tissue [174,170,176,177] cell death by both apoptosis and necrosis,[180,170,178,175,171,177,181] and decreased matrix synthesis rates in the remaining viable cells.[179] The exact amount of pressure required to produce disease in an *in vivo* situation is not yet known and needs to be understood to be able to develop an effective *in vitro* model for the study of cartilage pathology or for use to test various drugs.

OC was first described in 1888 by Konig.[182] OC was first described in the horse by Nilsson in 1947[183] but was not generally accepted as a clinically important problem in horses until the 1970s. Osteochondrosis (OC) is a disease of multifactorial etiopathogenesis which results in failure of normal endochondral ossification. OC refers to cartilage abnormalities which typically occur in specific locations within joints of affected species. In horses, articular cartilage changes associated with OC may spontaneously resolve, [184,185] or result in the formation of cartilage flaps which are characteristic of osteochondrosis dissecans (OCD), or in the formation of subchondral bone cysts (SBCs) [186]. Cartilage flaps often become calcified and may subsequently become detached loose bodies in the joint ("joint mice") [187]. OCD can be localized to a single joint, but is frequently bilateral, and may be a generalized condition. Though virtually any joint in the

horse can develop OCD, the most commonly affected sites are the hock, stifle, shoulder, fetlock and the cervical vertebrae [187]. OCD often results in incongruent articular surfaces which may cause secondary osteoarthritis.[188,42,189] Though predilection sites are variable among species, due to the similarity in the morphology of early lesions it is thought that the pathogenesis of osteochondrosis is the same, regardless of the species affected.[190]

Though the definitive cause is not known it is thought to have a multifactorial origin in which, nutrition, environment, heredity and trauma are thought to play roles in the etiopathogenesis of OC. Other factors such as ischemia and biomechanical forces are also considered to be involved [191-193] Gender may also be a major influence in the development of OC, as the incidence of OC in male horses is twice as high as in females which, in general, holds true across all species [194]. Exogenous corticosteroids and trauma or load have been implicated in causing OCD-like lesions in horses and rats although the mechanism of action is again unknown.[195-197]

Corticosteroids are commonly used not only in the treatment of joint injury, but are also used to treat ailments such as airway disease and shock dermatitis in various species, because they are the most potent anti-inflammatory drug available. They are known to have systemic effects, it is important for us to try and understand the effect of corticosteroids on cartilage tissues. Various studies have found that methylpredinsolone acetate (MPA) to be present in the joint and synovial fluid for periods ranging from 2- 39 days.[198,199] The effect of corticosteroids on cartilage appears to be influenced by the type and dose of the

corticosteroid used for treatment [200,201]. Intra-articular corticosteroids do have some undesirable side effects, and long term use can cause harm to the cartilage[202].

Corticosteroids reduce pain and swelling, but despite their clinical success, *in-vitro* and *in- vivo* studies have shown that intra-articular injections of corticosteroids may have detrimental effects on the cartilage matrix. Coricosteroids decrease proteoglycan synthesis[203-207,196,200] and have a generalized inhibitory effect on collagen type II synthesis[208,209,31]. Although there is eventual recovery in proteoglycan synthesis, the cartilage remains depleted of proteoglycan for several months.[205-207] Incubating articular cartilage explants in medium containing methylprednisolone acetate (MPA) at 10mg/ml for 24 hours has been reported to cause severe depression of proteoglycan synthesis which fails to recover after 13 days of culture in medium without MPA. [210]

Other pathological changes of intra-articular administration of corticosteroids include decreased collagen synthesis, increased water content and delayed healing[205-207, 202], thus making the cartilage more susceptible to mechanical injury [196,211]. Rats receiving corticosteroids and subjected to running exercises displayed fibrotic invasion and subchondral bone replacement of degenerated articular cartilage associated with areas of cell death, and loss of matrix staining when compared to rats that received running exercise or corticosteroids alone[196]Cartilage from joints of horses receiving MPA and subjected to running exercise had decreased compressive stiffness, permeability,

and shear modulus, and was 24% thinner than diluent treated control joints[197].These findings suggest a synergestic and/or potentiative interaction among the various risk factors that promote cartilage degradation. Corticosteroids are also known to affect gene expression of various matrix molecules and have been shown to affect the expression of procollagen and collagen II and and decrese matrix metalloproteinases (MMPs) 1, 3,and 13 and tissue inhibitor of metalloproteinases (TIMP)-1. [212,213] Though the exact mechanism or reason why corticosteroids along with exercise may cause OC is not known. From the above studies it may be possible to speculate that the decreased collagen and proteoglycan synthesis along with the increase in water content and thinning of cartilage, could alter the phenotype of the chondrocytes, thus causing an overall decrease in quality and quantity of matrix produced resulting in alteration of the mechanical properties of the cartilage. The change in the viscoelastic properties of the cartilage is accompanied by a change in the composition of the ECM and repeated stress on the damaged osteochondral location could produce the lesions observed in OC.

Other studies have indicated the chondroprotective effects of corticosteroids under certain conditions[214-216]. An *in vitro* study using various doses of MPA on equine articular cartilage explants state that lower doses increased protein and collagen synthesis whereas higher doses decreased total proteoglycan degradation in a dose dependent fashion[31]. Treatment with MPA has been shown to significantly reduce the incidence and size of osteophytes in

experimentally induced OA.[214] In an *in vivo* study of cartilage from horses with carpal osteochondral fragmentation, the histomorphological parameters were significantly better after intra-articular injections of triamcinolone acetonide (TA) than those which did not receive any treatment [201]

Comment [c1]: Not sure if I need to keep this paragraph.

Though the clinical importance of equine OC gained recognition in the 1970s, understanding the disease mechanisms has been advanced more recently by the development of methodologies that facilitate the *in vitro* and molecular study of OC. Efforts have been made to ascertain the cause of OC and also to understand the molecular and biochemical changes in the cartilage as the disease progresses. Various studies have looked at the effect of growth factors and changes in their concentration,[217] roles of cathepsins B and L [218] the effects of circulating insulin on chondrocyte maturation[219], the role of ischemia in initiation of OC [190], the levels of parathyroid hormone-related peptide (PTH-rP) and mRNA expression relating to delayed ossification [220], levels of matrixmetalloproteinases (MMP) [221-224] and bonemorphogenic enzymes [225] the rate of weight gain/growth rate [226,227],nutritional status of the dam [227,228], the morphology and characteristics of bone and cartilage [229-231] and its relationship to infection, [230] the effects of exercise. [184,232,231]

Various theories have been proposed to explain OC. A close association between MMP 13, Col X and chondrogenesis has been demonstrated. [233,234] Since MMPs are thought to play an important role in matrix

turnover and chondrogenesis[235,37] an imbalance could lead to delayed endochondrarl ossification resulting in OC. The rate of weight gain could also be important in the initiation of the disease a disparity in structural development and body weight could lead to the application of abnormal force to the physeal growth cartilage. A high calorie or rate of feed intake also contributes to rapid growth of the bones, but with low density resulting in a weak subchondral spongiosa. [129] It could be possible that this could be the reason for increased feed intake being thought of as a cause of OC.

Parathyroid hormone stimulates the production of Vit. D, which is important in the process of endochondal ossification. Infact studies have shown retained growth plate cartilage along with a disorganization of chondrocyte cell columns and inhibition of cartilage matrix calcification as a result of vitamin D deficiency. [236]

Cathepsins B and L have been shown to be involved in endochondral ossification. This may be relevant to developmental orthopaedic diseases (DOD) such as OC in that these enzymes have been reported to have the potential to degrade bone and extracellular matrix.[218] Regulation of growth factors in cartilage may be involved in OC; an increase in growth factors IGF-1 and TGF-beta1 was reported in OC cartilage compared to normal controls.[217] The authors of this study concluded that an increase in growth factors in OC was likely the result of attempted healing and that manipulation of growth factors in OC cartilage would be unlikely to alter the incidence or progression of disease.

Published data suggests that high amounts of circulating insulin from a high energy diet can affect chondrocyte maturation resulting in altered matrix metabolism and faulty mineralization.[219] There is also evidence that ischemia may be a key factor in the initiation of OC. [190] Results from another study showed elevated PTH-rP protein and mRNA expression suggesting a role for PTH-rP in delayed ossification and OC. [220] There is also evidence that ischemia may be a key factor in the initiation of OC. [190] Local ischemia to the epiphyseal cartilage of the articular-epiphyseal cartilage complex is thought to lead to the formation of highly vulnerable zones of necrotic epiphyseal cartilage which could cause a delay in endochondral ossification, with extension of necrotic cartilage into the subchondral bone.

Though exercise contributes positively to bone density, [237,238] depending on the circumstances it seems to have a dual role regarding developmental orthopaedic diseases (DOD). As a contributing factor to DOD, with the increased trauma of exercise non-clinical lesions present at an early age manifest into clinical signs.[239,187] Horses with access to free exercise or subjected to forced exercise (trotting five miles/day five days/week) in addition to free exercise tended to have less incidence and had less severity of DOD lesions, thus indicating a potential prophylactic role to the development and severity of DOD. [240-242]

Thus it is possible that any of these factors or a combination of them affect chondrocyte maturation and their ability to produce matrix components in the

desired quality and or quantity thus causing a failure in the process of enochondral calsification and an overall failure of the cartilage.

The extracellular matrix of normal articular cartilage is produced by chondrocytes and is composed of water, proteoglycans and collagens [243,244]. Proteoglycans are able to bind calcium so are thought to play an important role in ossification and thus may be important in the etiology and progression of OC [245]. GAG content has been shown to be altered in OCD lesions from several different species.[246-252] Abnormal GAG production could be the result of multiple factors, including genetic, nutritional, metabolic, or traumatic [250], and could occur either prior or subsequent to the completion of endochondral ossification and thus be either a cause of, or the effect/ sequel of, the pathogenesis of OCD. The decrease in GAG content could be due to a decreased production by chondrocytes, an increased loss of extracellular matrix, or both [253-257], and could occur either due to physiologic degradation of GAG in the absence of normal synthetic function due to genotypic or phenotypic abnormalities in cells, and imbalanced degradation of GAG secondary to pathologic enzymatic degradation that results form OCD induced osteoarthritis [253-256,250,257]. Abnormal GAG degradation is likely to be attributable to either a programmed or induced alteration in the balance of chondrocyte GAG synthesis and degradation.[253-256]. This could again be associated to genetic, nutritional, metabolic, or traumatic factors. In a previous study from our laboratory, chondrocytes from naturally occurring OC lesions were less viable and less capable of producing extracellular matrix molecules than chondrocytes from unaffected dogs [257], providing evidence

that decreased synthesis by chondrocytes may account for at least a portion of the decreased GAG consistently seen in OC.

As stated earlier collagen type II is the predominant collagen type in hyaline cartilage[258] and provides the tensile strength of the tissue [259] and aggregating proteoglycans form the main compression- resistant constituent [260]. Type-X collagen is predominantly produced by chondrocytes in the zone of hypertrophy and is thought to be associated with calcification of this zone of cartilage. [258,261, 228 ,229] Type X collagen is common in young horses and has been been demonstrated to be is limited to hypertrophic chondrocytes in horses <24 months of age [262], but in older horses it is present only in osteoarthritic articular cartilage [217].Thus the presence of collagen type X in OCD lesions could be due to the presence of hypertrophic chondrocytes.

Any defect in this unique extracellular matrix may result in weakening of the cartilage and the subsequent development of OC. Since proteoglycans and collagens are the critical components of the extracellular matrix, it is likely that one or both are critically involved in the etiopathogenesis. Increased degradative activity by MMP in OCD cartilage has also been reported.[223,224] Thus collagenases may not only play a role in the development of pathology of OCD lesions but may also be part of a final common pathway in the pathology of OCD and OA This could suggest that increased degradation of collagens and GAG could be the cause for GAG and collagen loss in OC, and pathologic matrix

degradation, could also be a component in disease initiation and/or progression. The deficiency of GAG and collagen type II and increased MMP activity associated with OC lesions may result in areas of mechanical weakness allowing subsequent fissure formation and release of cartilage fragments into the joint space.

One of the major factors for the lack of knowledge for the initiating causes of OCD is that most studies use tissues from advanced cases of OCD. It is thus possible that the changes observed in the tissue are due to pathology that has developed secondary to the development of OCD in the tissue, and therefore not a cause of the development of OCD. Further, it is difficult to differentiate causative changes in tissue metabolism from secondary changes in tissue metabolism that occur due to the development of OCD. It is thus important that studies be conducted at early stages of OC so as to be able to identify the main factors (genes/trauma/enzymes/hormones) involved in the initiation of disease and differentiate this pathology from pathology that develops later in the disease process secondary to OCD development. For example, long term studies of juvenile OCD in humans have shown that 50% of these cases progress to exhibit signs of OA after an average follow up of 33 years.[263] Similar results have been seen in other species. Since a large number of OC affected cases develop secondary OA, it is important that any study of OC differentiate the changes in the tissue due to primary and secondary pathologies. Therefore, studies designed to identify metabolic differences between tissues from OCD affected

individuals and OA affected individuals will be valuable in differentiating the pathological changes in OCD cases that are caused by OCD and those that are caused by the secondary development of OA in the tissue.

The most prevalent form of musculoskeletal disease is Arthritis. The term arthritis refers to disorder of one or more joints.There are over a 100 known types of arthritis, of which five account for 90 per cent of cases—OA, rheumatoid arthritis (RA), fibromyalgia, systemic lupus erythematosus and gout. OA is the most common of these and effects 10-15% (1995) of the population and this percentage is expected to increase to 18% by 2020.[264]

Over time the definition of OA has evolved form “hypertrophic arthritis” to “OA diseases are a result of both mechanical and biologic events that destabilize the normal coupling of degradation and synthesis of articular cartilage chondrocytes and ECM, and subchondral bone. Although they may be initiated by multiple factors, including genetic, developmental, metabolic and traumatic, OA diseases involve all of the tissues of the diarthrodial joints. Ultimately OA diseases are manifested by morphologic, biochemical molecular and biomechanical changes of both cells and matrix which lead to a softening, fibrillation, ulceration, loss of articular cartilage, sclerosis and eburnation of subchondral bone, osteophytes and subchondral cysts. When clinically evident OA diseases are characterized by joint pain, tenderness, limitation of movement, crepitus, occasional effusion and variable degrees of inflammation whithout systemic effects.”[265]

In other words OA is a complex interactive, degradative and repair process which involves cartilage, bone and synovium and is characterized by progressive and irreversible degradation of articular cartilage ECM.[266-270] Though various factors are involved in the initiation and progression of OA, it has been capsulized as " the application of abnormal stress to normal cartilage or the application of normal stress to abnormal cartilage."[271,272]

The etiopathogenesis of OA can be broadly divided into the following 3 stages:

Stage 1: Proteolytic breakdown of the ECM occurs. Chondrocyte metabolism is altered resulting in and increased production of enzymes, including MMPs (eg, collagenase, stromelysin) which destroy the ECM. The amount of TIMPs produced as a counter measure to the MMPs is insufficient to counteract the proteolytic effect.

Stage 2: In this stage proteoglycans and collagen fragments are released into the synovial fluid due to the fibrillation and erosion of the cartilage surface.[273,274]

Stage 3: The breakdown products of cartilage induce a chronic inflammatory response in the synovium. This causes production of cytokines, such as IL-1, tumor necrosis factor-alpha (TNF-α), and metalloproteinases by the synovial macrophages. These can diffuse back into the cartilage and directly destroy tissue or stimulate chondrocytes to produce more metalloproteinases. Osteorathritic cartilage has also been shown to produce increased amounts of IL-1.[275] Other pro-inflammatory molecules such as nitric oxide (NO),[276,277] an oxygen free radical[278] also may be a factor. Ultimately all these events alter the

joint architecture, and compensatory bone overgrowth occurs in an attempt to stabilize the joint. As the joint architecture is changed and further mechanical and inflammatory stress occurs on the articular surfaces, the disease progresses unchecked.

As stated earlier the healthy articular cartilage is being constantly remodeled and a strict balance is maintained between degradative and synthetic events in such a way that the quality and quantity of articular cartilage is maintained. In OA though a mostly succesful[279,280] the cytokines and free radicals which freely diffuse into cartilage can not only down regulate proteoglycan and collagen synthesis by chondrocytes, but also initiate the production of catabolic proteinases, cytokines, and free radicals such as NO thus contributing to further matrix degradation .[281-283] Therefore the degradative or catabolic activity is increased to levels much greater than the synthetic or anabolic activity resulting in an overall loss of proteoglycans and collagens.[38,273,274]

MMPs are a family of structurally related zinc-binding endopeptidases[284] and participate in the normal physiology of connective tissue during development, morphogenesis, bone remodeling, and wound healing, and it is believed that the unregulated activity of these enzymes leads to arthritis development. They are secreted as inactive proenzymes that require enzymatic cleavage in order to become activated. Once activated, MMPs become susceptible to the plasma-derived MMP inhibitor, alpha-2-macroglobulin, and to TIMPs which are also secreted by synovial cells and chondrocytes. There are

over 20 MMPs [235,284] and are known to play a vital role in cleavage and degradation collagens and aggrecans of ECM. [285-294] Of the 20 MMPs, MMP-1 (interstitial collagenase), MMP-3 (stromelysin-I), and MMP-13 (collagenase-3) are known to effect cartilage. The concentrations of several MMPs have been shown to be increased in cartilage, synovial membrane and synovial fluid of patients with arthritis, [295-298]and cartilage-specific overexpression of active human MMP-13 has been shown to cause OA in mice.[299] MMP 3 in addition to directly cleaving matrix molecules also is a member of the activation cascades of matrix degrading enzymes, including other MMPs.[300] Cytokines such as IL-1 and tumor necrosis factor (TNF)-α have also been shown to be increased in arthritic joints and are known to induce catabolic pathways leading to an enhanced expression of MMPs.[301-303,298,304] . Thus not only is inhibition of these proteases regarded as an important approach for reducing damage in arthritic tissues,[305,306] but are also thought of as markers for OA.[307-309]

Though controversy still exists, aggrecanases are thought to play an important role in degradation of proteoglycans. Aggrecanases are zinc metalloproteinases whose structure and domain arrangements are homologous to a disintegrin and a metalloproteinase domain with thrombospondin motifs proteins (ADAMTS) [310,311] Aggrecanase-1[312] was first reported in 1999 and is now known as ADAMTS-4 and aggrecanse-2[313] now known as ADAMTS-5 . More recently ADAMTS-1 has also been shown to have aggrecanase activity. [314] There is a wide variation in the results of

studies done in various species using various culture models using various stimulatory factors. [315-319] Some studies have shown an increase in mRNA expression of certain ADAMTS[316-319]but not of others where as others have shown no difference in all ADAMTS,[315] but a general increase in aggrecanase activity suggesting that enhanced aggrecanase activity may be regulated post-transcriptionally or that the increased activity is due to unidentified aggrecanases.[315] This variability could indicate that species and age of the tissue and culture conditions of isolated cells could play an important role in the regulatory mechanisms of ADAMTS-4 and ADAMTS-5 transcription and translation.

There is still a controversy as to wether MMPs or aggrecanseas cause aggrecan degradation in cartilage. Both MMP-generated G1-VDIPEN[341] fragment and the aggrecanase-generated G1-NITEGE[373] fragment are found in cartilage,[34] and synovial fluids[320-322] from patients with OA. In in vitro models where cartilage explants stimulated with IL-1, TNF-α or retinoic acid, aggrecanases appeared to be the primary enzymes responsible for aggrecan degradation in the first week,[323,294] and though the mRNA levels of MMP-3 and MMP-13 are elevated,[323] they are thought to play little if any role in the degradation process. However, after 3 weeks MMP-dependent cleavage of aggrecan core protein can be detected, collagen breakdown also starts to occur at this point.[294]

Articular cartilage also contains TIMPs [324,325]which inhibit all three proteinases and are the main regulators of MMPs in cartilage as they bind 1:1 with MMPs[326-328].

In earlier studies it has been shown that though TIMP was not elevated, proteinases were elevated about threefold in OA patellar cartilage.[329,325] This may lead to an imbalance in OA so that the proteinases escape control by the inhibitor and cause excessive matrix degradation. A more recent study[330] has shown that deficienct of TIMP-3 results in changes similar to those seen in OA patients giving further credibility to the theory that an imbalance between MMP and TIMP may play a pathophysiologic role in the development of OA. The ability of TIMP-1 and 2 to inhibit MMPs has also been shown in chondrocyte and explant cultures.[331-333]

It is well understood that inflammation is an integral part of OA.[334] The list of proinflammatory mediators in animals and humans is long and not yet complete. Some of these are histamine, bradykinin, prostanoids (such as prostaglandin E2), leukotrienes, platelet-activating factor, nitric oxide, cytokines (some are anti-inflammatory), endothelin and oxygen radicals. [335]

Various cytokines (pro- and antiinflammatory), antagonists, and growth factors, in particular the proinflammatory cytokines are thought to play a role in the pathophysiology of OA. Of the proinflammatory cytokines IL-1β and TNFα are of major importance to cartilage destruction.[336,337] IL-1β and TNFα not only stimulate their own production, but also induce chondrocytes and synovial cells to produce other cytokines, such as IL-8, IL-6, and leukocyte inhibitory factor

(LIF), as well as stimulate proteases and prostaglandin E_2 (PGE_2) production. IL-1 a proinflammatory cytokine and can be secreted from almost every nucleated cell in the body.[335] Extensive research has and is being done to understand the effects of IL-1 on cartilage. They are thought to be produced by the synovium and activated chondrocytes [338,293] but can diffuse freely into cartilage. IL-1 plays an important role in the pathophysiology of OA [339] and stimulates the production of nitric oxide and PGE2 in a concentration-dependent manner[340,275,341] IL-1β is synthesized as a precursor, and is converted to the mature form by IL-1β-converting enzyme (ICE), or caspase 1.[342] Specific cell-surface receptors (IL-1R) mediate the biologic activation of cells by IL-1.[343]The levels of ICE [344] and IL-1R [345,346] have been shown to be up-regulated in both OA synovium and cartilage giving these cells a higher sensitivity to stimulation by IL-1β.[345] This phenomenon could be responsible for potentiating the effect of this cytokine and up-regulating the gene expression of a number of catabolic factors, which, in turn, enhances cartilage destruction. The main isoform produced in the horse is IL-1β.[347] IL-1 receptor antagonist (IL-1Ra) is a competitive inhibitor of IL-1R and can inhibit various catabolic pathways related to OA, including PGE_2 synthesis, collagenase and NO production by chondrocytes, and cartilage matrix degradation. Though a higher level of IL-1Ra is found in OA tissues, the ratio of IL-1Ra to IL-1β is insufficient to deal with the increased level of IL-1β found in OA,[338,348] and though studies have shown that intraarticular injections of the IL-Ra gene can prevent the progression of structural changes in OA,the exact role of IL-1 antagonists and their ability to neutralize increased level of active IL-1β are not known.

IL-1β stimulates various enzymes (particularly MMPs) that result in cartilage degradation, [349-352] and also inhibits the compensatory synthesis pathways used by chondrocytes to restore the integrity of the degraded ECM.[353] The differential regulation of metalloprotease and TIMP syntheses by IL-1 suggests that this cytokine, during inflammatory conditions, may promote cartilage degradation by creating an imbalance between the level of these enzymes and their inhibitors.[354] When IL-1 was injected into mouse joints a single injection produced only mild inflammation, but resulted in substantial inhibition of proteoglycan synthesis and enhanced breakdown, repeated injections however had very seviour degradative effects on the cartilage and produced profound inflammation.[355,356] An increase in release of IL-1β in osteoarthritic cartilage has also been shown. [339] IL-1 causes degradation of proteoglycans and collagens. IL-1 suppresses collagen synthesis at two levels : a pretranslational level which is NO independent, and a translational or post-translational level which is NO-mediated.[357]

Thus the importance of IL-1 in cartilage metabolism results from its ability to suppress the synthesis of type II collagen and promote the synthesis of type I collagen thus giving the tissue fibroblasts; induce the production of enzymes involved in matrix degradation; and suppress the ability of chondrocytes to synthesise new proteoglycan, and efforts are being made to use this as a potenitial marker for diagnosis or a candidate for treatment of disease.

NO is formed by the conversion of L-arginine to citrulline, with oxygen serving as an electron acceptor. The removal of the terminal guanidino nitrogen is catalyzed by an enzyme nitric oxide synthase (NOS).[358] NOS exists in three isoforms,

NOS1 (nNOS),[359,360] NOS2 (iNOS)[361] and NOS3 (eNOS).[362] It is the iNOS isoform, which is predominantly responsible for NO production in articular cartilage[363] and can be induced by endotoxin, cytokines, and microbial products.[364-367,282,368,369] In the diarthrodial joints chondrocyte are the major source of NO,[370-373] with the cells in the superficial zone being the best source.[374] It is not clear if osteoarthritic cartilage spontaneously produces nitrites[375,376] or if these are induced by cytokines.[377,371,372,378] Though there is acute inflammation in osteoarthritis, resulting in induction of iNOS, but its detection could be time dependent and also be based on the viability of the cells.[334] Previous studies have shown mechanical stress to be an important modulator of NO production[109,379]. In a study done on porcine articular cartilage explants, an increase in NO synthesis by both static and intermittent compression was identified which could play an important role in cartilage degradation[380]. An increased production of nitrite in monolayer cultures of bovine articular chondrocytes, due to mechanical loading in the form of fluid-induced shear stress corresponding to the duration and magnitude of the physical stimulation has also been reported[109] NO has been shown to suppress proteoglycan synthesis, and induce chondrocyte apoptosis in articular chondrocytes[381-383]. NO has also been implicated in rheumatoid and osteoarthritis in both clinical cases and experimental models[384-386]. NO can play both a protective and a degradative role in cartilage *in vitro*,[387,282] and this could depend on the oxygen tension as the ratio of NO to O_2 may determine the amount of various NO derivatives that are formed. Endogenously generated NO has been shown to suppress the

biosynthesis of aggrecan in various species, [388] [381] and is also thought to play a role in collagen degradation. [357] Since NO plays such an important role in the mediation of inflammation and cartilage degradation various iNOS inhibitors have been developed and tested and could play a potential role in prevention and treatment of OA.

Prostaglandins exert diverse and complex modulatory roles during physiologic and pathophysiologic conditions, including OA. The regulation of prostaglandins is the subject of intensive investigation. There are two isoforms of COX, which are designated COX-1 and COX-2 [389] and are the enzymes which catalyze the rate-limiting step in prostaglandin synthesis, converting arachidonic acid into PGG_2 and PGH_2. COX-1 is expressed in many cells and is important in maintaining homeostasis, where as COX-2 is induced by various stimuli including cytokines in inflammatory cells and tissues. PGE synthase (PGES) acts downstream of COX to catalyze the conversion of PGH_2 into PGE_2. [390,391] In many cells the main prostaglandin produced is PGE_2, and studies with a specific monoclonal antibody to PGE_2 have indicated that this prostaglandin contributes to inflammation.[392] The expression of the inducible cyclooxygenase, COX-2, is increased in OA chondrocytes that spontaneously produce PGE_2 ex vivo,[376] thus suggesting that COX-2 has a key role in the process of inflammation. Increased PGE_2 production in response to stimulation by proinflammatory cytokines coincides with the upregulation of COX-2 expression. In addition, *in vivo* studies have shown that COX-2 inhibitors reduce PGE_2 synthesis more markedly than other prostaglandins.[393]

Since both OC and OA are a major problem in both veterinary and human medicine it is necessary that we try and understand the mechanism of initiation and progression of these diseases. The key to any cure for these painful conditions will only be possible if we understand the initial changes that occur in the complex organ called the cartilage. For this it is important that appropriate *in vivo* and *in vitro* models be developed and validated. Since load plays a critical role in cartilage development, homeostasis and degradation the aim of the studies presented here was to try and understand the role that load plays in the initiation and progression of these diseases.

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Biochemical characterization of cartilage affected by osteochondrosis dissecans in the tarsocrurual joints of horses

Introduction

Osteochondrosis (OC) is a disease of multifactorial etiopathogenesis which results in failure of normal endochondral ossification. OC refers to cartilage abnormalities which typically occur in specific locations within joints of affected species. In horses, articular cartilage changes associated with OC may spontaneously resolve (1, 2), or result in the formation of chondral or osteochondral flaps which are characteristic of osteochondrosis dissecans (OCD), or in the formation of subchondral bone cysts (SBCs) (3). Cartilage flaps often become calcified and may subsequently become detached loose bodies in the joint ("joint mice") (4). OCD is commonly observed in young, rapidly growing and early maturing horses. OCD can be localized to a single joint, but is frequently bilateral, and may be a generalized condition. Though virtually any joint in the horse can develop OCD, the most commonly affected sites are the hock, stifle, shoulder, fetlock and the cervical vertebrae (3,4). OCD often results in incongruent articular surfaces which may cause secondary osteoarthritis (5-7). The economic cost of equine OC is attributable to a number of associated features including treatment costs, lost earnings, loss of training time due to lameness, and the decline in value associated with chronic lameness. Though the definitive cause of OC is not known, it is thought to have a multifactorial origin in which nutrition, heredity and trauma are thought to play roles (3). Other factors

such as ischemia and biomechanical forces are also considered to be involved (8-10). Gender may also be a major influence in the development of OC, as the incidence of OC in male horses has been reported to be as much as twice as high as in females which, in general, holds true across all species (11). Breed may also be a factor in the development of OC as Standardbred, Warmblood, and Thoroughbred breeds have a reported incidence from 10-35% in selected joints, whereas OC is rarely reported in ponies and feral horses (3).

Though the clinical importance of equine OC gained recognition in the 1970s, understanding the disease mechanisms has been advanced more recently by the development of biochemical and molecular biology techniques that facilitate the study of OC. Efforts have been made to ascertain the cause of OC and also to understand the molecular and biochemical changes in the cartilage, and the effect of various factors on the initiation and progression of the disease (2, 12-22).

The extracellular matrix of normal articular cartilage is produced by chondrocytes and is composed of water, proteoglycans and collagens (16, 17). Proteoglycans consist of a core protein with covalently attached glycosaminoglycan side chain(s). Proteoglycans are able to bind calcium so are thought to play an important role in ossification and thus may be important in the etiology and progression of OC (18). Collagen type II is the predominant collagen type in hyaline cartilage (19) and provides the tensile strength of the tissue (20) while aggregating proteoglycans form the main compression- resistant constituent (21). Type-X collagen is predominantly produced by chondrocytes in

the zone of hypertrophy and is thought to be associated with calcification of this zone of cartilage (19, 22). Any defect in this unique extracellular matrix may result in weakening of the cartilage and subsequent development of OC. Since proteoglycans and collagens are the critical components of the extracellular matrix, it is likely that one or both are critically involved in the degenerative changes associated with OC. Determining the concentration of sulfated glycosaminoglycans (GAG) provides an estimate of the proteoglycan content of the extracellular matrix. Hydroxyproline (HP) is present in the Y position of the Gly-X-Y repeating tripeptide of collagen, and the determination of HP concentration has been used as a measure of total collagen content in tissue specimens(23, 24).

The present study was designed to contribute to our understanding of the changes that occur in cartilage secondary to the development of OC. The specific purpose of this study was to compare the concentrations of glycosaminoglycan (GAG) and hydroxyproline (HP) and the immunohistochemical staining characteristics of type I, II and X collagen from normal cartilage and cartilage associated with osteochondrosis dissecans (OCD) in the tarsocrural joints of horses.

Methods

Horses included in this study were those presented to the University of Missouri Veterinary Medical Teaching Hospital between June 2001 and September 2003 diagnosed with OCD, based on clinical and radiographic

evaluations. Osteochondral fragments and cartilage samples were collected via arthroscopic or arthrotomy approach to the joints of OCD affected horses (n = 20). Control samples (n = 13) were obtained from horses which died for reasons unrelated to OCD. Normal articular cartilage samples were collected from the distal intermediate ridge of the tibia (n= 11), the lateral trochlear ridge of the talus (n=1) and the medial malleolus of the tibia (n=1). Each sample was divided into two portions perpendicular to the site of attachment, dividing the site of attachment and the articular cartilage into two approximately equal portions. The wet weight of one portion was determined prior to storage in Hanks buffered salt solution (HBSS) at -80^{o}C for subsequent GAG and HP analysis. The other portion was placed in 10% neutral buffered formalin prior to histological processing and examination.

Glycosaminoglycan assay: The total sulfated GAG content of articular cartilage was measured using dimethylmethylene blue (DMMB) spectrophotometric analysis (25). Frozen samples were thawed and digested in 1.0 ml of papain (14 U/mg; 0.5 mg/ml) in distilled de-ionized water at 65^{o}C for 12 hours. A 5 µl aliquot of the digest solution was combined with 240 µl of DMMB solution and absorbance was determined at 525 nm spectrophotometrically. Bovine trachea chondroitin sulfate A was used to construct a standard curve. The results were standardized by correcting for differences in sample weights. Total GAG content for samples was recorded in micrograms per milliliter per gram of sample wet weight.

Hydroxyproline assay: Articular cartilage HP content was determined colorimetrically (24). A 50 µl aliquot of papain digested articular cartilage was combined with 4N sodium hydroxide (50 µl) and hydrolyzed by autoclaving at 120^{o}C for 20 minutes. Chloramine T reagent (450 µl) was added to the hydrolysate, mixed gently and oxidized at room temperature for 25 minutes. Ehrlich aldehyde reagent (450 µl) was then added to each sample. Samples were incubated at 65^{o}C for 20 minutes to develop the chromophore. Known concentrations of HP standard were used to construct a standard curve. Absorbance (550 nm) was determined using a spectrophotometer. Hydroxyproline content was recorded in micrograms per milliliter per gram.

Histologic assessment: After routine histologic processing, 5 µm sections were stained with hematoxylin and eosin (H&E) and toluidine blue (TB). Sections were examined by one investigator (JLC) who was unaware of the origin of each section. Sections were evaluated for tissue morphology, cell and matrix content, and proteoglycan staining.

Immunohistochemistry: Immunohistochemical staining was performed on unstained sections in two batches for each collagen type. The antibodies used in this study have been used in other species (26). All staining was performed using a commercially available streptavidin - biotin- peroxidase kit.[a] Endogenous peroxidases were quenched with 3% hydrogen peroxide in water. For collagen X,

testicular hyaluronidase digestion for 30 minutes was followed by a serum block, and the primary antibody (1:800 dilution mouse anti-deer monoclonal type-X collagen)[b] was applied for 18 hours at 4^{o} C. For collagen types I and II, a 30 minute trypsin digestion was performed, a serum block was used and the primary antibody (1:150 dilution goat anti- bovine collagen type I) [c] for collagen type I was applied overnight at 37^{o}C. For Collagen type II, a 1:400 dilution of rabbit anti-bovine collagen type II[c] was applied and kept overnight at 4^{o} C. Positive (equine growth plate) and negative [non-immunised serum (serum lacking antibody) of the same species as the primary antibody] controls were included in the immunohistochemistry staining for each batch to ensure specificity of staining. All specimens were then counter-stained with hematoxylin. Stained sections were examined microscopically by two investigators (JLC & KK) who were unaware of the origin of each section. The sections were subjectively evaluated and scored by a method previously developed and used in our laboratory (26) for the presence and intensity of staining for the respective collagen types on the basis of the following scale: 0, no staining evident in the tissue section; 1, low intensity staining of < 25% of the tissue section; 2, low to moderate intensity staining of > 25% of the tissue section; 3, high intensity staining of > 25% of the tissue section. Sections were then subjectively evaluated for the location of the staining.

Statistical analysis: Data were categorized by group: control or affected (OCD). Medians and means (±SD) were calculated for each group. Data were compared

using a Mann-Whitney rank sum test with significance at $P < 0.05$. Spearman rank order correlation was used to determine correlation between groups.

Results

Thirty-four cartilage samples were obtained from 20 horses with tarsocrural joint OCD with a mean age ± SEM of 19.6 ± 3.1 months. The breed distribution for horses with OC was Standardbred (8), Clydesdale (4), Quarter Horse (3), Warmblood (2), and other (3). Of the 20 horses with OCD, 14 were affected bilaterally, 4 had lesions only in the left tarsus and 2 had a lesion only in the right tarsus. Lesions were located on the distal intermediate ridge of the tibia (n= 30) and the lateral trochlear ridge of the talus (n=4). All osteochondral fragments were attached to the parent bone at the time of collection however, a clear defect in the cartilage was apparent to identify the extent of the OC fragment. The entire osteochondral fragment was removed and collected for evaluation. Twenty-six cartilage samples from 13 control horses with a mean age ± SEM of 14.6 ± 4 months were analyzed. The breed distribution for the control horses was Quarter Horse (4), Thoroughbred (2), Warmblood (3), and other (4).

Glycosaminoglycan (GAG) content: The mean GAG content of cartilage samples from control horses (919.2 ± 126.7 mg/ml/g) was significantly higher than for samples collected from horses with OCD (209.9 ± 48.8 mg/ml/g), $P < 0.001$ (Fig 1.1).

Hydroxyproline (HP) content: The mean HP content of cartilage samples collected from control horses (170.8± 37.9 mg/ml/g) was significantly higher ($P < 0.001$) than for horses with OCD (56.8± 6.7 mg/ml/g)(Fig 1.1).

Histology: The histologic appearance of cartilage from the control horses was consistent with normal hyaline cartilage, with little variation among sections. The TB stained sections had normal zonal arrangement from superficial to deep, lacked fibrillation and necrosis and exhibited strong staining of proteoglycan; a characteristic of normal hyaline cartilage. The tissue sections from OCD horses exhibited a wide variation in histologic characteristics (Fig 1.2). The affected tissue ranged from nearly normal-appearing hyaline cartilage fragments to small pieces of proliferative fibrocartilage with no evidence of normal hyaline cartilage to sections that were completely mineralized. Variable loss of proteoglycan staining was consistently observed with the staining being most intense in sections with hyaline like cartilage appearance. Subjectively less intense proteoglycan staining was noticed in sections with proliferative fibrocartilage, and chondrocyte necrosis was observed in many OCD cartilage samples. Areas of necrotic cartilage were detected in some sections. Many sections contained tissue that comprised only hypertrophic chondrocytes, which was consistent with areas of cartilage that had not undergone the normal endochondral ossification process. Histologic evidence of cartilage injury was present in many sections and consisted of necrosis, fissures, cleft formation, fragmentation, chondrocyte hypertrophy and clone clusters.

Collagen immunohistochemistry: Subjective determination of staining intensity for type I collagen was increased for OCD samples compared to controls. Staining for type I collagen was diffusely distributed throughout the matrix of tissues that had a fibrocartilaginous or osteoarthritic appearance histologically. Cells in these samples were oval to fusiform and of high cell density, with clone clusters.

Control samples had a consistent staining pattern for collagen type II and X. Type-II collagen was located throughout each section and could be identified in pericellular, territorial, and interterritorial regions of each zone of the matrix in the control horses. Type II collagen staining intensity was decreased in samples from OCD horses compared to those from the control group. In the OCD samples, the pattern of staining varied from a normal distribution to patchy areas associated with islands of staining within the pericellular and territorial matrix of remaining chondrocytes.

Type X collagen staining in OCD samples was observed primarily in the pericellular and territorial matrix of hypertrophic chondrocytes, but in some sections diffuse staining was also observed in the interterritorial matrix of tissue with many oval to fusiform cells, with multiple clone clusters.

The mean immunoreactivity scores (mean ± SEM) for types I, II and X collagen for normal horses were 0.5 ± 0.15, 2.16 ± 0.21, and 0.16 ± 0.09 respectively, whereas for OCD cartilage samples the scores were 0.71 ± 0.11, 1.645 ± 0.16, and 0.548 ± 0.11 (Fig 1. 3). Although the difference in the staining

intensity for collagen type I was not statistically significant (P = 0.169), the differences in collagen types II and X between OCD and control groups were significant (P<0.039 and P<0.029, respectively). No significant correlations were found between collagen types I, II or X within OCD or control groups.

Discussion:

The objectives of the present study were to determine relevant biochemical characteristics of cartilage affected by OCD in comparison to normal cartilage in horses to contribute to understanding cartilage pathology associated with OC. The data from the present study are consistent with data from a similar study from our laboratory performed on dogs (26). In the canine study, we reported that cartilage affected by OCD contains less GAG and collagen type X and more type I collagen which provided initial evidence stimulating a series of studies characterizing cellular, molecular, and biomechanical aspects of OCD in dogs (27, 28). The present study showed similar changes in major ECM constituents to the initial canine study, as did another study investigating middle and late stage OCD in Dutch Warmblood foals (29). Importantly, these data suggest that OCD has a common disease pathway among species that involves resident cells' synthesis and turnover of major matrix constituents. The next steps are to investigate the initiating events and the temporal nature of the biochemical and histologic changes reported here.

In the present study, GAG content in samples from horses affected by OCD was significantly lower than that found in controls. These data support

earlier studies (26, 28, 29, 30-33) where GAG content was altered in OCD lesions from several different species. Abnormal GAG production could be the result of multiple factors, including genetic, nutritional, metabolic, or traumatic (26), and could occur either prior or subsequent to the completion of endochondral ossification and thus be a cause of, or the effect/ sequel of, the pathogenesis of OCD. This lower GAG content could be due to a decreased production by chondrocytes, an increased loss of extracellular matrix, or both (28, 34-37). Mechanisms by which these changes may occur include physiologic degradation of GAG in the absence of normal synthetic function due to genotypic or phenotypic abnormalities in cells, and imbalanced degradation of GAG secondary to pathologic enzymatic degradation that results from OCD induced osteoarthritis (26, 28, 31, 34-37). Abnormal GAG degradation is likely to be attributable to a programmed or induced alteration in the balance of chondrocyte GAG synthesis and degradation (34-37). This could again be associated with genetic, nutritional, metabolic, or traumatic factors. In a previous study from our laboratory, chondrocytes from naturally occurring OC lesions were less viable and less capable of producing extracellular matrix molecules than chondrocytes from unaffected dogs (28), providing evidence that decreased synthesis by chondrocytes may account for at least a portion of the decreased GAG consistently seen in OC. Further investigation into the genotypic and phenotypic characteristics of chondrocytes from horses affected by OC needs to be done to determine the etiopathogenesis and occurrence of decreased GAG

concentrations in cartilage affected by OC and also to determine whether this is a primary change or the result of secondary degradation.

Increased degradative activity by MMP in OCD cartilage has also been reported (27, 38). These data suggest that the second mechanism discussed for GAG loss in OC, pathologic matrix degradation, is also a component in disease initiation and/or progression. The deficiency of GAG and increased MMP activity associated with OC lesions may result in areas of mechanical weakness allowing subsequent fissure formation and release of cartilage fragments into the joint space (38).

The staining pattern seen in the sections in our study was similar to those seen in other species (26). Immunoreactivity for collagen type I was greater in OCD samples than in control samples, though this difference was not statistically significant. A possible reason for this could be that although collagen type I is detectable in early chondrogenesis, it later becomes undetectable by standard methods (39). However, type-I collagen was detectable in the cartilage of adult pigs after prolonged digestion procedures (40). These data are in agreement with an earlier study where collagen type I was found to be increased in OCD cartilage when compared to normal samples (41). During endochondral ossification, hypertrophic chondrocytes replace type-II collagen with type-I collagen (42, 43). Therefore, the type-I collagen found in cartilage from horses with OCD in our study could be the result of normal production by hypertrophic chondrocytes. However, type-I collagen produced by normal chondrocytes usually occurs in the area of calcification and mineralization, which would not be

expected to be part of an OCD lesion and was not identified in any of our samples.

The control samples in our study were collected from horses with a mean age of 15 months. The fact that horses of this age are still growing and maturing could explain the presence of collagen type I in control samples. Collagen type I is produced by hypertrophic chondrocytes, as well as fibroblasts. It is thus possible that the detection of type I collagen in OCD cartilage samples was a result of alterations in the phenotype of chondrocytes in response to OCD induced injuries (26). This is supported by the findings of the presence of fibrocartilage, and changes associated with osteoarthritis seen on histologic evaluation in these samples.

The predominant collagen in both normal and OCD samples was type II, which is consistent with reported findings (19, 26), Collagen type II plays an important role in maintaining the tensile strength of cartilage and any loss in collagen type II integrity, due to either decreased production or increased breakdown, will affect the ability of cartilage to bear load. Immunoreactivity for collagen type II was significantly lower in OCD samples compared to control samples which suggest degradation of type II collagen.

Type X collagen is present at the surface of normal articular cartilage (22). It is produced by hypertrophic chondrocytes and is associated with calcification (19, 22, 26). The expression of type II, VI and X collagen has been demonstrated in physeal cartilage and type VI and X collagens have been shown to be developmentally regulated (44). Type X collagen is common in young horses but

in older horses it is present only in osteoarthritic articular cartilage (41). The data from our study shows a significant increase in immunoreactivity of type X collagen in OCD cartilage samples when compared to normal. This could be due to the presence of hypertrophic chondrocytes in OCD lesions. This finding is in agreement with earlier data which shows that the expression of type X collagen mRNA in equine epiphyseal cartilage is limited to hypertrophic chondrocytes in horses <24 months of age (44). Another study revealed an increase in collagen type X transcription but a decrease in collagen type X protein in the matrix (45). A subsequent study reported no difference in the expression of type X collagen mRNA between control and OCD groups (41).

In osteoarthritic cartilage, type X collagen is produced by clusters of hypertrophic cells (46). OCD samples from our study showed chondrocyte clone clusters. All sections with clone clusters stained for type I and type X collagen. These data are in agreement with a study on dogs performed in our laboratory (26). As collagen types I and X are produced by hypertrophic cells, the increased concentration of these collagen types in OCD samples seems to be consistent across species (26) and could indicate a defect in cartilage development which could either be a cause of or the result of secondary changes due to OCD or joint damage. There was no significant correlation between immunoreactivity scores for the different collagen types (I, II and X).

The significant decrease in HP content of OCD tissues is reflected in a significant decrease in collagen type II staining observed in the tissues. However, the results of this study also indicate that OCD tissues produce

increased levels of collagen types I and X, which may change the biomechanical properties of OCD cartilage tissue and propagate the changes observed in the tissues affected by OCD.

This study evaluated the changes that occur in OCD affected cartilage including articular cartilage degradation and increased activity of hypertrophic chondrocytes as evidenced by an increase in type X collagen. Further work needs to be directed at delineating the causes of these and other reported changes, and to delineate whether these changes are similar to the changes observed with OA or are more specific to the cartilage degradation associated with OCD.

Footnotes

a) Vectastain, Vector Laboratories, Inc, Burlingame, Calif.

b) Dr. Gary Gibson, The Henry Ford Hospital, Detroit, Mich.

c) Chemicon International, Inc, Temecula, Calif.

Legend

Figure 1 - GAG and HP content in OCD and normal cartilage. ⊨ Normal, ■ = OCD affected, * = Significant P ≤ 0.05.

Figure 2 - Toluidine blue staining of normal (A) and OCD affected (B) articular cartilage.

Figure 3 - Collagen types I, II and X content in OCD and normal cartilage. ⊨ Normal, ■ = OCD affected, * = Significant P ≤ 0.05

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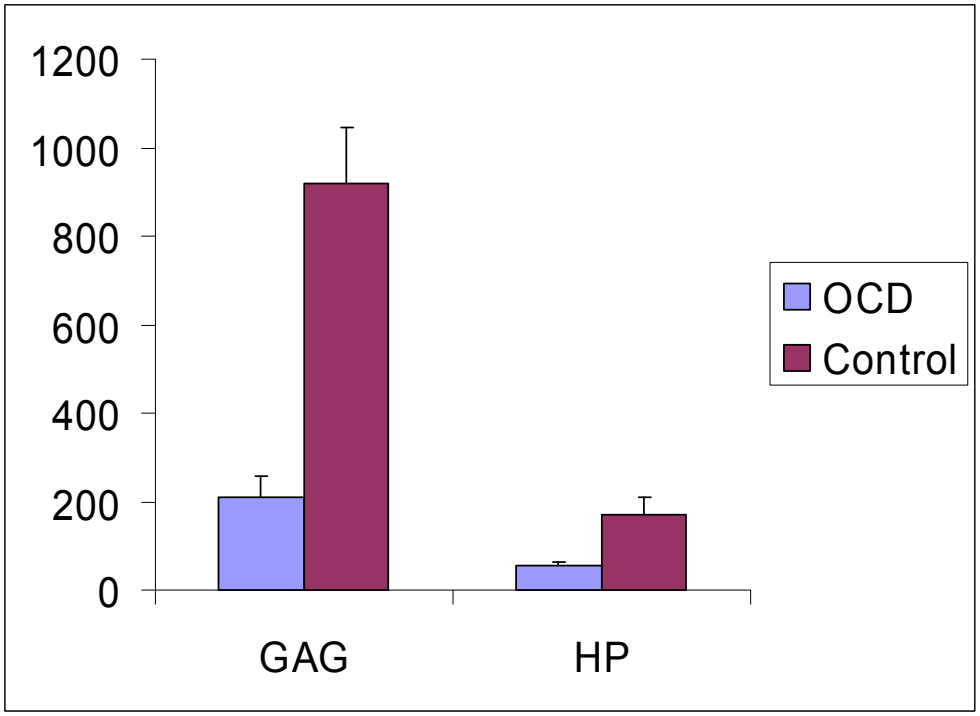
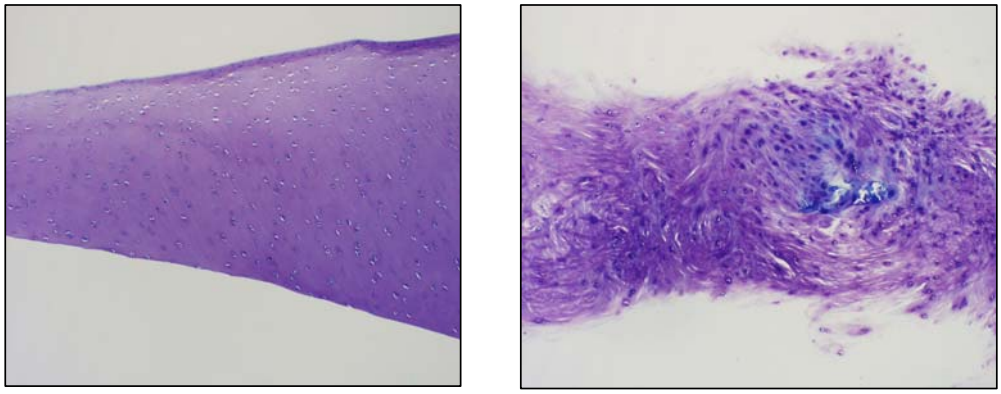
Fig 1.1 Tissue GAG and HP content.



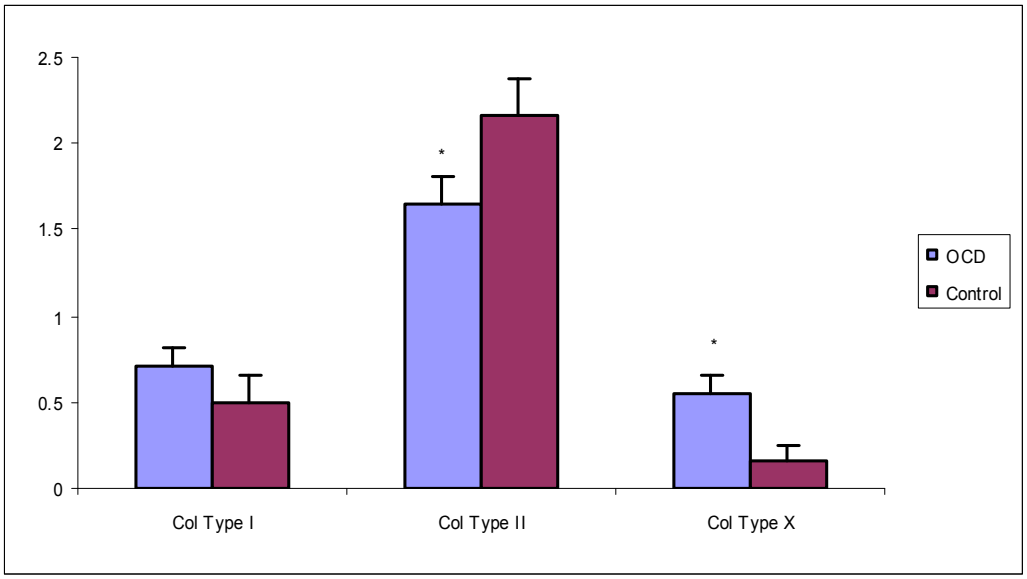
* Significantly different from control.

Fig 1.2 A B



Pictomicrograph of Toluidine blue staining of normal (A) and OCD affected (B) articular cartilage. Note loss of proteoglygan staining and hypertrophic chondrocytes in OCD affected cartilage.

Fig 1.3 Collagen immunohistochemistry.



* Significantly different from control.

The effects of corticosteroids on chondrocytes. A biochemical study.

Lameness results in more loss to the equine industry than any other single medical problem, primarily due to loss of use. The majority of lameness problems are due to affections of the joints and under the best circumstances joints heal slowly. Continued training or delay in treatment after injury can result in damage to the articular cartilage.

Corticosteroids are the most common treatment for joint injury in horses. Corticosteroids are used because they are the most potent anti-inflammatory drug available. The effect of corticosteroids on cartilage appears to be influenced by the type and dose of the corticosteroid used for treatment[1,2] The controversy associated with steroid treatment is not in the use of steroids for treatment, but rather the dose and duration of treatment in a horse. Steroids do have some undesirable side effects, and long term use can cause more harm than good to the cartilage. [3]

Being such efficient anti-inflammatory drugs they reduce the pain and swelling, corticosteroids have a generalized inhibitory effect on collagen type II synthesis [4-6] *in-vitro* and *in- vivo* studies have shown that intra- articular injections of corticosteroids may have detrimental effects on the cartilage matrix and decrease proteoglycan synthesis.[7-12,1] Though there is an eventual recovery in proteoglycan synthesis the cartilage remains depleted of proteoglycan for several months. [9,10] In a study done on articular cartilage explants when the explants were incubated in medium containing methylprednisolone sodium succinate

(MPS) at 10mg/ml for 24 hours, proteoglycan synthesis was severly depressed and failed to recover after 13 days of culture in medium without MPS. [13] Other pathological changes of intra-articular administration of corticosteroids include decreased collagen synthesis, increased water content and delayed healing [9-11] thus making the cartilage more susceptible to mechanical injury. [12,14] Certain other studies have indicated the chondroprotective effects of steroids under certain conditions.[15-17]

Because steroids are used to treat not only joint diseases in horses and other species, but also other ailments such as airway disease, shock dermatitis, it is important for us to try and understand the effect of these drugs on various tissues, using *in vitro* systems that closely mimic the *in vivo* environment.

Previous studies have used models that have mostly ignored the influence of biomechanical stress on chondrocytes and its influence in mediating disease to analyze the effect of corticosteroids on articular cartilage. To the authors knowledge only one study has been done in this regard on canine cartilage explants, [14] and no such studies have been reported using agarose constructs seeded with chondrocytes.

Agarose gel cultures have been shown to maintain chondrocyte viability, phenotype and extra cellular matrix production (ECM). [18-22] where as in mono culture the chondrocytes change their phenotype and produce primarily collage type 1 and non aggregating proteoglycans[23] [24]

An acceptable *in vitro* model should resemble the *in vivo* environment, be cost effective, reproducible and minimize sacrifice of horses. The authors contend that incorporating dynamic physiologic load to suspension cultures of equine chondrocytes would be an improved *in vitro* model for the study of chondrocyte response to steroid treatment.

Hypothesis: - subjecting chondrocytes treated with steroids to dynamic compressive load will influence chondrocyte viability and ECM metabolism drastically in comparison to chondrocytes treated with steroids but not subjected to load and only load groups.

The overall goal of this study was to understand the effect of load on chondrocytes treated with steroids. To achieve these goals the following experiments were conducted 1) peak dynamic load, 2) duration in culture, 3) dose of steroids.

Research design.

Equine chondrocytes were cultured in Dulbeco's Modified Eagle Media (DMEM) with 20 % foetal bovine serum (FBS) 4mM L- glutamine, pencillin (57 /ml), streotomycin (57 µg/ml) and ascorbic acid (50 ug/ml), pH 7.2. These chondrocytes/agarose hydrogel discs were subjected to treatment with MPA and dynamic compressive stress in the FX- 400 C^{TM} Flexercell Compression Plus bioreactor for 24 hours once a day starting 24 hours after their formation. Tratment groups will be formed for the evaluation of 2 doses of MPA (0, 0.4 mg/ml and 4.0

mg/ml) and 3 levels of sinusoidal peak dynamic compressive stress (0, 10kPa, 25 kPa) delivered at 2 frequencies (0, 0.1 Hz) for 3 or 10 days. 2 agarose constructs per horse were subjected to each treatment and loading regime combination of peak load and frequency for 3 and 10 days. At the end of testing 1 construct each was subjected to evaluation as follows:- 1 section was used for viability testing . 1 construct was subjected to digestion for sulfated Glycosaminoglycan (GAG), hydroxyproline and DNA content determination. The media from each chonrocyte/ agarose contruct was collected at the time of each media replacement and construct harvest and was stored at – 20^0c for subsequent biochemical analysis.

Selection of doses of MPA was done on the basis of previous studies. Todhunter *et al*,. reported a severe depression in proteoglycan synthesis and increased proteoglycan degradation with doses of 0.4 and 4.0 mg/ml of MPA.[6] The selections of load values were to some extent empirical. The compression bioreactor in our laboratory is configured to deliver defined forces up to 14 lbs and applied stress is calculated (1 MPa = 145.038 psi) The daily contact stress on a human joint has been reported to range from approximately 3 to 10 MPa, [25] and though no data is available for the stresses on equine stifle joints, the stress in the equine distal interhalangeal joint at a trot has been estimated to be as high as 5 MPa. [26] Experience gained during other studies in our laboratory suggest that peak force applied to chondrocytes/agarose constructs should be restricted to kPa range to prevent the disruption of constructs.

Methods

Chondrocyte isolation:– near full thickness articular cartilage was aseptically removed from the femeropatellar joints of 2-10 year old hores within 2 hours of euthenesia at the University of Missouri Veterinary Medical Teaching Hospital. Cartilage slices were washed in Earle's balanced salt solution (EBSS) and transferred to tissue cultured dishes containing DMEM (compostion of which is described earlier). Tissue samples were minced and subjected to collagenase (1 %) digestion at 37^0C, 16 hours using standard procedure (21). Chonrocytes were washed twice with DMEM, counted using a hemcytometer , assessed for viability by trypan blue exclusion assay and resuspended in media at a concentration of 20 X 10^6 cell/ml.

Preparation of constructs:- chondrocyte suspensions were added to an equal volume of 3%(w/v) agarose type VII (Sigma Chemical Company, St. Louis, MO) in EBSS to yield a final concentration of 10 X 10^6 chondrocytes in 1.5 % (w.v) agarose. Chondrocyte/agarose suspensions were poured into Perspex moulds approximately 40 X 35 X 5 mm and allowed to harden at 4^0C for 20 minutes. Cylindrical chondrocyte/agarose gel plugs were cut using sterile 5 mm diameter skin biopsy punches and cultured in DMEM (supplemented as previously described) to which was added 0.4 and 4.0 mg/ ml of MPS. The media was replenished every 3 days.

Application of cyclic compression:- constructs were subjected to cyclic compressive load using FX- 400 C^{TM} Flexercell Compression Plus unit (Flexcell

international). Cyclic compression driven by air pressure was monitored by an inline manometer and controlled by solenoid valves using Flexsoft^{TM} software. The application of biomechanical force to the constructs while in culture is enabled by flexible- bottom culture plates. Constructs were subjected to cyclic compression over a range of frequencies and peak magnitudes for 20 minutes once a day for 8 days. No load and no treatment groups were subjected to the described culture conditions but without cyclic compressive loading and/or treatment with MPA.

Chondrocyte viability:- chondrocyte/ agarose gel slices (~ 1.5 mm thick) were prepared with a scalpel blade and stained with ethidium homodimer-1 (13 µl/ml phosphate buffered saline (PBS)) and calcein acetoxymethylester (AM) (0.4 µl/ml PBS) fluorescent satin (LIVE/DEAD Viability/Cytotoxicity ki), Molecular Probes, Eugene, Orgon). Cell viability was determined by confocal microscopy. Sections were incubated for 30 minutes at room temperature, placed on glass slides, and moistoned with several drops of PBS. A confocal laser microscope (BioRad Radiance 2000 confocal system coupled to an Olympus I X 70 inverted microscope) equipped with Krypton- Agron and red diode lasers will be used with a triple labeling technique. The method of determining the location of surviving cells is based on the knowledge that viable and non- viable cell differ in their ability to exclude fluorescent dyes. [27]The cell membranes of dead, damaged or dying cells are penetrated by ethidium homodimer-1 to satin their nuclei red. Living cells with intact plasma membranes and active cytoplasm metabolise calcein AM and show green fluorescence.

Glycosaminoglycan Analysis:- total sulfated GAG was quantified using 1-9-dimethlymethylene blue (DMMB) spectrophotometeric assay. [28] Stored media and chondrocyte/agarose constructs were thawed and digested in solutions of 2.8 unit/ml papain or 2.8 unit/ml papain and 10 units/ml agarase respectively (Sigma Chemical Co., St. Louis, MO). A 10 µl aliquot of the digested solution was mixed with 240 µl of DMMB solution and absorbance will be determined at 525 nm spectrophotometrically (Beckman DU-65 spectrophotometer, Beckman Instruments, Inc., Fullerton, CA). A standard curve was constructed using bovine tracheal chondroitin sulfate A. The results were corrected for differences in sample weight and normalized by DNA content. Total sulfated GAG content is reported in µg GAG/ wet weight.

PGE_2 Analysis:- Total PGE_2 was determined in conditioned media by an enzyme immunoassay systems (Amersham International, PLC, Buchinghamshire, England). The stored media was thawed and assayed for PGE_2 content according to the manufacturer's instructions. All samples were run in duplicate. Sample concentrations were determined by comparison with the manufacturer supplied standard curves.

NO Analysis:- Nitric oxide (NO) content in media was determined by measuring nitrite concentration, which is one of the two stable products from the breakdown of NO. Stored samples were thawed and nitrite concentrations determined using the Griess Reaction (Promega, Madison WI) and evaluation of

spectrophotometric (Beckman DU-65, Beckman Instruments, Inc., Fullerton CA) absorbance at 520-550 nm.

Statistical Analysis:- All statistical analysis will be performed using a computer software program (SigmaStat, Jandel Scientific, San Rafael, CA). One way ANOVA will be performed to determine differences among treatment groups with respect to each assay at each time collection time. When significant differences among groups are detected, an all pair-wise multiple comparison (Tukey test) will be performed. Differences compared to day 0 controls with respect to each assay at different collection times will be analyzed similarly. Significance was be established at $p < 0.05$.

Results:

Chondrocyte Viability:-Chondrocyte viability decreased with increasing load and with the application of corticosteroids. Cell viability was significantly ($P<0.030$) decreased at day 3 in the 10 kPa 0.4 mg/ ml and both the MPA groups loaded at 25 kPa when compared to the day 3 untreated controls. A significant ($P<0.030$) decrease in cell viability was seen in the unloaded group treated with 4 mg/ml of MPA at day 10 when compared to day 3 untreated controls. A similar decrease was also noticed in both the MPA treated groups loaded at 25 kPa. Although a general trend of decreased viability was noticed in all groups with the application of load or MPA these were not significant when compared to their respective controls. Fig 2.1

Nitric oxide assay:-Release of nitric oxide to media decreased with time. Media NO content increased with load applied and also with the use of load and corticosteroids. A significant ($P<0.030$) increase in release of NO to media was seen in the loaded groups on day 3 when compared to the unloaded groups. Application of load and corticosteroids significantly ($P<0.030$) increased the release of NO to media when compared to their respective no load samples. A significant decrease was seen in the grous loaded at 10 kPa on day 6 when compared to the corresponding day 3 samples. Fig 2.2.

PGE_2 Assay:- The PGE_2 levels showed a different trend where an increase was seen with load but a decrease was seen with the addition of MPA. PGE_2 release to media increased with load but this increase was not significant. A decrease in PGE_2 content was seen in all groups treated with MPA and was significantly ($P<0.030$) decreased in the no load groups. A significant ($P<0.030$) decrease in the group treated with 0.4 mg/ml of MPA and loaded at 25 kPa was seen at all time points (days 3, 6 and 10) when compared to their corresponding no MPA group loaded at the same levels. Fig 2.3.

Construct GAG content : Although an increase in GAG content was seen in the group loaded at 10 kPa this was not significantly different from any of the load only groups, but was significantly higher when compared to the 0.4 mg/ml ($p=0.04$) and 4 mg/ml($p=0.030$) MPA groups loaded at the same level. Fig 2.4

Media HP and GAG: HP and GAG content in media was not significantly different with the application of either load or MPA.

Discussion:

Corticosteroids are used extensively in the equine industry not only for conditions affecting the joints but also for various other conditions. Since corticosteroids are known to have systemic effects it becomes important to understand their affects on chondrocyte viability and ECM metabolism. Also a joint is constantly experiencing loads of various degrees and this factor should be taken in to consideration as load in itself has been shown to have both beneficial and detrimental effects. [29,30]

A significant decrease in chondrocyte viability was seen in the loaded groups treated with MPA. Previously a decrease in chondrocyte viability at a dose of 2000 µg/ml of MPA [31] has been reported and thus decreased viability at 0.4 and 4 mg/ml 4 mg/ml MPA would be expected. We did observe a decrease in chondrocyte viability in the MPA only groups also but this was significant only at 4 mg/ml of MPA. Chondrocytes are the only living component of the cartilage [32] and produce matrix components such as collagens and proteoglycans. Thus their viability is very important for the health of the cartilage. This data is in agreement with previously reported studies where corticosteroids have been shown to cause chondrocyte cytotoxicity, hypocelularity and necrosis. [9,33,34] Evidence suggests that corticosteroid treatment causes chondrocyte apoptosis in both cultures and arthritis models.[35]

Proteoglycans are important for cartilage health. Moderate levels of exercise have been shown to increase proteoglycan production and retention of

ECM molecules [36,37,30]. We saw a moderate increase in proteoglycan content in the 10 kPa group on day 10. This could indicate an increase in proteoglycan synthesis by the chondrocytes. Where as a significant decrease was seen in the same group when treated wit MPA. This data is in agreement with other studies where doses of 0.1 and 1 mg of methylprednisolone depressed proteoglycan synthesis, [13] but they did see an increase in proteoglycan synthesis 2 days after methylprednisolone was removed. We would not see such an increase because our constructs were constantly exposed to methylprednisolone. In the same study they did see a significant decrease in proteoglycan synthesis at 10 mg/ml which failed to increase even after 13 days in culture. In another study doses of as little as 1 mg/ml reduced proteoglycan synthesis 49 % after 262-266 hrs following exposure to 1 mg/ml of MPS for 20 hrs.[14] Todhunter *et al*,. reported a severe depression in proteoglycan synthesis with doses of 0.4 and 4.0 mg/ml of MPA, which is closer to our study. [6]

The increase seen in media NO with the addition of MPA could be as a response to load, but this is unlikely as the levels of NO in the load only groups were lower than their corresponding load plus MPA groups. Another probable reason could be that in a normal joint chondrocytes are embedded in a matrix which protects them and the cells would probably be exposed to the MPA in a gradual manner. In this situation the chondrocytes did not have any matrix and thus lacked protection. Further the fact that in the no load group a dose dependent decrease was seen in the NO content where as in the load groups a dose dependent increase was seen, further gives credence to this theory that the

increase seen could probably be due to interaction of the chondrocytes with the MPA as load would increase construct permeability thus increasing the supply of MPA to the chondrocytes. A good example to support the importance of cell matrix interactions is that this is that chondrocytes in agarose cultures do not show significant response to load until they have synthesized enough/measurable ECM. [38,39] Given the short duration of our cultures one would not expect the chondrocytes to produce a lot of ECM and this is supported by the lack of detection of collagen by the HP assay and the low numbers of our GAG assay.

The initial increase in both NO and PGE_2 in all loaded groups compared to the unloaded controls were probably the response of chondrocytes to initial trauma of loading. NO and PGE_2have been shown to act independent of each other in proteoglycan synthesis,[40] and degradation and could thus be expressed differently. Since both NO and PGE_2 are produced by activated chondrocytes [41] and NO has been suggested to play a role in chondrocyte apoptosis[42] and since cell viability was decreased in the day 10 samples, it could explain the progressive decrease in their release to media.

Rats receiving corticosteroids and subjected to running exercises displayed fibrotic invasion and subchondral bone replacement of degenerated articular cartilage associated with areas of cell death, and loss of matrix staining when compared to rats that received running exercise or corticosteroids alone. [12] The findings of this stuydy and such previous studies suggests a synergestic and /or potentiative interaction between load and corticosteroids.

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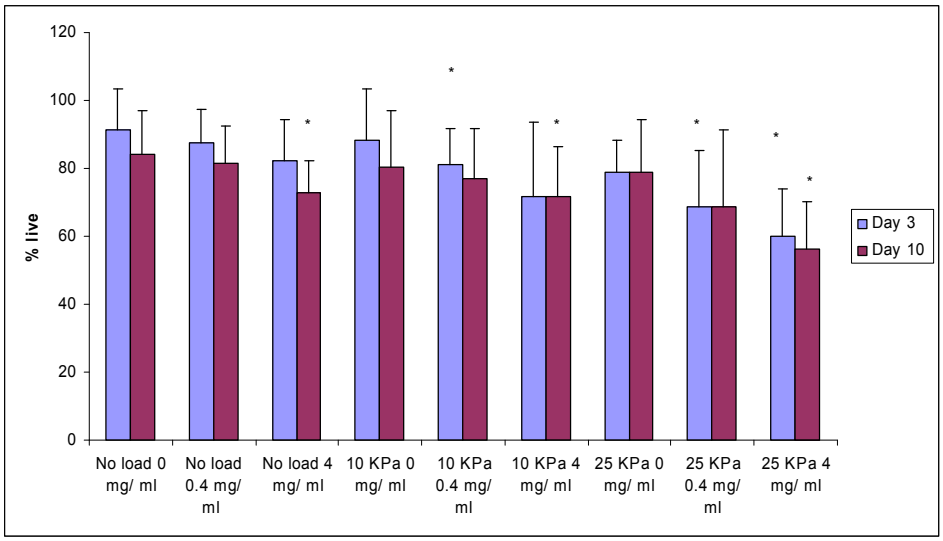
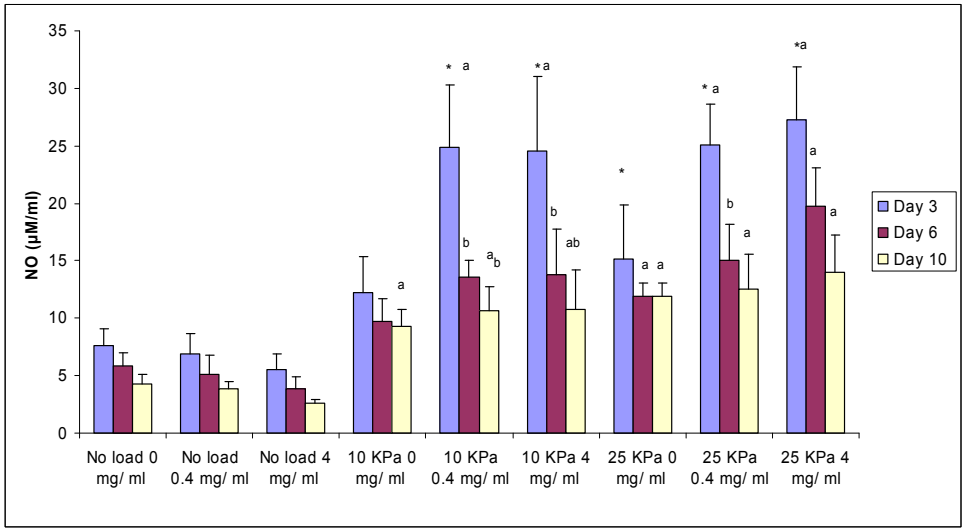
Figure 2.1 Cell viability



* Significantly different form no load 0 mg/ml day 3.

Fig 2.2. Meidia NO content.

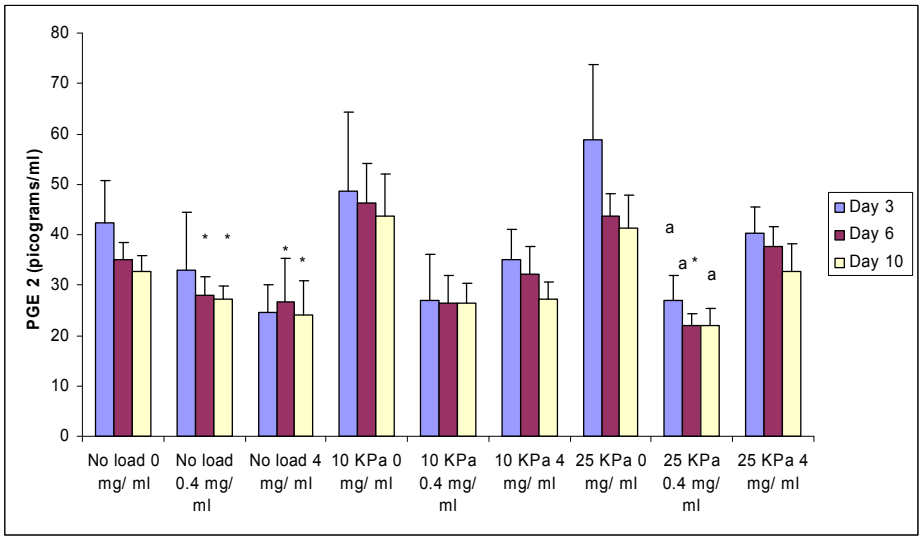


* Significantly different from no load 0 mg/ ml day 3.

a Significantly different from corresponding no load sample.

b Significantly different from corresponding day 3 sample.

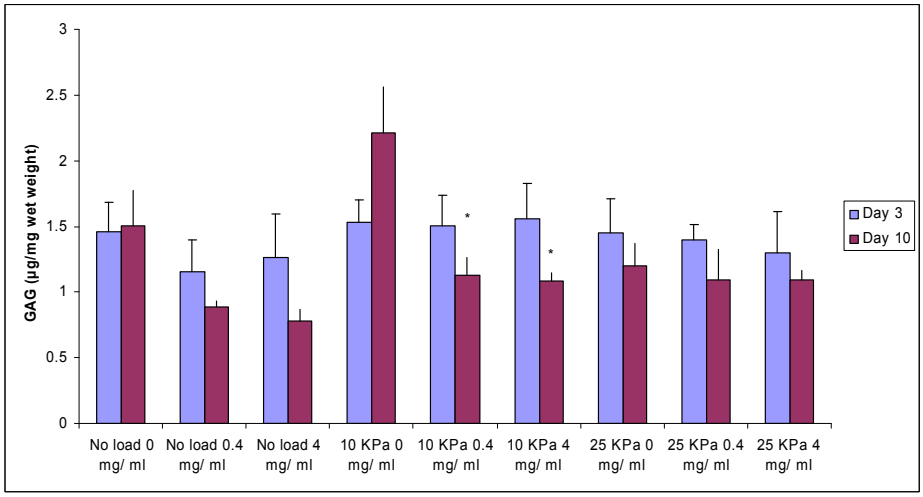
Figure 2.3. Media PGE_2 levels.



* Significantly different from no load day 0 mg/ ml day 3

a Significantly different from corresponding no MPA group

Figure 2.4. Construct GAG content.



* Significantly different from corresponding no MPA group.

An in vitro model of cartilage degradation: comparison of two loading regimes.

Articular cartilage, acts as a cushion for the bones and, along with synovium, provides a low friction gliding surface for the joints. Articular cartilage is exposed to, and required to withstand, large cyclical stresses and strains. The ability to withstand these stresses and strains depends on the extracellular matrix (ECM). The cartilage ECM is mainly composed of water, collagen and proteoglycans.[1-5] These components work together in a delicate and complicated mechanism to provide cartilage the ability to withstand the large stresses and strains observed. Any disease of cartilage (such as osteoarthritis (OA) or osteochondrosis (OC)) affects the ECM and reduce its ability to bear weight. These conditions are a major problem in both humans and animals causing immense pain and loss of function and have significant economic costs in terms of lost use and treatment.

Collagens provide cartilage with its tensile strength and proteoglycans play a role in the resistance of compressive forces by attracting water and creating swelling pressure. Chondrocytes are the cellular component of cartilage and, although sparse, are the sole producers of the ECM. The chondrocytes play a central role in the production, organization and maintenance of the ECM and are in turn protected by the ECM from the biomechanical forces exerted on the cartilage.[2,4]

Biomechanical stress plays a major role in cartilage health by enhancing fluid movement into and out of the cartilage matrix thus allowing distribution of matrix components synthesized by the chondrocytes and also nutrient/waste exchange.[7] Biomechanical forces also directly influence chondrocyte ECM through mechanotransduction.[8,9] Various regions within a joint experience various degrees of load and it is likely that topographical differences in matrix synthesis and metabolism [10-14], cartilage morphology[15,16], biochemical and mechanical properties,[6,17,18]are seen in different regions of the cartilage in a joint because of these various degrees of load. Thus biomechanical load plays an important role in cartilage development and health, but it could also well be that a disparity between stress and cartilage biomechanical properties could be a cause for cartilage degeneration.[19,20]

The magnitude and frequency of loading may also play an important role in influencing *in vitro* chondrocyte response. Application of relatively physiologic loads elicit an ECM sparing response by chondrocytes including decreased nitric oxide (NO) and prostaglandin E_2 (PGE_2) synthesis,[21,22]and increased glycosaminoglycan (GAG), total protein and DNA synthesis.[23] Application of supraphysiologic (excessive frequency/magnitude) loads elicit a net ECM catabolic response by chondrocytes characterized by increased expression of matrix metalloproteinases (MMP)-1, 3, 9 and 13, interleukin 1β (IL- 1 β), tumor necrosis factor α, and cycloxygenase-2 (COX 2), increased chondrocyte cell death and increased synthesis of NO and PGE_2,

reduced expression of type II collagen and aggrecan, and inhibited synthesis of DNA, proteoglycan, collagen and protein.[24-28]

Moderate levels of exercise have been shown to increase proteoglycan production and retention of ECM molecules [29,30,20] whereas static load results in degreased proteoglycan synthesis and increased protein loss. [31-34] Various other *in vitro* and *in vivo* studies in different species have shown that prolonged strenuous exercise leads to loss of proteoglycans and changes in cartilage matrix composition.[35-38,20] A more recent study looked at cartilage deformation after different levels of physical activity and found that the deformation of cartilage was directly proportional to the intensity of loading which is dependent on activity.[39]

The daily contact stress on human joints range from approximately 3 to 10 MPa[40] and though no data is available for the stresses on equine stifle joints, the stress in the equine distal interhalangeal joint at a trot has been estimated to be as high as 5 MPa. [41] At this time the effects of both load and frequency in physiologic levels is unclear. Currently there is no consensus on the dynamic loading profiles which closely resemble the *in vivo* situation, but it is important to understand the *in vivo* environment in order to better develop *in vitro* models. Though various models have been proposed including single impact models,[38, 39] an ideal model should accurately reflect the course of naturally occurring disease.

The goal of this study was to develop a loading regimen suitable to cause cartilage degeneration, but which would be within the physiological range of load that joints are typically exposed to.

We hypothesized that cartilage damage will be directly proportional to the magnitude, frequency and duration of the load applied, and that a combination of increasing magnitude, frequency, and duration will cause increased cell death, increased expression of MMPs and altered matrix synthesis as seen in naturally occurring cartilage degeneration.

Methods:

Cartilage explants: - Articular cartilage samples were collected aseptically from the femoropatellar joints of 6 horses aged 2-10 years within 24 hours of death at the University of Missouri Veterinary Medical Teaching Hospital. Cartilage slices were washed in Earle's balanced salt solution (EBSS) and transferred to tissue culture dishes containing Dulbeco's Modified Eagle Media (DMEM). Tissue samples were cut into cylindrical plugs using sterile 3 mm diameter skin biopsy punches and cultured in DMEM with 10 % fetal bovine serum (FBS), 4mM L- glutamine, penicillin (57 U/ml), streptomycin (57 µg/ml) and ascorbic acid (50 ug/ml), and pH adjusted to 7.2.

Application of cyclic compression: - Explants were subjected to cyclic compressive load using a FX- 400 C^{TM} Flexercell Compression Plus unit (Flexcell international, Hillsborough, North Carolina). Cyclic compression, driven by air pressure, was monitored by an inline manometer and controlled by solenoid valves using Flexsoft^{TM} software. The application of biomechanical force to the explants while in culture was enabled by flexible- bottom culture plates. Explants were subjected to cyclic

compression over a range of frequencies (0.1 Hz or 1 Hz) and peak magnitudes (2 MPa or 6 MPa) for 20 minutes either once a day or three times a day for 3 and10 days. No load control groups were subjected to the described culture conditions but without cyclic compressive loading.

Chondrocyte viability:- Explants (~ 1.5 mm thick) were prepared with a scalpel blade and stained with ethidium homodimer-1 (13 µl/ml phosphate buffered saline (PBS)) and calcein acetoxymethylester (AM) (0.4 µl/ml PBS) fluorescent stain (LIVE/DEAD Viability/Cytotoxicity kit), Molecular Probes, Eugene, Oregon). Cell viability was determined by confocal microscopy. Sections were incubated for 30 minutes at room temperature, placed on glass slides, and moistened with several drops of PBS. A confocal laser microscope (BioRad Radiance 2000 confocal system coupled to an Olympus I X 70 inverted microscope) equipped with Krypton-Argon and red diode lasers were used with a triple labeling technique. The method of determining the location of surviving cells was based on the knowledge that viable and non-viable cells differ in their ability to exclude fluorescent dyes.[42] The cell membranes of dead, damaged or dying cells were penetrated by ethidium homodimer-1 to stain their nuclei red. Living cells with intact plasma membranes and active cytoplasm metabolize calcein AM and showed green fluorescence.

RNA extraction:- Snap frozen explant samples were pulverized, transferred to 0.5 ml screw cap tubes filled with 1.0 mm diameter Zirconia Beads (BioSpec Products) and Trizol reagent (Invitrogen, Carlsbad, CA), and homogenized using a mini- bead beater (BioSpec Products) at 5000 rpm for 30 seconds. RNA was extracted from the homogenates using the TRIspin methods as described.[43] Briefly, homogenates were

chloroform extracted and separated by centrifugation. Ethanol was added to the aqueous phase to a final concentration of 35%, and subjected to RNeasy mini column chromatography (Qiagen Inc., Valencia, CA). The column was washed with RW1 buffer and subjected to an on column DNase 1 digest (Qiagen Inc., Valencia, CA) for 15 minutes at room temperature. Following serial washes with RW1 and RPE buffers, RNA was eluted with 30 µl of water. Isolated RNA was stored at - 80^0C following determination of concentration and purity.

***Reverse Transcription (RT)*:** Eight hundred ng of total RNA was reverse transcribed in 20 µl reactions using 0.5 µM of random hexamers and Stratascript reverse transcriptase(Stratagene, LaJolla, CA) according to the manufacturer's instructions. For each sample a No-RT control was run in parallel to assess DNA contamination. The RT profile was 42^0C for 2 hours, 68^0C for 10 minutes, 4^0C hold. Two µl of the RT reaction was used for subsequent Polymerase Chain Reaction (PCR).

Polymerase Chain Reaction (PCR): An assessment of steady state mRNA concentrations corresponding to genes of interest was made using Real-Time PCR. Primer pairs were designed (PrimerSelect, DNASTAR, Madison WI) for amplification of the following gene sequences: collagen (COL) types I, II and X, matrix metalloproteinases (MMPs) 1 and 13, tissue inhibitor of metalloproteinases (TIMP) - 1, Cyclo oxygenase 2 (Cox-2), Aggrecan, and glyceraldehyde 3- phosphate dehydrogenase (GAPDH). Real-Time PCR was performed with the Rotor-Gene RG-3000 (Corbett Research, Sydney, Australia) using the Quantitect SYBR green PCR kit (Qiagen) following the manufacturers guidelines. The PCR profile for all tests

consisted of an initial incubation of 94^0C for 15 minutes, followed by 55 cycles of 5 seconds at 94^0C, 10 seconds at 57^0C and 20 seconds at 72^0C. After the PCR profile, a melt curve analysis was done to ensure specific amplification for each sample. SYBR green fluorescence was monitored during the extension step of the PCR profile, and take off values and amplification efficiencies were determined using the Rotor-Gene software. Target gene expression was normalized to GAPDH expression and determined using Q-gene.[45] No-RT controls were tested for each primer set utilized to ensure that there was no contamination genomic DNA in the sample.

Glycosaminoglycan Analysis: Total sulfated GAG was quantified using 1-9-dimethlymethylene blue (DMMB) spectrophotometric assay.[44] Stored media and explants were thawed and digested in solutions of 2.8 units/ml papain (Sigma Chemical Co., St. Louis, MO). A 10 µl aliquot of the digested solution was mixed with 240 µl of DMMB solution and absorbance was determined at 525 nm spectrophotometrically (Beckman DU-65 spectrophotometer, Beckman Instruments, Inc., Fullerton, CA). A standard curve was constructed using bovine tracheal chondroitin sulfate A. The results were corrected for differences in sample weight and normalized by wet weight. Total sulfated GAG content was reported in µg GAG/ Wet Wt.

Hydroxyproline assay: The HP assay was used to estimate total collagen content in cartilage explants. Articular cartilage HP content was determined colorimetrically.

[45] A 50 µl aliquot of papain digested articular cartilage was combined with 4N sodium hydroxide (50 ul) and hydrolyzed by autoclaving at 120^{o}C for 20 minutes. Chloramine T reagent (450 µl) was added to the hydrolysate, mixed gently and oxidized at room temperature for 25 minutes. Ehrlich aldehyde reagent (450 µl) was then added to each sample. Samples were incubated at 65^{o}C for 20 minutes to develop the chromophore. Known concentrations of HP standard were used to construct a standard curve. Absorbance (550 nm) was determined using a spectrophotometer. Hydroxyproline content was reported in microgram per milliliter per gram.

PGE_2 Analysis: Total PGE_2 was determined in conditioned media by an enzyme immunoassay system (Amersham International, PLC, Buchinghamshire, England). The stored media was thawed and assayed for PGE_2 content according to the manufacturer's instructions. All samples were run in duplicate. Sample concentrations were determined by comparison with the manufacturer supplied standard curves.

NO Analysis: Nitric oxide (NO) content in media was determined by measuring nitrite concentration, which is one of the two stable products from the breakdown of NO. Stored samples were thawed and nitrite concentrations determined using the Griess Reaction (Promega, Madison WI) and evaluation of spectrophotometric (Beckman DU-65, Beckman Instruments, Inc., Fullerton CA) absorbance at 520-550 nm. Briefly, the standard was prepared according to the manufacturers' recommendation from the provided standard. A 50 µl sample was added to each of the test wells followed by the addition of 50 µl of sulfanilamide solution to all wells and incubation for 10 minutes. Fifty µl of N-1-naphthylethylenediamine dihydrochloride (NED) solution was then added to all

wells and incubated for 10 minutes at room temperature and absorbance was read at 520-550 nm.

Statistical Analysis: All statistical analyses were performed using a computer software program (SAS 9.1, SAS institute, Cary, North Carolina). One way ANOVA and paired student T tests were performed to determine differences among treatment groups with respect to each assay at each collection time. When significant differences among groups were detected, an all pair-wise multiple comparison (Tukey test) was performed. Differences compared to day 0 controls with respect to each assay at different collection times were analyzed similarly. Significance was established at $p < 0.05$.

Results:

Gene expression:

Collagen I gene expression was decreased in all loaded groups when compared to the unloaded control group at both days 3 and 10; however this decrease was not significant. When compared to the groups loaded at a similar regimen once a day a significant decrease was seen in collagen I gene expression at day 10 in the groups loaded at 2 MPa 1 Hz ($p=0.039$) and 6 MPa 1 Hz ($p= 0.013$) in the groups loaded three times a day. In general the maximum decrease was seen in the groups loaded three times a day and at a frequency of 1 Hz. Fig. 3.1

Collagen II gene expression showed a significant decrease ($P<0.034$) in the groups loaded at frequencies of 1 Hz 3 times a day on day 3 and in all the groups on day 10. In the groups loaded once a day a significant ($P=0.037$) decrease was seen in the groups

loaded at 1 Hz on day 3, and 10 when compared to the day 3 controls. A significant ($P<0.042$) decrease was also seen between the groups loaded once a day and three times a day in the groups loaded at 2 MPa 1 Hz and 6 MPa 0.1 Hz. No significant differences were seen as a result of duration of loading or load and frequency on any of the groups loaded either three times a day or once a day when compared to groups loaded for similar time periods, except the group loaded at 6 MPa 1 Hz loaded once a day which was significantly ($P=0.030$) lower than the group loaded for the same duration at 6 MPa 0.1 Hz on day 10. Fig.3.2

Collagen X Gene expression showed no significant differences in any of the groups. Data not shown.

Aggrecan gene expression was significantly ($P<0.036$) decreased in the 2 MPa 1 Hz group and at both frequencies at 6 MPa on day 3 in the groups loaded three times a day and in the groups loaded at a frequency of 1 Hz in the groups loaded once a day when compared to the no load day 3 group. On day 10 aggrecan gene expression was significantly ($P<0.008$) decrease in all groups loaded three times a day and in the groups loaded at frequencies of 1 Hz ($P< 0.024$) once a day. A significant ($P<0.042$) decrease was seen in both the groups loaded at a frequency of 0.1 Hz three times a day on day 10 when compared to the their corresponding day 3 samples as well as the corresponding day 10 samples loaded once a day. Fig.3.3

Though MMP1 gene expression was increased in all groups loaded 3 times a day, this increase was significant ($P=0.005$) only in the 6 MPA 1 Hz group. This group was significantly ($P< 0.010$)higher when compared to all the other groups loaded either once

a day or three times a day except the group loaded at 6 MPa 0.1 Hz. The greatest increase in this group at day 10 was observed in the 6 MPA 0.1HZ group but again this was not significant when compared to the other day 10 samples. MMP 1 gene expression remained elevated in all groups loaded at 3 times a day except for the day 10 6MPa 1Hz group. In the groups loaded once a day there were no significant difference in MMP 1 gene expression in any group loaded at a frequency of 0.1 Hz,, but at 1Hz, MMP 1 gene expression was greater than at 0.1Hz, but still insignificant. Fig.3.4

Although no significant differences were seen MMP 13 gene expression was increased at day 3 in the group loaded at 6 MPa 1Hz in both loading regimens when compared to the unloaded group on day 3. At day 10 the largest increase was seen at 2 MPA 1HZ and was remarkably decreased at 6 MPA 1HZ in the groups loaded three times a day. Though the levels of gene expression at day 3 in the groups loaded once a day were below those of the groups loaded 3 times a day, this trend was reversed at day 10. Data not shown

Gene expression patterns for TIMP 1 showed a dose dependent pattern with 3 times a day loading showing the maximum decrease in all groups loaded at 6 MPa 1 HZ in all loading regimens. TIMP 1 Gene expression was significantly ($P=0.018$) decreased by three times a day loading in the 6 MPa 1 Hz group and in all ($P< 0.013$) groups loaded 3 times a day on day 10 when compared to the day 3 no load control. In the groups loaded once a day a significant ($P<0.036$) decrease was observed only in the groups loaded at 1 Hz on day 10 when compared to the day 3 no load control. No such

differences were seen when any of the groups were compared to the day 10 no load controls. TIMP 1 gene expression significantly (P= 0.041) decreased by day 10 in the group loaded at 6 MPa 1 Hz when compared to the corresponding day 3 sample. Fig 3.5

Cox 2 gene expression showed a different trend with greater increase observed in day 10 groups. The groups loaded 3 times a day at 1 Hz showed a significant (P<0.036) increase at both time points when compared to the respective unloaded control groups. A slight increase in relative COX 2 gene expression was also seen in the unloaded groups at day 10. In the groups loaded once a day there was minimal to no increase in any of the groups compared to the respective controls at either time points. Fig 3.6

Hydroxyproline assay.

A significant (P<0.005) decrease in HP content was observed in cartilage explants loaded at 6MPA 1 HZ on day 3 and 2 MPa 1 Hz and both 6 MPA groups on day 10 (P<0.045) with three times a day loading. A general trend of decreased collagen was also seen in the higher (1 Hz) frequency groups across all loading regimens. HP content was slightly elevated in samples loaded at 2 MPa 0.1 HZ for duration of 20 minutes once a day at both days 3 and 10 when compared to the respective unloaded controls, but this increase was not significant. In the groups loaded once a day a significant (P=0.035) decrease was observed only in the 6 MPa 1 Hz group on day 10 when compared to the no load day 3 group. Loads of 6 MPa at a frequency of 1 Hz

significantly ($P<0.030$) decreased tissue collagen content when compared to similar loads at frequencies of 0.1 Hz on day 3, but not at day 10. Fig. 3.7

Glycosaminoglycan content: showed a slight but insignificant increase in GAG content at both time points (days 3 and 10) in the samples loaded at 2 MPa 0.1 HZ for duration of 20 minutes once a day at both time points when compared to the respective unloaded controls. In the samples loaded 3 times a day, the GAG content was significantly ($P<0.030$) reduced in the groups loaded at 1 Hz on day 3 and also in the 6 MPa 0.1 Hz group on day 10 ($P<0.005$) when compared to the day 3 controls. Although similar decreases were seen in the samples loaded once a day, these were significant only at day 10. Though not significant a general trend of decreased GAG content in all samples was observed at day 10 when compared to their respective day 3 samples. Fig.3.8

DMMB Assay: The GAG content in media from the unloaded samples remained fairly constant at all points of collection, but in the groups loaded once a day there was an insignificant initial increase at day 3 in all groups and the levels remained above the control groups on day 6, but returned to control levels on day 10 and were significantly lower than the previous levels ($P<0.036$). In the group loaded 3 times a day, the media GAG content was mildly elevated in the groups loaded at 0.1 Hz at day 3 when compared to the corresponding unloaded controls, whereas in the groups loaded at 1 Hz the levels remained below the corresponding control levels on all days. Fig 3.9

Nitric oxide content: Release of NO to the media showed a significant ($P<0.019$) increase in all groups loaded three times a day except the 2 MPa 0.1 Hz group, an in the groups loaded once a day a significant ($P<0.036$) increase was seen in the groups loaded at a frequency of 1 Hz when compared to the unloaded group at day 3. By day 6 the levels of the samples loaded 3 times a day were still elevated when compared to the controls, but were below the levels of their corresponding once a day load samples. A significant ($P<0.030$) decrease was seen on day 6 when compared to day 3 in significantly elevated groups loaded three times a day. Fig. 3.10

Media PGE_2: Release of PGE_2 to media was significantly ($P<0.019$)increased at day 3 when compared to the unloaded controls in all groups other than the 2 MPa 0.1 Hz group loaded three times a day. The PGE_2 levels remained significantly ($P<0.03$) elevated in all groups, subjected to this loading regimen, on day 6, but were significantly ($P<0.019$) increased only in the 0.1 Hz groups on day 10 when compared to the respective unloaded controls. Though an insignificant increase in PGE_2 content was observed in the groups loaded once a day at day 3, these decreased to unloaded control levels by day 6 and remained stable through day 10. A significant ($P<0.006$) increase in media PGE_2 content was seen in all groups loaded three times a day except the 6 MPa 1 Hz group when compared to their corresponding once a day load group. The levels of PGE_2 from media loaded three times a day remained elevated across all time points, but also showed a decrease over time. Fig.3.11.

Cell Viability. In our study cyclic loading at frequencies of 1 Hz at all loading regimens caused significant ($P<0.019$) cell death, irrespective of the loading protocol when compared to the respective unloaded controls at both days and compared to the day 3 unloaded control. Cell viability was relatively better in the groups loaded once a day when compared to their respective groups in the samples loaded three times a day. Superficial cell death was seen in all loaded groups at all time points, but at the groups loaded at 6 Mpa 1 Hz cell death was seen in all zones in all loading regimens. In the groups loaded at 2 MPa 0.1 Hz, increased cell death was observed in the groups loaded 3 times a day when compared to the corresponding group loaded once a day. Significantly ($P= 0.030$) higher cell death was observed at 6 MPA 0.1 Hz only on day 10 when compared to the unloaded controls. Cell viability decreased in explants loaded once a day from days 3 to 10 and was significant ($P<0.030$) in the 2 MPa 1 Hz and 6 MPa 0.1 Hz groups. Fig 3.12.

Discussion:

Various models have been proposed for the *in vitro* study of chondrocytes, either by seeding them in various construct materials or using explant cultures. Explant cultures have a theoretical advantage as they include the ECM which is an important component of articular cartilage. Hopefully, using explant cultures to evaluate the effects of load magnitude, frequency, and duration will provide a better understanding of the cartilage as a whole.

Various models have been created to study the responses of cartilage to physiologic and supraphysiological stresses. Some have used isolated

chondrocytes while others have used cartilage explants, with or without the subchondral bone attached. Explants have been subjected to wide range of load regimens ranging from single-impact,[46-48] static[31,32,49] to cyclic loading[20] at various frequencies in either confined [50] or unconfined [20,51] conditions. The response of cartilage in *in vivo* animal models of exercise[36,37,14] and trauma[52] have also been developed.

It is important to understand how mechanical factors in the physiological range effect cartilage to be able to further develop models of cartilage damage and degeneration as would happen in a living animal. Currently there is no consensus on the dynamic loading profiles which closely resemble the *in vivo* situation.

In our study superficial cell death was observed in all loaded samples. Similar results have been reported in other studies done in our laboratory and by other investigators. [53,54,20,55] [55]One of the reasons for superficial cell death could be that it is due to contact of tissue with our loading platen. This could be a possible and relatively likely cause for superficial cell death as the platens used in our study are relatively smooth, non-porous, rigid and hard. Similar patterns of cell death have been reported in other loading studies[20] and also in studies where two cartilage pieces[53] were placed opposing each other. It could well be possible that synovial fluid which is present between the opposing cartilage ends in an *in vivo* environment provides a cushion to the cartilage and the absence of synovial fluid in *in vitro* studies could result in the increased cell death. Another possible explanation could be the absence of subchondral bone, which would

provide further support *in vivo*. Further the compositional differences among the different layers of the cartilage could also play a role in cell death.[20] Previous studies[20] have also shown an increase in cell death due to mechanical trauma and it could be possible that the cells in the superficial zone of the cartilage are directly exposed to the force and hence the increased cell death in this zone. Further we see a pattern of increasing cell death even in the deeper zones with increased frequency over a period of 10 days in our explants. This indicates that higher frequency loading can cause cartilage damage. This may not be all that true in an *in vivo* situation where other factors which support the cartilage are involved. There is also much more cartilage and thus weight probably gets distributed over a wider surface and shear forces are minimized. Also one could argue that in an *in vivo* situation the likelihood of the same section of cartilage being exposed to loads at a frequency of 1 Hz for 20 minutes would be unlikely if not impossible, but even in an *in vivo* situation long term strenuous exercise has been shown to cause osteoarthritis like changes in cartilage.

The goal of our study was to create a model of cartilage degeneration in a reasonable time period to mimic an *in vitro* situation, but still be within the physiological range. Further investigation is needed to determine if this decrease in chondrocyte viability is due to apoptosis or necrosis. Also, the exact mechanism leading from trauma/ excessive load to cell death needs to be investigated and it could well be that certain other factors such as caspases[56] are activated and involved in the actual process of initiating cell death rather than the trauma itself.

Proteoglycans are an important component in cartilage health and are responsible for maintaining the hydration of the cartilage. Proteoglycans and collagens act together in maintaining cartilage hydration in that the negatively charged proteoglycans attract water into the cartilage and the collagen network prevents the expansion of the cartilage to a maximum of 20 % of its volume.[57] Cartilage swelling indicates a breakdown of this mechanism.[46,57] Though we did not include this parameter in our study, casual visual examination during sample collection noted an increase in the size of the tissues in some of the loaded groups when compared to the unloaded controls. Cartilage swelling has been reported in a range of studies looking at mechanical trauma/forces. [57,58]

There is a controversy as to the levels of proteoglycan in early stages of OA. Some report a decrease in proteoglycan content and an increase in water content as one of the earliest changes in OA [59,60] whereas other studies have shown an increase in proteoglycan synthesis[61] in early stages of OA. It is possible that breakdown of the collagen network and a corresponding lack of restriction on the level of tissue expansion, proteoglycans are able to attract large quantities of water into the tissue, resulting in increased tissue hydration and cartilage swelling.

In our study we found a moderate increase in proteoglycan content in the group loaded once a day at 2 MPa 0.1 Hz. Light to moderate exercise has been shown to increase proteoglycan production. Brama *et al*.,[62] reported a significant

increase in proteoglycan content in cartilage from horses kept on pasture compared to boxed horses, but not compared to an exercise group.

Aggrecan gene expression correlated with tissue proteoglycan content in that similar patterns were observed with the groups loaded 3 times a day showing decreased protein and gene expression when compared to the groups loaded once a day.

The results of the DMMB assay showed a similar trend of increased GAG content in the groups loaded once a day when compared to the groups loaded three times a day. Increased GAG content in media could be either due to increased synthesis or increased degradation. We feel that in our study the increased loss of GAG to media in the samples once a day could be due to a balance between synthesis and natural proteoglycan degradation. One might expect to see a significant increase in GAG content in media from the loaded groups, but it could be possible that since our explant size (3 mm) was small the release of GAG into media due to degradation could not be detected by our assay and requires a more sensitive assay. Also, since there was a gradual decrease in media GAG content in the groups loaded three times a day rather than a drastic sudden decrease, it could be possible that there was a gradual loss in proteoglycans over time.

Gene expression for collagens I, II and X was decreased in the loaded groups. Collagen II is the main collagen found in cartilage and collagen I and X are seen in young growing animals and in hypertrohic chondrocytes.[63,64] Our cartilage samples were collected from horses below 2 years of age and were still

growing which could explain the presence of these collagens in our samples. A decrease in the expression of these collagens correlates well with a similar pattern of decrease seen in the results of our hydroxyproline assay, though the marked decrease in gene expression does not correlate well with the subtle difference seen in the tissue. A possible explanation for this could be that collagen turnover in tissue is exceptionally long (> 100 years).[65] Therefore, although a decrease in gene expression was observed, a corresponding decrease in total collagen content in tissues was not observed in groups other than the group loaded at 6 MPa 1 HZ. The results of our study are similar to the results of another study where loading at peak stresses between 3.5 and 14 MPa caused an immediate dose dependent denaturation of cartilage collagen. [66]

MMPs are the major degrading enzymes affecting collagens and has been reported to be increased in animal models of OA[67] and in animals affected with OC.[55] [68] The increased expression in samples loaded at 6 MPa 1 HZ in both loading regimens at day 3 could be indicative of cartilage degradation and could be the cause for the severe decrease seen in the collagen gene expression patterns in these samples. The decrease in MMP expression in groups loaded at this regimen 3 times a day by day 10 could be due to increased cell death, which could also be the cause for the decrease in gene expression of other components and related protein content. The similar pattern of decrease in TIMP 1 gene expression may confirm that these changes are associated with decreased cell viability thus affecting their ability to produce matrix molecules.

The increase in MMP in the group loaded at 6MPa 1 Hz at day 3 and the simultaneous decrease in the TIMP 1 in this group could indicate tissue degradation. TIMPs are the inhibitors of MMPs and a balance between them is essential for cartilage health. The decrease in TIMP as early as day 3 is intriguing because one would expect a compensatory increase in TIMP to counteract the increase in MMP expression. Further work is needed to evaluate the protein expression of these molecules, however the present data indicates that cartilage degradation could be either due to the increase in MMP or the decrease in TIMP. Further work needs to determine which occurs first.

Media NO and PGE_2 were elevated in all loaded samples to various degrees at all time points, indicating an inflammatory process. However NO elevation was observed more in the groups loaded once a day compared to the groups loaded three times a day. NO and PGE_2have been shown to act independent of each other in proteoglycan synthesis,[69] and degradation and could thus be expressed differently. The initial increase in both NO and PGE_2 in all loaded groups compared to the unloaded controls were probably the response of chondrocytes to initial trauma of loading. Since both NO and PGE_2 are produced by activated chondrocytes [70] and NO has been suggested to play a role in chondrocyte apoptosis[71] and since cell viability was decreased in the day 10 samples, it could explain the progressive decrease in their release to media.

COX-2 gene expression showed a different trend with the maximum increase seen in the groups loaded three times a day at 6 MPA 1 Hz at both time points. The discrepancy in the results of gene expression and PGE_2 release to

media needs to be investigated further, but it could also be possible that other mechanisms are involved in the production of PGE_2.

The results of this study indicate that high levels of load at high frequencies for prolonged duration may cause progressive cartilage damage even if applied within physiological limits. Although the amount of load applied is important in producing cartilage damage, the frequency seems to play a more significant role as indicated by the results of our loading regimen at 2 MPa 1 Hz vs 6 MPa 0.1 Hz. Based on the results of our study, we feel that a loading regimen of 2 or 6 MPa at a frequency of 1 Hz delivered three times a day would be ideal for producing models of cartilage degeneration within physiological limits. Further work needs to be done to understand the mechanism of initiation of cell death as not only has cell death been accepted as a hall mark of inflammatory and degenerative joint disease,[72] but chondrocyte senescence [73] is also thought to play a major role in primary OA.

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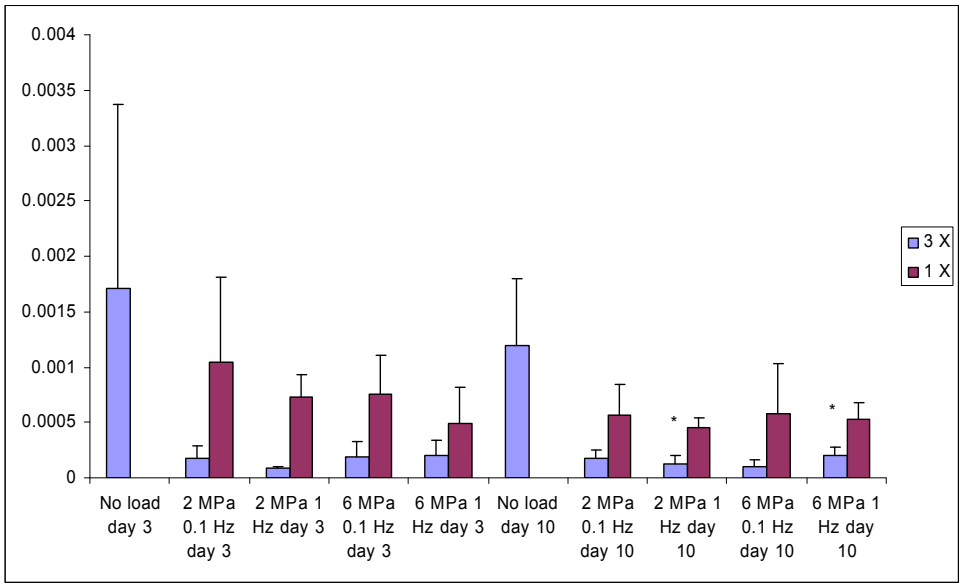
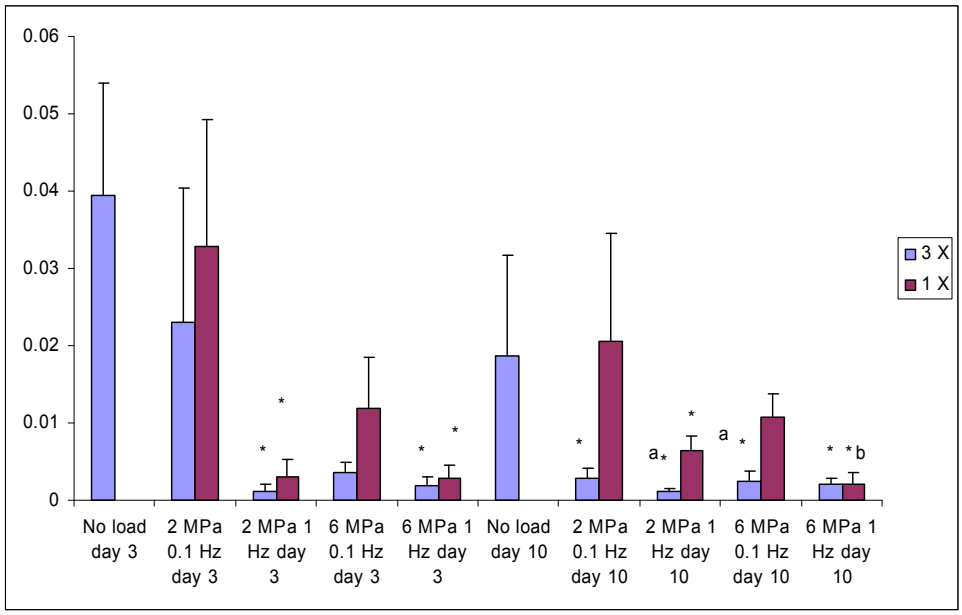
Figure 3.1. Relative gene expression for collagen 1



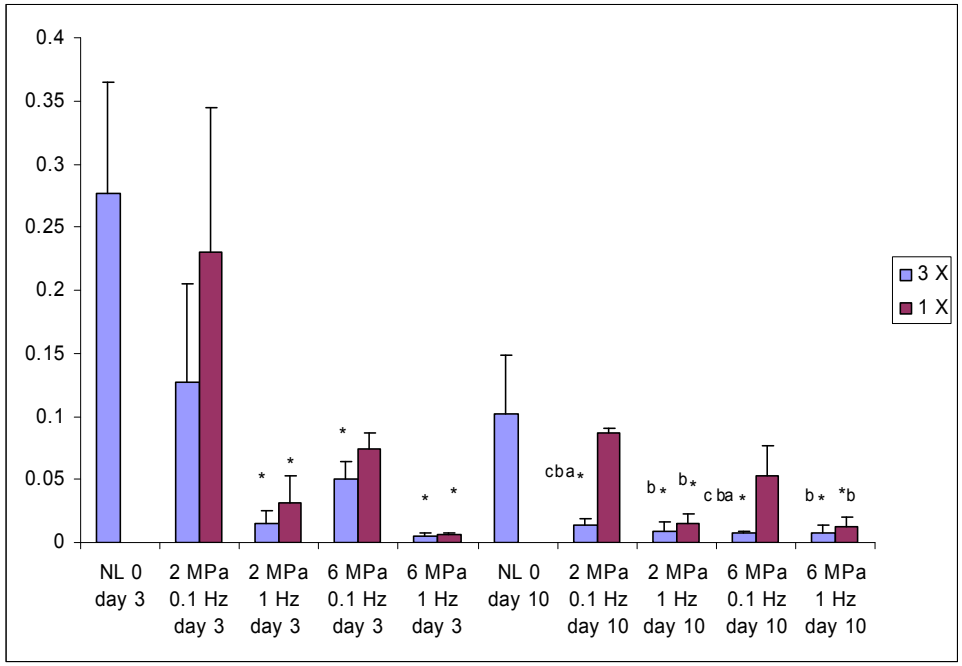
* Significantly different from corresponding group loaded once a day.

Figure 3.2. Relative gene expression collagen II



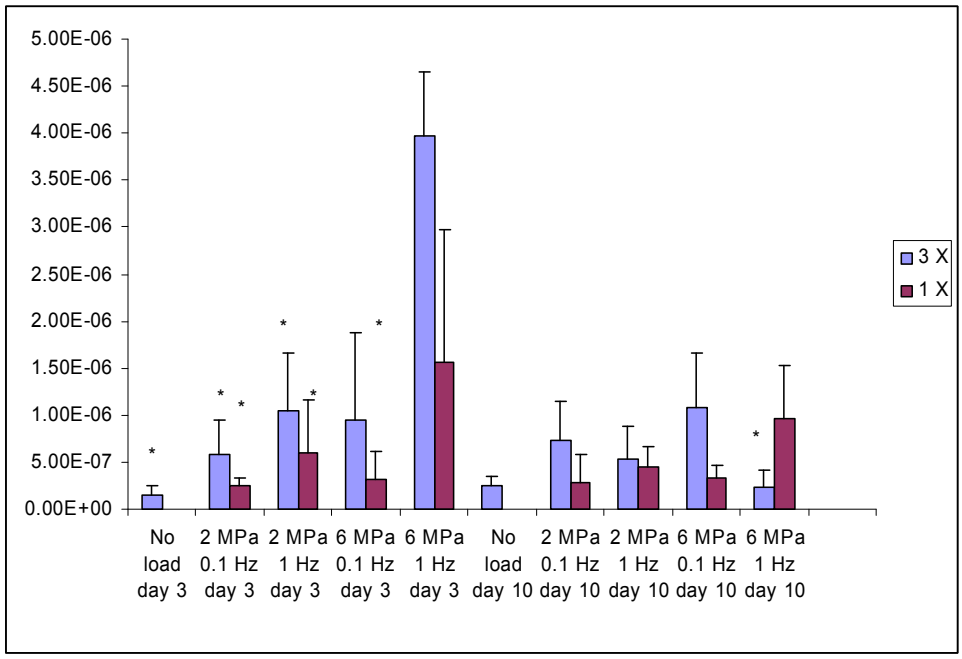
* Significantly different from no load day 3.
a Significantly different from group loaded at similar regimen once a day.
b Significantly different form 6 MPa o.1 Hz once a day.

Figure 3.3. Relative gene expression for Aggrecan



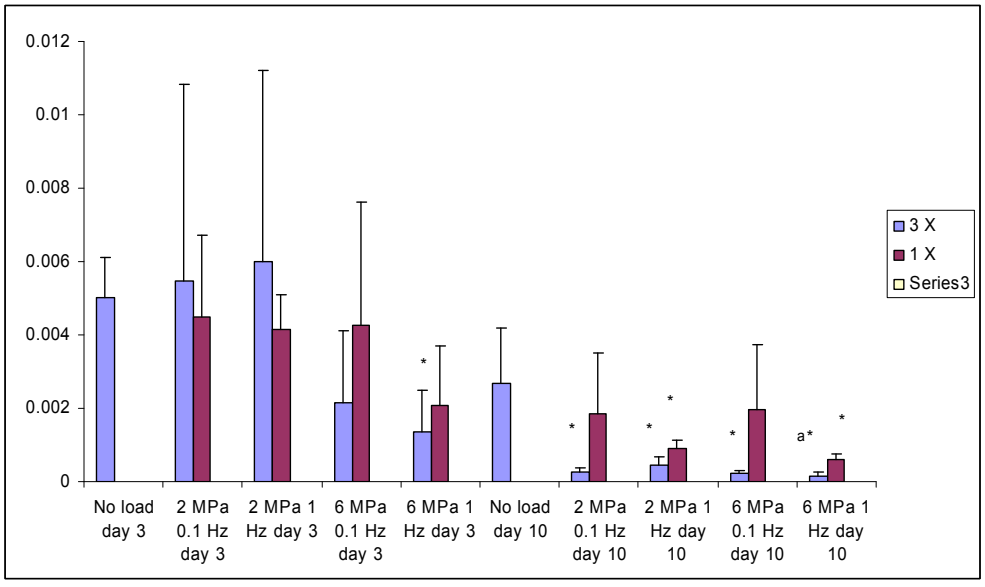
* Significantly different from no load day 3
a Significantly different from corresponding day 3
b Significantly different from no load day 10.
c Significantly different from corresponding sample loaded once a day.

Figure 3.4. Relative gene expression for MMP 1



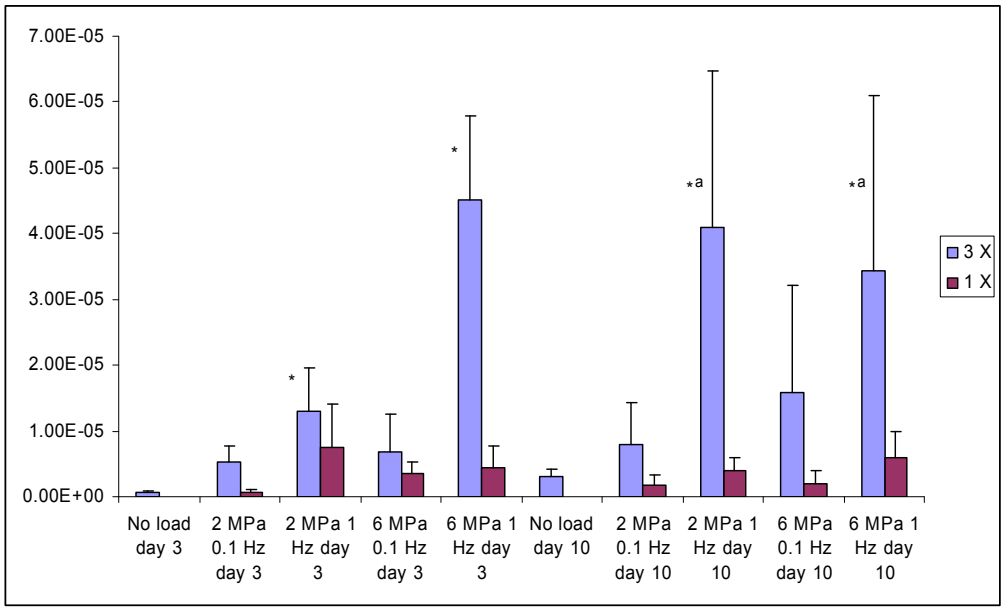
* Significantly different form 6 MPa 1 Hz day 3

Figure 3.5. Relative gene expression for TIMP 1



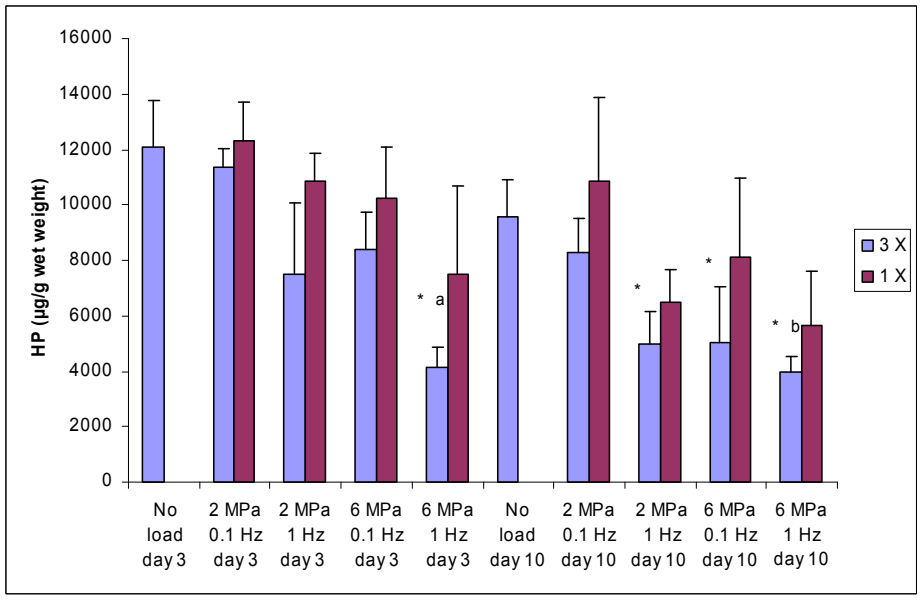
* Significantly different from no load day 3.
a Significantly different from corresponding day 3 sample.

Figure 3.6. Relative gene expression for COX 2



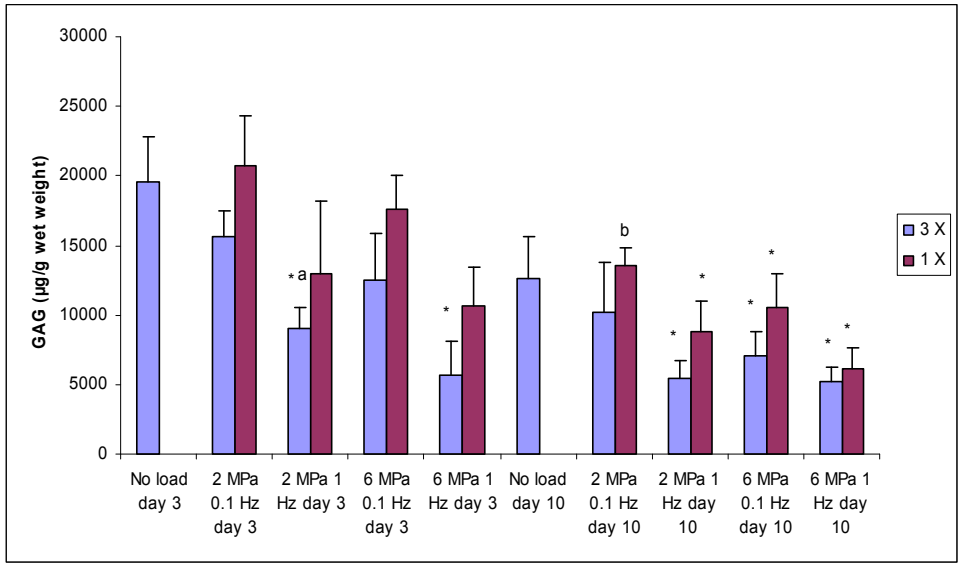
* Significantly different from no load day 3.
a Significantly different from no load day 10

Figure 3.7. Total tissue hydroxyproline content.



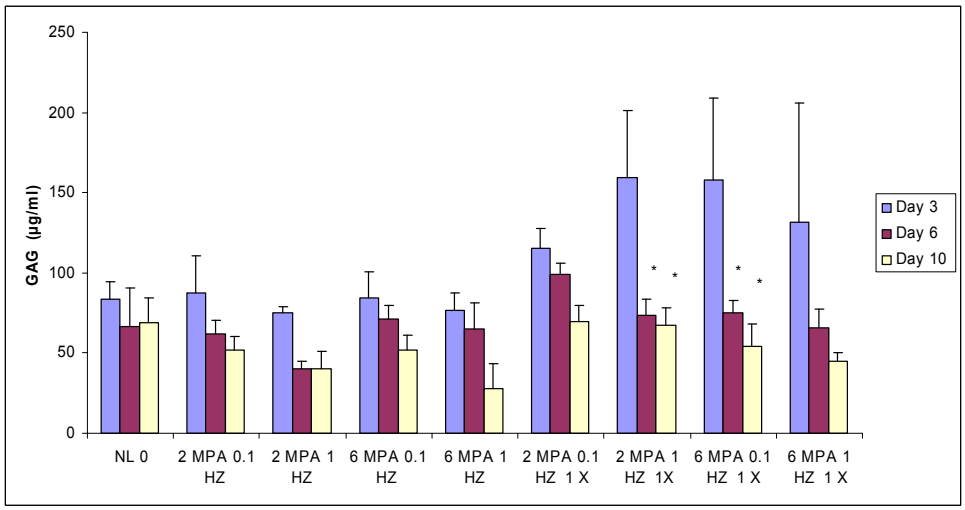
* Significantly different from no load day 3
a Significantly different from no load day 10
b Significantly different from group loaded at similar MPa but lower frequency.

Fig 3.8 Total tissue glycosaminoglycan content.



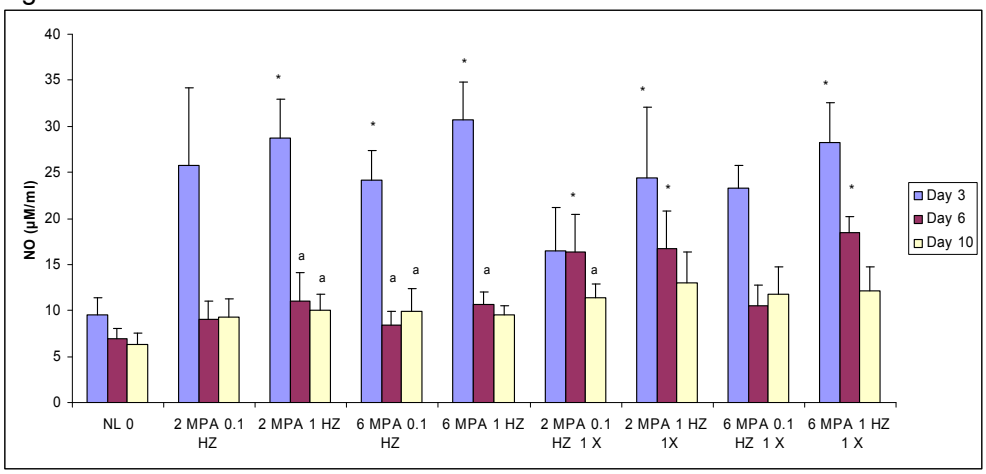
* Significantly different from no load day 3
a Significantly different from lower frequency at same MPa
b Significantly different from corresponding day 3 group.

Fig 3.9 Total media glycosaminoglycan content.



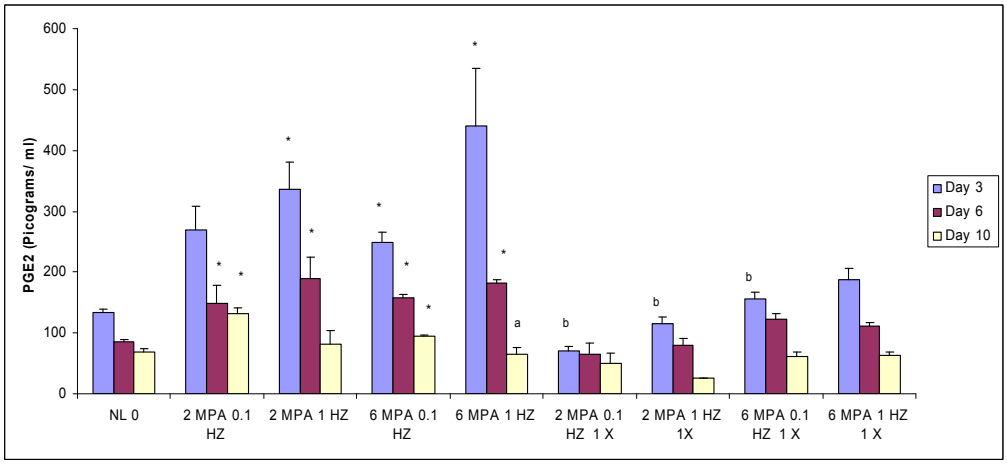
* Significantly different from corresponding day 3.
Note: 1X: samples loaded once a day

Figure 3.10 Media NO content.



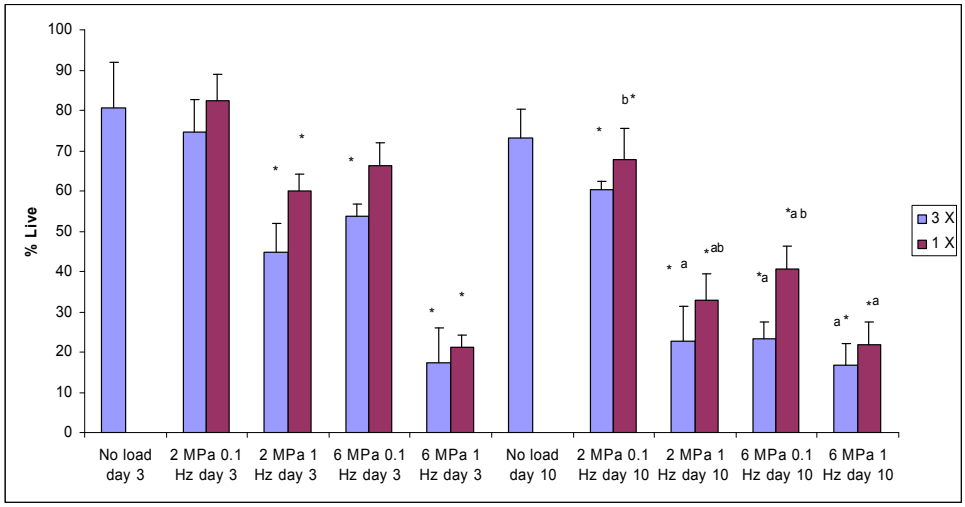
* Significantly different from corresponding day no load
a Significantly different from corresponding day 3
Note: 1X: samples loaded once a day.

Figure 3.11. Media PGE_2content.



* Significantly higher than corresponding no load sample
a Significantly lower than corresponding day 3 sample
b Significantly lower than corresponding sample loaded three times a day.
Note: 1X: samples loaded once a day.

Figure 3.12. Cell viability (% live)



* Significantly different from no load day 3.
a Significantly different from no load day 10.
b Significantly different from corresponding day 3 group.

Effect of Corticosteroids and Load on Articular Cartilage Explants.

Corticosteroids are potent anti-inflammatory agents commonly used in the treatment of joint injury in horses. [1] Their effects on cartilage appear to be dependent on the type and dose of agent used for treatment. [2,3] For horses, the major controversy of intra-articular therapy is not the use of steroids, but rather the dose and duration of intra-articular treatment. Steroids are also used to treat ailments such as airway disease, shock and dermatitis in horses for which they have systemic effects.

Despite their clinical success in the reduction of pain and swelling, corticosteroids have a generalized inhibitory effect on collagen type II synthesis.[4-6] *In vitro* and *in vivo* studies have shown that intra-articular injections of corticosteroids may have detrimental effects on cartilage matrix and decrease proteoglycan synthesis.[7-12,2] Although there is eventual recovery of proteoglycan synthesis, the cartilage remains depleted of proteoglycan for several months.[9-11] In a study of articular cartilage explants were incubated in medium containing methylprednisolone acetate (MPA) at 10mg/ml for 24 hours, proteoglycan synthesis was severely depressed and failed to recover after 13 days of culture in medium without MPA. [13] Other undesirable side effects resulting from long term intra-articular administration of corticosteroids include decreased collagen synthesis, increased water content and delayed healing, [9-11,14] thus making the cartilage more susceptible to mechanical injury[12,15] Rats receiving corticosteroids and subjected to running exercise displayed fibrotic invasion and subchondral bone replacement of degenerated articular

cartilage associated with areas of cell death, and loss of matrix staining when compared to rats that received running exercise or corticosteroids alone. [12] The latter finding suggests a synergestic and /or potentiative interaction between corticosteroids and exercise. Carter et al.,[16] reported a decrease in the formation of repair tissue and synovial inflammation with the long term use of MPA.

Using an *in vitro* system that closely mimics the *in vivo* environment, the responses of cartilage to steroid treatment can begin to be determined. By providing biomechanical stimulus to cartilage in the culture system, we can determine if loading of cartilage causes a differential response to steroid treatment, and whether any or both of these agents are involved in the progression of cartilage pathology.

We hypothesized that subjecting articular cartilage to dynamic compressive stress and corticosteroids influences ECM metabolism.

Material and methods:

Equine articular cartilage was collected aseptically within 24 hrs of death from the medial and lateral trochlear ridges of the femurs in grossly normal femoropatellar joints with grossly normal articular cartilage of 7 adult horses that died or were euthanized for reasons unrelated to joint disorders. Three mm explants were made using a biopsy punch, and cultured in Dulbeco's Modified Eagle Media (DMEM) with 10 % fetal bovine serum (FBS) 4mM L- glutamine, penicillin (57 U/ml), streptomycin (57 µg/ml) and ascorbic acid (50 ug/ml), pH 7.2. These cartilage explants were subjected to treatment with methylprednisolone acetate (MPA) and dynamic

compressive stress in the FX- 400 C^{TM} Flexercell Compression Plus bioreactor for 20 minutes three times a day starting 24 hours after their formation. Treatment groups were formed for the evaluation of 2 doses of MPA (0, 0.4 mg/ml and 4.0 mg/ml) and 3 levels of sinusoidal peak dynamic compressive stress (0, 2 MPa and 6 MPa) delivered at 3 frequencies (0, 0.1, and 1 Hz) for 10 days. Three cartilage explants per horse were subjected to each treatment and loading regime combination of peak load and frequency for 3 or 10 days. At the end of testing 1 explant each was subjected to evaluation as follows: 1 section used for viability testing; 1 explant was subjected to digestion for sulfated GAG and hydroxyproline determination; and 1 explant was processed for protein and PCR analysis. The media from each explant was collected at the time of each media replacement and explant harvest and was stored at –20^{0}C for subsequent biochemical analysis.

Selection of doses of MPA was based on previous studies. Todhunter *et al*,. reported a severe depression in proteoglycan synthesis and increased proteoglycan degradation with doses of 0.4 and 4.0 mg/ml of MPA.[6] The selections of load values were to some extent empirical. The compression bioreactor in our laboratory is configured to deliver defined forces up to 14 lbs and applied stress is calculated (1 MPa = 145.038 psi) The daily contact stress on a human joint has been reported to range from approximately 3 to 10 MPa,[17] and though no data is available for the stresses on equine stifle joints, the stress in the equine distal interphalangeal joint at a trot has been estimated to be as high as 5 MPa.[18]

Application of cyclic compression: - Explants were subjected to cyclic compressive load using FX- 400 C^{TM} Flexercell Compression Plus unit (Flexcell International, Hillsborough, North Carolina). Cyclic compression driven by air pressure was monitored by an inline manometer and controlled by solenoid valves using Flexsoft^{TM} software. The application of biomechanical force to the explants while in culture is enabled by flexible- bottom culture plates. Explants were subjected to cyclic compression over a range of frequencies and peak magnitudes for 20 minutes every eight hours for 3 or 10 days. No load and no treatment control groups were subjected to the described culture conditions but without cyclic compressive loading and/or treatment with MPA. The loads applied to the cartilage explants in this study were 5.6 and 16.8 lb for 2 and 6 MPa respectively calculated on the formula: pressure = force/area.

RNA extraction: Total RNA was extracted using a modified TRIspin method.[19] Snap frozen explant samples were powdered by crushing, transferred to 1ml of Trizol (Invitrogen, Carlsbad, CA), and homogenized using 3.2mm steel beads (BioSpec Products) and a mini- bead beater (BioSpec Products) set at 5000 rpm for 30 seconds. Homogenates were chloroform extracted and separated by centrifugation. The RNA was precipitated using isopropanol, and the pellet was resuspended in 100µl of DEPC water. The RNA was then further cleaned using the RNeasy Minelute cleanup kit (Qiagen Inc., Valencia, CA) following the manufactures protocol. Total RNA was eluted with 14µl of water and contaminating DNA was digested using the Turbo DNase kit

(Ambion). Isolated RNA was stored at – 80^{0}C following determination of concentration and purity.

***Reverse Transcription (RT)*: Five hundred** ng of total RNA was reverse transcribed in 20 µl reactions using 0.5µM of random hexamers and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For each sample a No-RT control was run in parallel to assess DNA contamination. The RT profile was: 42^{0}C for 2 hours, 68^{0}C for 10 minutes, 4^{0}C hold. The cDNA was diluted with 180µl of water, and 4µl of the diluted cDNA was used for subsequent real-time Polymerase Chain Reaction (PCR).

Polymerase Chain Reaction (PCR): An assessment of steady state mRNA concentrations corresponding to genes of interest was made using Real-Time PCR. Primer pairs have been designed (PrimerSelect, DNASTAR, Madison WI) for amplification of the following gene sequences: collagen types I, II and X, matrix metalloproteinases (MMPs) 1, 3 and 13, tissue inhibitor of metalloproteinases (TIMP) - 1, aggrecan, cyclooxygenase (COX) 2, inducible nitric oxide (iNOS), and glyceraldehyde 3- phosphate dehydrogenase (GAPDH). Real–Time PCR was performed with the Rotor-Gene RG- 3000 (Corbett Research, Sydney, Australia) using the Quantitect SYBR green PCR kit (Qiagen, Inc.) following the manufacturers guidelines. The PCR profile for all tests consisted of an initial incubation of 94^{0}C for 15 minutes, followed by 55 cycles of 5 seconds at 94^{0}C, 10 seconds at 57^{0}C and 20 seconds at 72^{0}C. After the PCR profile, a melt curve analysis was done to ensure

specific amplification for each sample. SYBR green fluorescence was monitored during the extension step of the PCR profile, and take off values and amplification efficiencies were determined using the Rotor–Gene software. Target gene expression were normalized to GAPDH expression and determined using Q-gene.[20] No-RT controls were tested for each primer set utilized to ensure that there was no contamination genomic DNA in the sample.

Glycosaminoglycan Analysis: Total sulfated GAG was quantified using 1-9-dimethlymethylene blue (DMMB) spectrophotometeric assay.[21] Stored media and explants were thawed and digested in solutions of 2.8 units/ml papain (Sigma Chemical Co., St. Louis, MO). A 10 µl aliquot of the digested solution was mixed with 240 µl of DMMB solution and absorbance was determined at 525 nm spectrophotometrically (Beckman DU-65 spectrophotometer, Beckman Instruments, Inc., Fullerton, CA). A standard curve was constructed using bovine tracheal chondroitin sulfate A. The results were corrected for differences in sample weight and normalized to dry weight of the sample. Total GAG content in cartilage explants was reported as GAG/weight (µg/g). GAG concentrations in the liquid media were reported as a percentage of total GAG in the cartilage explant.

Hydroxyproline Assay: Total collagen content in cartilage explants was determined by measuring the HP content using a colorimetric procedure,[22]as previously described.[23] HP content wasreported in HP/weight (µg/g).

PGE_2 Analysis: Total PGE_2 was determined in conditioned media by an enzyme immunoassay system (Amersham International, PLC, Buchinghamshire, England). The stored media was thawed and assayed for PGE_2 content according to the manufacturer's instructions. All samples were run in duplicate. Sample concentrations were determined by comparison with the manufacturer supplied standard curves.

NO Analysis: Nitric oxide (NO) content in media was determined by measuring nitrite concentration, which is one of the two stable products from the breakdown of NO. Stored samples were thawed and nitrite concentrations determined using the Griess Reaction (Promega, Madison WI) and evaluation of spectrophotometric (Beckman DU-65, Beckman Instruments, Inc., Fullerton CA) absorbance at 520-550 nm. Briefly, the standard was prepared according to the manufacturers' recommendation from the provided standard. Fifty µl of sample was added to each of the test wells followed by the addition of 50 µl of sulfanilamide solution to all wells and incubated for 10 minutes. Fifty µl of N-1-naphthylethylenediamine dihydrochloride (NED) solution was then added to all wells and incubated for 10 minutes at room temperature and absorbance was read at 520-550 nm.

Chondrocyte viability:- Explants (~ 1.5 mm thick) were prepared with a scalpel blade and stained with ethidium homodimer-1 (13 µl/ml phosphate buffered saline (PBS)) and calcein acetoxymethylester (AM) (0.4 µl/ml PBS) fluorescent stain (LIVE/DEAD Viability/Cytotoxicity kit), Molecular Probes, Eugene, Oregon). Cell viability was determined by confocal microscopy. Sections were incubated for 30 minutes at room temperature, placed on glass slides, and moistened with several

drops of PBS. A confocal laser microscope (BioRad Radiance 2000 confocal system coupled to an Olympus I X 70 inverted microscope) equipped with Krypton-Argon and red diode lasers were used with a triple labeling technique. The method of determining the location of surviving cells was based on the knowledge that viable and non-viable cells differ in their ability to exclude fluorescent dyes.[42] The cell membranes of dead, damaged or dying cells were penetrated by ethidium homodimer-1 to stain their nuclei red. Living cells with intact plasma membranes and active cytoplasm metabolize calcein AM and showed green fluorescence.

Statistical Analysis: All statistical analyses were performed using a computer software program (SAS 9.1, SAS institute, Cary, North Carolina). One way ANOVA and paired student *t* tests were performed to determine differences among treatment groups with respect to each assay at each collection time. When significant differences among groups were detected, an all pair-wise multiple comparison (Tukey test) was performed. Differences compared to day 0 controls with respect to each assay at different collection times were analyzed similarly. Significance was established at $p < 0.05$.

Results:

Tissue hydroxyproline and GAG content: A significant ($P < 0.045$) decrease was observed in collagen content on day 3 in the groups treated with 4 mg/ ml MPA which were either unloaded or loaded at a frequency of 1 Hz, when compared to day 3 untreated controls. On day 10, a significant ($P < 0.030$) decrease was observed in collagen content in the load only groups at frequencies of 1 Hz at both load levels

when compared to the day 3 unloaded controls, but not the day 10 unloaded controls. Whereas with the use of MPA, a significant ($P < 0.045$) decrease was seen in the 4 mg/ml group in the 2 MPa 1 Hz group and in the 6 MPa groups, a decrease was also seen in the 0.4 mg/ml group in addition to the 4 mg/ml group when compared to the unloaded controls on day 3. When these were compared to the day 10 unloaded controls a significant ($P < 0.045$) decrease was only seen in the 6 MPa 1 Hz 4 mg/ml group when compared to the untreated control and the no load 0.4 mg/ml group. Though a general trend of decrease in collagen content was observed with the addition of MPA in all groups when compared to their respective controls, this was not statistically significant ($p=0.080$. power=0.57). Figure 4. 1

Total GAG content was significantly ($P < 0.030$) decreased in the groups loaded at a frequency of 1 Hz in the load only group at day 3, but was significant only in the groups loaded at 6 MPa at day 10 when compared to the day 3 untreated controls ($P < 0.030$). In the corticosteroid only group, a significant ($P = 0.037$) decrease was seen with 4 mg/ ml of MPA on day 3 and 10 when compared to the day 3 untreated controls.

In groups where a combination of load and MPA were used, a significant ($P < 0.013$) decrease was seen at all combinations of load and MPA in the groups loaded at 1 Hz on day 3, but at day 10, in addition to these groups, all doses in the groups loaded at 6 MPa 0.1 Hz were significant ($P = 0.005$) when compared to the day 3 untreated controls. When compared to the day 10 untreated controls, although a decrease was seen in other groups, this was significant ($P = 0.030$) only at 6 MPa 1 Hz 4 mg/ ml MPA. Total GAG content was decreased in the no load groups

exposed to 0.4 mg/ml, but was significant only at 4 mg/ml of MPA (p = 0.037). The decrease in GAG content was further increased with application of load on day 3 in the groups loaded at frequencies of 1 Hz. Although a general trend towards decrease was seen in all loaded groups and the decrease was more in the groups where corticosteroids were used when compared to their respective loaded controls these were not significant at any time point. The greatest decrease was seen in the groups loaded at 6 MPa 1 Hz. Figure 4.2

Media GAG: Release of GAG to media was decreased by addition of load and corticosteroids either alone or in combination in all groups except in the load only group at 2 MPa 0.1 Hz, where a mild and insignificant increase was seen on day 3 but not on days 6 or 10. Release of GAG to media further decreased on days 6 and 10 in all groups. Release of GAG to media was decreased in all groups where a combination of MPA and load was applied on day 3 but was significant (P< 0.036) only in the groups loaded at 2 MPA and 6 MPA (0.4 and 4 mg/ml) at frequencies of 1 Hz when compared to the no load no MPA control group at 3. Release of GAG to media in these groups was further decreased on days 6 and 10 when compared to the no load no MPA control group on these day. Figure 4.3

Gene expression: The use of MPA suppressed collagen I gene expression on day 3 in all groups except the group loaded at 6 MPa 1 Hz and treated with 0.4 or 4 mg/ml of MPA which was significantly increased when compared to the no load 4 mg/ml group (P=0.008) at the same time point. A significant (P<0.041) increase at this dose level was seen when compared to all the

other groups at day 3, but not when compared to the untreated control. On day 10 gene expression for collagen I was significantly increased in the samples treated with MPA and loaded at 2 MPa 1 HZ when compared to the day 3 samples (p = 0.04 and 0.007) for the 0.4 mg/ml and 4 mg/ ml groups respectively and (p= 0.04, 0.002) respectively for the 0.4 mg/ml and 4 mg/ ml when compared to the untreated control on day 10.These groups were significantly higher (p= 0.03, 0.004) respectively for the 0.4 mg/ml and 4 mg/ ml when compared to the load only group at this load level. In the other groups an increase of about 2-3 times was seen on day 10 when compared to the day 3 samples, but this was not significant. Figure 4.4

Collagen II gene expression was decreased in a dose dependent manner in the load only groups at day 3, but was significant only in the group loaded at 6 MPa 1 Hz (p = 0.0143). In the group loaded at 2 MPa with a similar frequency this was not significant (p = 0.0546) when compared to the day 3 control. A sharp but insignificant decrease was seen in the MPA only groups at doses of 0.4 mg/ml (p = 0.0596) and a significant (p=0.0120) decrease was seen in the groups exposed to 4 mg/ ml of MPA at day 3. In groups where a combination of load and MPA were used a significant decrease was seen in all groups at doses of 4 mg/ ml of MPA and also at 0.4 mg/ ml of MPA at 6 MPa on day 3 (P<0.036). By day 10 a significant (P<0.045) decrease was seen in all groups where MPA and load were used. The addition of load at 1 Hz in samples treated with 4 mg/ ml of MPA significantly (P<0.036) decreased collagen II gene expression by day 10 when compared to the day 3 unloaded sample exposed to the same

concentration of MPA. A significant (0.021) decrease was seen in the group loaded at 6 MPa 0.1 Hz when MPA was added at any concentration on day 3 but not at day 10. Figure 4.5

Though no significant differences were found in collagen X gene expression between any of the groups, an initial dose dependent decrease was seen in all groups treated with MPA except the 6 MPA 1 Hz group where an increase was seen on day 3. On day 10 this trend was reversed and a dose dependent increase was observed in the MPA treated groups in all groups except the 6 MPa 1 Hz group. The maximum increase was seen in the 2 MPa 1 Hz 4 mg/ ml group. Figure 4.6

Aggrecan gene expression was significantly ($P<0.021$) decreased in all groups treated with MPA and load. In the load only groups a significant ($P< 0.012$) decrease was seen in the groups loaded at a frequency of 1 Hz when compared to the unloaded controls on day 3. When compared to the day 3 control a significant ($P<0.035$) decrease was seen in all groups on day 10 except the day 10 untreated controls. In the group treated at 4 mg/ ml of MPA a significant ($P<0.018$) decrease in aggrecan gene expression was seen with the addition of load on day 10 in all loading regimens with similar concentrations of MPA when compared to the day 3 and 10 no load 4 mg/ ml MPA group. Increasing the frequency and magnitude of load from 2 MPa 0.1 Hz to 2 or 6 MPa 1 Hz caused a significant ($P<0.020$) decrease in aggrecan gene expression

on day 3. A significant ($P<0.033$) decrease in aggrecan gene expression was also seen in the 2 MPa 0.1 Hz group at day 10 with or without the addition of MPA when compared to the day 3 load only group with a similar loading regimen. In the loaded groups on day 10 doses of MPA further decreased aggrecan gene expression. Figure 4.7

MMP 1 gene expression was significantly ($P< 0.034$) increased in the groups loaded at 6 MPa 1 Hz at both doses of MPA on day 3 when compared to the no treatment group. These groups were also significantly ($P<0.031$) higher than their corresponding no load groups exposed to similar levels of MPA. When compared to the no treatment group on day 10 a significant increase was seen in the groups treated with MPA and were not subjected to load ($P<0.031$). In the groups where a combination of load and MPA were used the levels were above the untreated control group, but were below the MPA only group at frequencies of 0.1 Hz. Where as at frequencies of 1 Hz this increase was more pronounced irrespective of the load applied, although it was slightly higher in the 6 MPa group. The levels returned to normal in groups treated with 0.4 mg/ml of MPA except in the group where no load was applied, but remained elevated in the groups treated with 4 mg/ml MPA even at day 10. Figure 4.8.

Gene expression of MMP 13 gradually increased and was dependent on dose, load and frequency. A significant increase was seen ($P<0.010$) in the groups loaded at frequencies of 1 Hz and exposed to doses of 4 mg/ ml of MPA at both load levels when compared to the untreated controls at day 3. When the day 3 untreated controls were compared to the day 10 MMP gene expression

levels a significant ($P<0.022$) increase was seen in all groups where a combination of load and 4 mg/ml of MPA was used except in the 6 MPa 1 Hz group. A significant ($P<0.043$) increase was seen with the addition of load of 2 MPa to groups treated with 0.4 mg/ml of MPA by day 10 when compared to the unloaded group at day 3. Although a slight increase was seen in the no load groups with the addition of MPA this was not significant at day 10 when compared to the corresponding day 3 samples. A significant ($P<0.022$) increase in MMP 13 gene expression was seen in the groups treated with 4 mg/ml MPA when compared to the unloaded group treated with a similar concentration of MPA at day 3 at loads of 6 MPa 1 Hz, and at 2 MPa 1 Hz and 6 MPa 0.1 Hz on day 10. No significant differences were seen when the corresponding day 10 unloaded sample was compared to these groups. MMP 13 gene expression was significantly ($P< 0.010$) lower in the 6 MPa 1 Hz group at day 10 when compared to the 2 MPa 0.1 Hz group and also when compared to its corresponding sample from day 3. A significant ($P<0.043$) increase was also seen in the samples loaded at 2 MPa 1 Hz and 6 MPa 0.1 Hz and treated with 0.4 and 4 mg/ ml of MPA when compared to the untreated controls on day 10. Such an increase was also seen on day 10 in the groups treated with 0.4 mg/ml of MPA and loaded at 2 MPa when compared to the corresponding unloaded sample at day 10. (Table 4.9.B). Figure 4.9.

Expression of TIMP 1 gene was generally reduced at day 10 when compared to day 3 in all groups but was significant only in the no treatment group ($P<0.018$). In the only load groups TIMP-1 gene expression was maintained to

levels of the control group at day 3 in the groups loaded at 2 MPa, but a significant ($P<0.022$) decrease was seen at day 10 in these as well as the 6 MPa groups. In groups where a combination of load and corticosteroids was used an immediate decrease was seen across all groups on day 3 when compared to the control group on day 3 but was significant ($P<0.030$) at a frequency of 1 Hz at both load levels and at 0.1 Hz in the 6 MPa group . MPA seemed to cause generalized inhibition of TIMP 1 gene expression irrespective of the dose although a slightly greater decrease was seen with doses of 4 mg/ml this was not significant. Also a combination of load and MPA caused decreased TIMP 1 expression in all groups when compared to load or MPA alone and was significantly ($P<0.035$) different at day 10, thus indicating a synergistic effect.. Figure 4.10

Cox 2 gene expression was elevated in all groups loaded at 1 Hz by day 10 except in the group loaded at 6 MPa 1 Hz, in this group an increase was seen on day 3 followed by a decrease at day 10, this increase was dependent on the dose of MPA used. The maximum increase in COX 2 gene expression was seen in the 6 MPa 1 Hz no MPA group on day 3 and although it was decreased by day 10 the significance ($P< 0.041$) varied when compared to the no load controls and the groups loaded at 0.1 Hz. Cox 2 gene expression was highest within all groups on day 10 in the samples exposed to 4 mg/ml of MPA. Figure 4.11

PGE $_2$ Assay: PGE $_2$ content in the media decreased with dose of corticosteroids used when compared to the respective controls in each group. A significant (P< 0.021) increase in PGE $_2$ content was seen with increasing frequency and load in all groups but was more dependent on frequency. PGE $_2$ content decreased over time in all samples. Although the PGE $_2$ content in media decreased over time in the loaded groups these levels remained above the unloaded controls at all time points. Figure 4.12.

Media NO assay: Release of NO to the media was inhibited by MPA in all groups in a dose dependent manner. A significant (P<0.037) increase in media NO content was seen in all groups loaded at 6 MPa and though increased in the 2 MPa group the levels this was not significant. Figure 4.13

Cell viability assay: Cell viability decreased progressively in a dose dependent manner. A significant (P<0.030) decrease in cell viability was seen in all groups treated with MPA on day 10. On day 3 a significant (P<0.030) decrease was seen in the no load group and all groups where a combination of MPA and load above 2 MPa 1 Hz was used. In the load only groups a significant decrease in cell viability was seen in the 1 Hz groups at both time point s and the 6 MPa 0.1 Hz group on day 10 when compared to the untreated control. Increasing the frequency significantly decreased cell viability in the 2 MPa group. Figure 4. 14.

Discussion:

In this study, we had the opportunity to study the effects of corticosteroids, with and without load, on articular cartilage explants in culture. Corticosteroids are used extensively in the equine industry not only for conditions affecting the joints but also for other inflammatory conditions in horses. Since corticosteroids have both local and systemic effects, it is vitally important to understand their effects on chondrocyte viability and ECM metabolism for ethical use of these products in clinical veterinary medicine. The fact that equine joints constantly experience loads of varying degrees should also be considered as joint loading has been shown to have both beneficial and detrimental effects. [24,25] The data from this study delineate the negative effects of corticosteroids with and without load, on cartilage.

Chondrocytes are the only living component of healthy articular cartilage [26] and produce functional matrix components including collagens and proteoglycans. Thus chondrocyte viability is critical to cartilage health and joint function. Our data are in agreement with previously reported studies where corticosteroids have been shown to cause chondrocyte cytotoxicity, hypocellularity and necrosis. [9,27,28] A significant decrease in chondrocyte viability was seen in groups treated with 4 mg/ml of MPA. Decreases in chondrocyte viability at doses as low as 2 mg/ml of MPA [29] have been previously reported and thus decreased viability at 4 mg/ml MPA would be expected. Evidence suggests that corticosteroid treatment causes chondrocyte death through apoptosis.[30] Apoptosis may have been the principal cause of cell death observed in

corticosteroid alone treated cartilage in our study, while the increased cell death see in loaded samples suggests a combination of necrosis and apoptosis for loss of cell viability. Previous studies in our laboratory using canine cartilage explants revealed similar trends. [31]

To survive and function properly, chondrocytes need an attachment to either the ECM or another cell. In cartilage degradation there is extensive loss of proteoglycans and a breakdown of the collagen network, resulting in a loss of chondrocyte anchorage to the ECM.[32] This could also be an explanation for the cell death observed in our study as we did observe a decrease in proteoglycan and collagen content in our samples. Further investigation needs to be done to investigate if cell death is due to apoptosis or necrosis or both and if it precedes ECM degradation of is a sequel of this process.

Proteoglycans are an important component in cartilage health and are responsible for maintaining the hydration of the cartilage as well as resistance to compressive loads. Proteoglycans and collagens act together in maintaining cartilage hydration in that the negatively charged proteoglycans attract water into the cartilage, and the collagen network prevents the expansion of the cartilage to a maximum of 20% of its volume.[33] Cartilage swelling indicates a breakdown of the collagen network.[34,33] We noticed an increase in the size of the tissues in some of the loaded groups during sample collection when compared to the unloaded controls. Cartilage swelling has been reported in a range of studies looking at mechanical trauma/forces. [33,35] Corticosteroids are known to suppress

collagen synthesis [28] and this could exacerbate the effects of load leading to increased collagen degradation. An increase in tissue hydration following corticosteroids has been reported and this breakdown in collagen network could be the cause of the increased tissue water content. [9]

A decrease in proteoglycan synthesis and an increase in degradation have been previously reported. [13] In this study, doses of 0.1 and 1 mg of methylprednisolone depressed proteoglycan synthesis, but they did see an increase in proteoglycan synthesis 2 days after methylprednisolone was removed. We would not have observed such an increase because our explants were constantly exposed to methylprednisolone. In the same study they reported a significant decrease in proteoglycan synthesis at 10 mg/ml which failed to recover even after 13 days in culture. In another study doses of as little as 1 mg/ml reduced proteoglycan synthesis 49 % after 262-266 hrs following exposure to 1 mg/ml of MPS for 20 hrs.[15] Todhunter *et al*,. reported a severe depression in proteoglycan synthesis with doses of 0.4 and 4.0 mg/ml of MPA, which is closer to our study. [6] Although we did not see significant decrease with a dose of 0.4 mg/ml we did see a significant decrease with 4 mg/ml MPa.

The results from the GAG assay correspond to our aggrecan gene expression results wherein we observed a significant decrease in GAG and aggrecan when exposed to MPA and loading frequencies of 1 Hz. The ability of MPA to suppress proteoglycan synthesis is important as a decrease in total tissue proteoglycan results in decreased compressibility of the cartilage.

Proteoglycans attract water into the cartilage to maintain its hydration and compressibility.

One would expect to see an increase in proteoglycan loss to the media, but in our study we actually saw a decrease of release corresponding to a decrease in total proteoglycan in tissue. A possible reason could be due to decreased synthesis due to increased cell death. Also in these samples the aggrecan gene expression was suppressed which could be another reason for the decrease in proteoglycan synthesis. Another likely cause could be that the size of our explants was 3 mm and the amounts we detected in our media were at the outer limits of detection by our instrument.

A decrease in total collagen could indicate either suppression of synthesis or increased degradation. It is possible the decrease in total collagen observed in our samples could be a result of both. We observed a decrease in collagen II gene expression which is the principal collagen in cartilage which could indicate a decrease in synthesis. The simultaneous increase in MMPs could lead to increased degradation.

Collagen type II plays an important role in maintaining the tensile strength of cartilage and any defects will affect the ability of cartilage to bear load. We 0bserved a decrease in collagen II gene expression in our samples with doses of as little as 0.4 mg/ml of MPA. Our data is in agreement with other studies where a decrease in type II procollagen with concentrations of 10^5 pg/ml was reorted.[28]

Robion et al., reported an inhibition of procollagen II synthesis by repeated injections of MPA.[36]

The increase in collagen I and X gene expression is interesting and to the authors knowledge has not been previously reported. Although we did not see an increase in gene expression of collagen I and X at day 3 except in the groups loaded at 6 MPa 1 Hz and treated with corticosteroids, an increase was seen in all the other groups at day 10 when compared to the no load no corticosteroid group and the respective untreated groups in each loading regimen. Type I and X collagen are thought to be produced by hypertrophic chondrocytes.[37] The presence of COL I and X in our control samples could probably be due to the fact that our samples were collected from yearlings which are still growing. The simultaneous increase of MMP 13, which has also been shown to be produced by hypertrophic chondrocytes,[38-40] could indicate a shift in chondrocyte morphology. Corticosteroids have been shown to alter chondrocyte phenotype [28] and it is possible the expression of Type I collagen and MMP 13 in corticosteroid treated groups could be due to corticosteroid therapy. Further, the increase observed on day 3 in the 6 MPA 1 Hz groups exposed to corticosteroids indicates a synergistic effect of these factors in combination, and the subsequent decrease seen in these groups on day 10 was most likely due to cell death. In support of an earlier *in vivo* study using rats receiving corticosteroids and subjected to running exercise, fibrotic invasion and subchondral bone replacement of degenerated articular cartilage associated with areas of cell death, and loss of matrix staining was observed when compared to rats that

received running exercise or corticosteroids alone. [12] Another study comparing MPA and hyaluronate (HA) in osteoarthritic cases found that patients receiving HA showed a better response when compared to the MPA group. [41] Shoemaker *et.al*., reported that cartilage from horses treated with MPA had morphologic changes, which included chondrocyte cluster formation, loss of architecture, and cell necrosis.

The half life of MPA in synovial fluid has been estimated to be 10.3 hours.[42,43] If 80 mg of MPA (40 mg/ml) was injected into a midcarpal joint and that joint typically contains 6 mls of synovial fluid, the initial concentration of the drug would be 12.5 mg/ml (33mM). This concentration would decrease to 6.25mg /ml (16.5 mM) after 10.3 hrs and further decrease over time. Literature suggests that the concentrations would remain above 2 mM for more than 36 hrs.[28] In our model, the explants were exposed to concentrations of 0.4 mg/ml (1mM) and 4 mg/ml (10 mM) for a period of 10 days. Furthermore, methylprednisolone, which is the active form of MPA,[44] has been detected in samples taken from the joint for as long as 39 days after intra articular administration and MPA has been detected for 6 days after administration in horses.[42] This proves that despite a single shot of MPA, concentrations would be maintained at some level for a long time and thus repeated treatment with MPA could result in some of the changes seen in our study. Such a situation may not arise in a living horse as the pharmacokinetics would be different in an actual joint. However, the changes in collagen I and X, and MMP 1 and13 gene expression observed in our study would be something to consider as these

changes could indicate a shift towards hypertrophic chondrocytes. It is possible that this shift may have occurred and is a possible reason for the changes reported in other studies.[9,11,12,14]

Another interesting finding was the increase in MMP 1 and 13 gene expression in the MPA treated groups. Though various studies indicate that corticosteroids decrease MMP synthesis in cartilage and chondrocytes, most of these studies were done using affected cartilage. Although one study used normal chondrocytes the culture period in this study was one day.[45] The fact that corticosteroids seemed to have beneficial effects at day 3 when compared to their respective untreated groups, but caused an increase at day 10 needs to be further investigated. It also needs to be investigated if the changes seen in the gene expression pattern also translate into protein activity or are a compensatory mechanism by the tissue. MMP1 has also been reported to be present in hypertrophic chondrocytes. [46,47]

Various studies have reported the decrease in synthesis and increase in degradation of proteoglycans with the use of corticosteroids.[6,28] It is generally accepted that MMPs 1 and to some extent 13 [48] play a role in proteoglycan breakdown. It is possible that the sudden surge in MMP1 and 13 could be responsible for the increased proteoglycan degradation.

TIMPs are the natural inhibitors of MMPs and an imbalance between TIMPs and MMPs in affected cartilage has been suggested.[31] We observed

significant changes in TIMP 1 gene expression with the addition of MPA and load. Previous reports have suggested corticosteroids can suppress TIMP.[49] This phenomenon needs further investigation as an imbalance in MMPs and TIMPs in the tissue can damage the tissue.

NO and PGE 2 release to the media decreased with increasing dose. This data is in agreement with various other studies where a similar decrease was observed with the use of corticosteroids.[50] However, this data does not agree with COX 2 gene expression. COX-2 is highly inducible by a number of stimuli including cytokines and is associated with inflammation.[51] Although various reports have suggested the inhibitory effects of corticosteroids on COX 2, we found a slight but insignificant increase in COX 2 gene expression in samples on day 10 which was dependent on dose and load. Although a dose dependent decrease was seen on day 3. This shift in COX 2 expression needs to be further investigated. Previous studies have reported decreased healing and synovial inflammation after prolonged use of corticosteroids.[16] The increase in COX 2 gene expression could indicate the start of an inflammatory process or the beginning of chondrocyte induced arthropathy.

In our study, cartilage was exposed to corticosteroids for 10 days. Such a situation would not be likely to arise in a living animal. Another consideration is that in our model, a 3 mm tissue was exposed to a supraphysiologic amount of MPA and load, but our goal was to see the effects of long term corticosteroid treatment in horses. As this increase was seen only at day 10 could mean that the tissue at this stage is changing its morphology, such a change has been

previously reported. [28] The cyotoxic nature of corticosteroids as seen in our study has been previously reported [28] and could be a potential cause of some of the changes seen in our study. Also the results of this study must be taken with caution given the low numbers of our samples and the weak powers which were below the desirable power of 0.800

From this study we could conclude that long term use of corticosteroids would alter cartilage metabolism. When used in combination with exercise these changes are exacerbated, decreasing the collagen and proteoglycan content of the cartilage tissue and could result in similar changes in cartilage gene expression observed after cartilage damage. These results are similar to changes previously reported in studies combining exercise and corticosteroid treatment. Further investigation is needed to determine if these changes in gene expression translate into changes in protein production and activity, as well as changes in cartilage biomechanical properties. Previous studies have reported changes in protein production and cartilage tissue matrix composition similar to the gene expression and biochemical data obtained in this study.[9,11,12]

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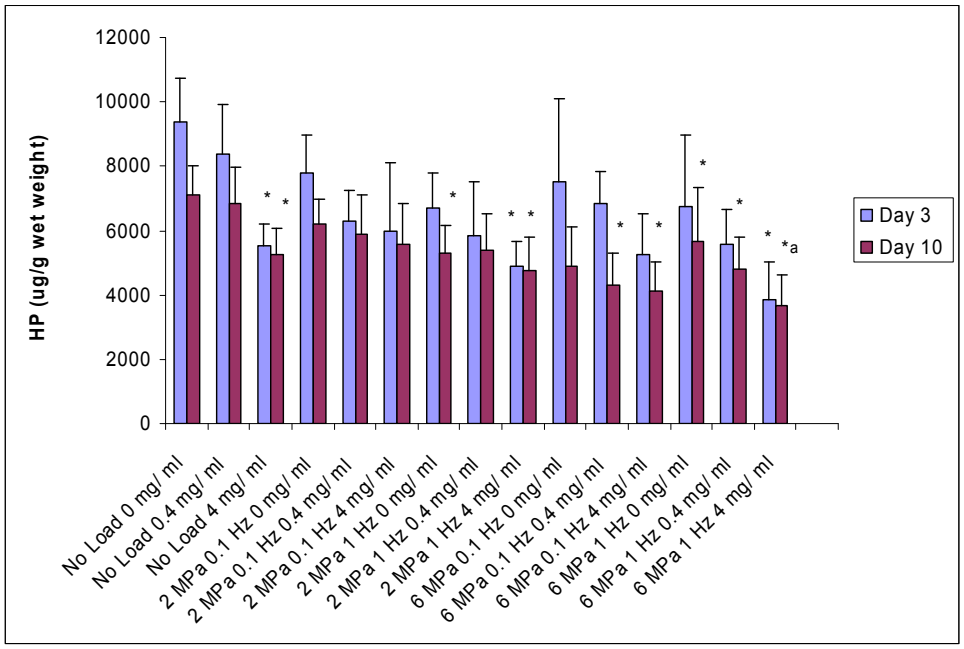
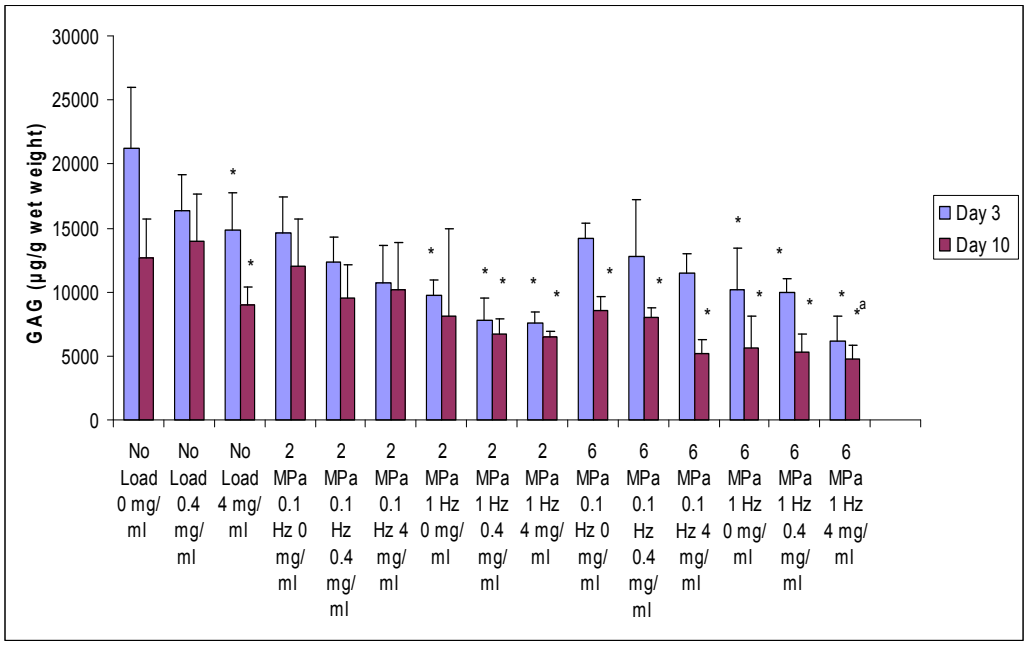
Figure 4. 1 Total tissue collagen content



* significantly different form no load 0 mg/ ml day 3.

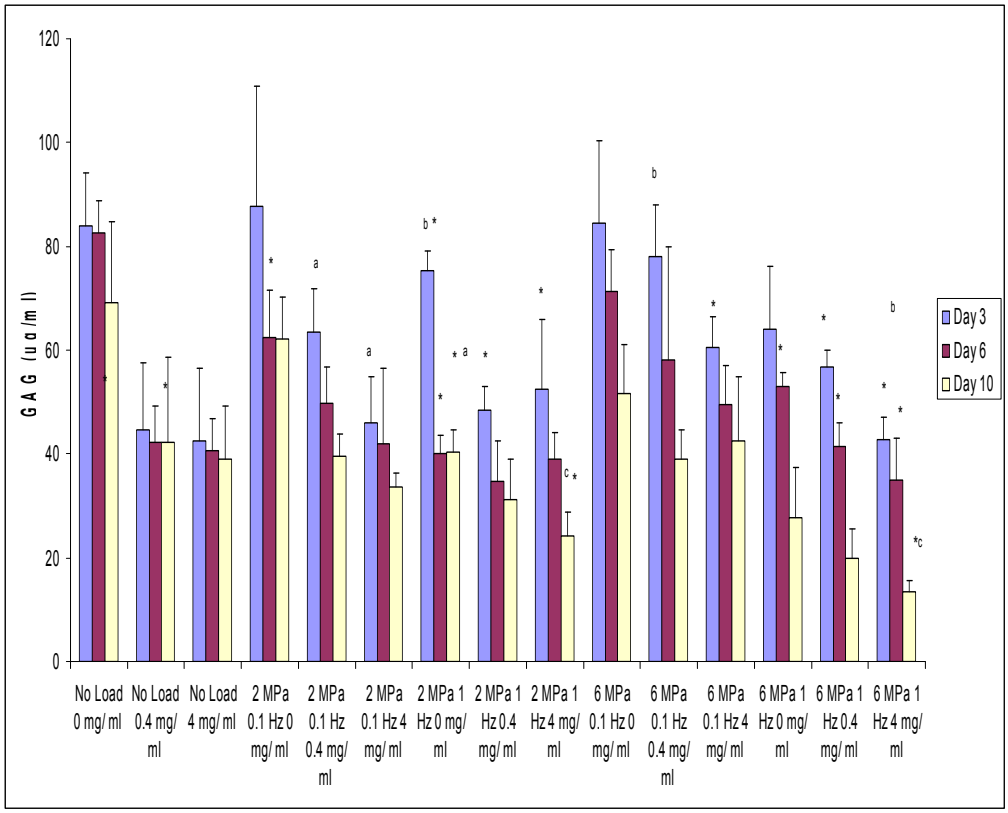
a significantly different form no load 0 mg/ ml day 10

Figure 4.2 Total tissue glycosaminoglycan content



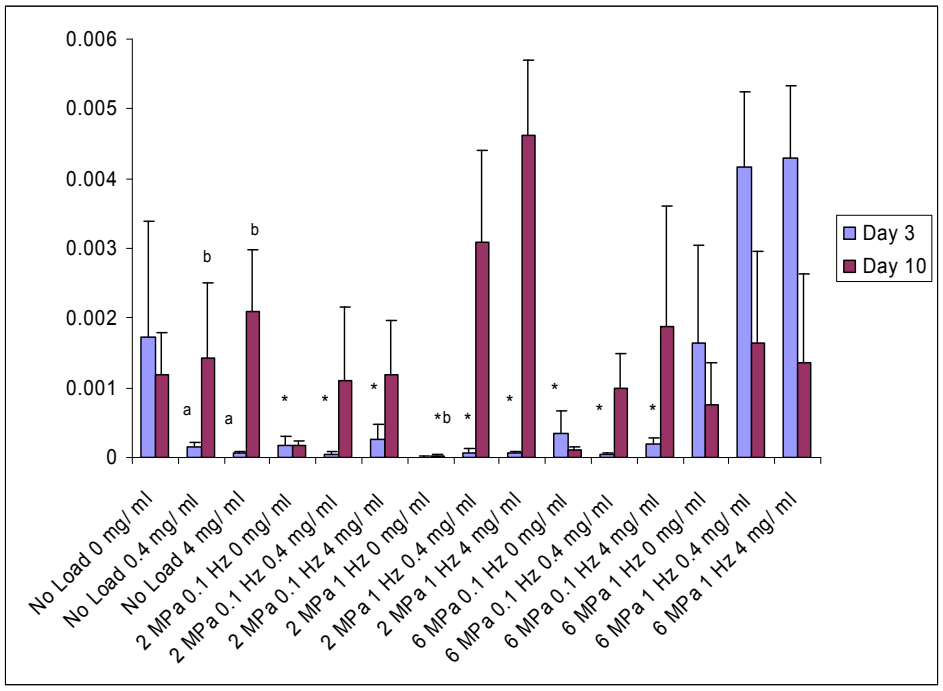
* Significantly different form no load 0 mg/ ml day 3.
a Significantly different from no load 0 mg/ ml day 10.

Figure 4.3 Media GAG content.



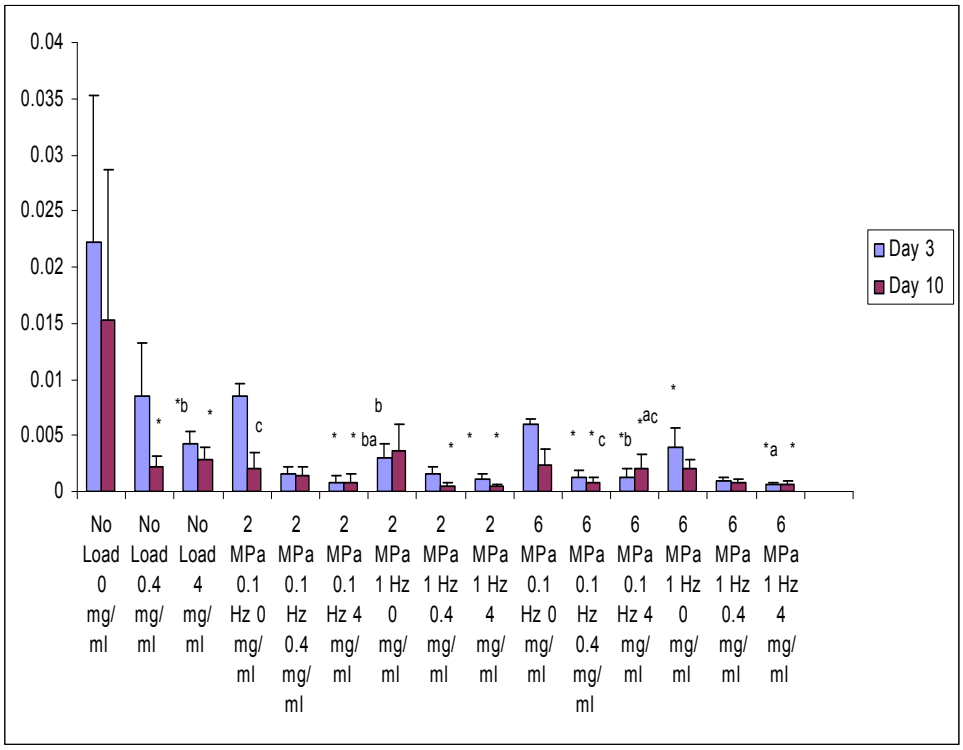
* Significantly different from corresponding no load 0 mg/ ml.
a Significantly different from corresponding no MPA group.
b Significant differences between days
c significantly different from corresponding no load group.

Figure 4.4 Relative gene expressions for Collagen I



* Significantly different from both 6 MPa 1 Hz 0.4 and 4 mg on day 3.
a Significantly different from only 6 MPa 1 Hz 4 mg on day 3.
b Significantly different from both 2 MPa 1 Hz 0.4 and 4 mg on day 10.

Figure 4.5 Relative gene expression collagen II



* significantly different from untreated control day 3.
a significantly different form no load 4 mg/ ml day 3.
b significantly different from 2 MPa 0.1 Hz 0 mg/ ml day 3.
c significantly different from its corresponding no MPA group.

Fig 4.6 Relative gene expression for collagen X

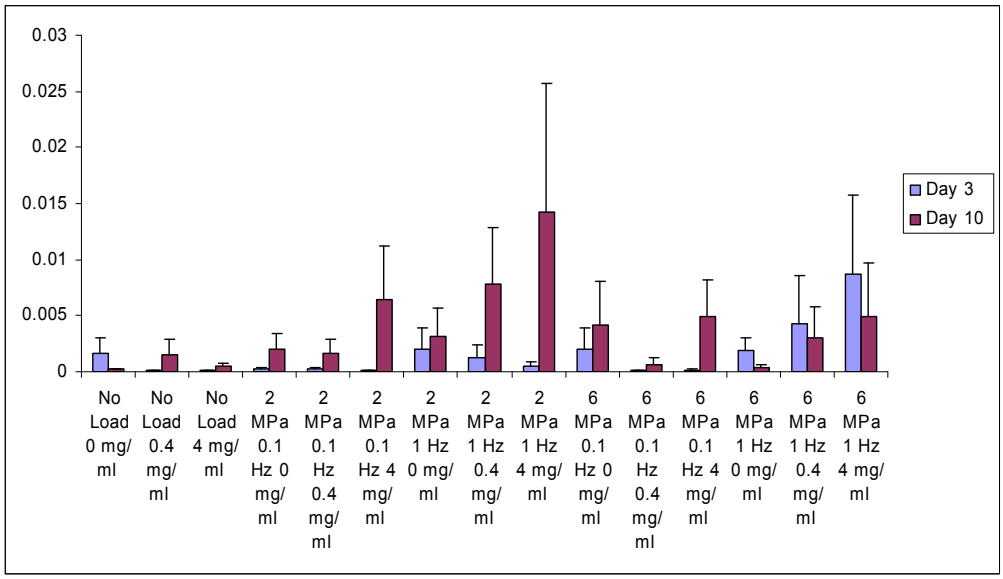
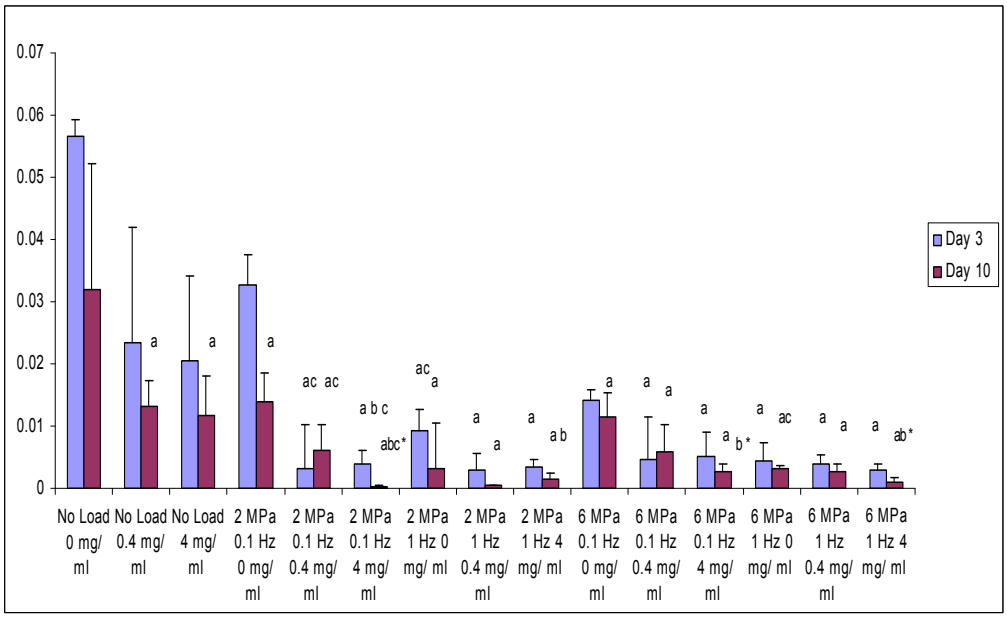
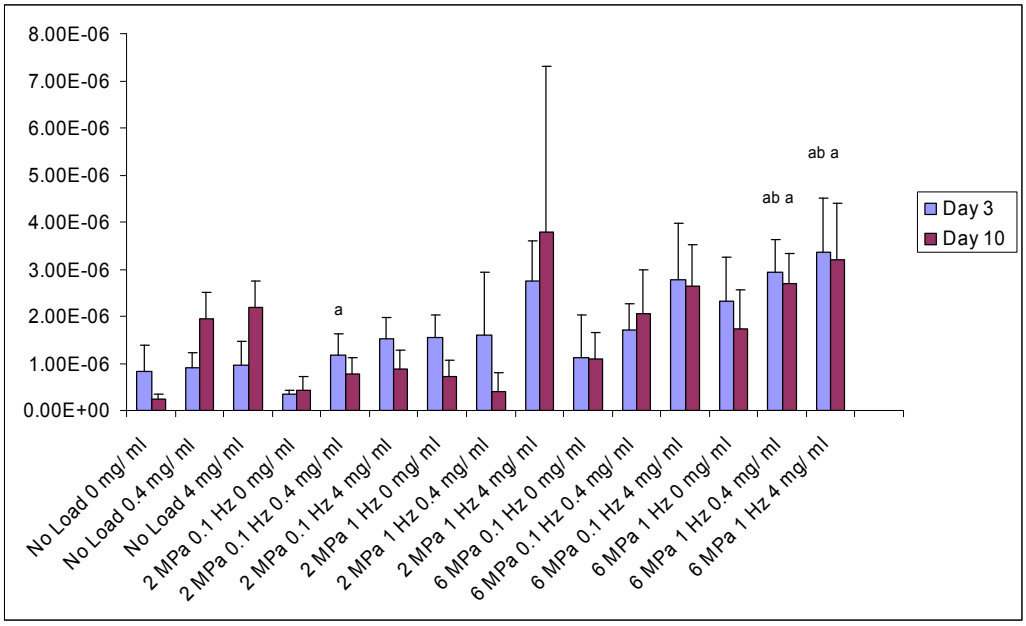


Figure 4.7 Relative gene expression Aggrecan



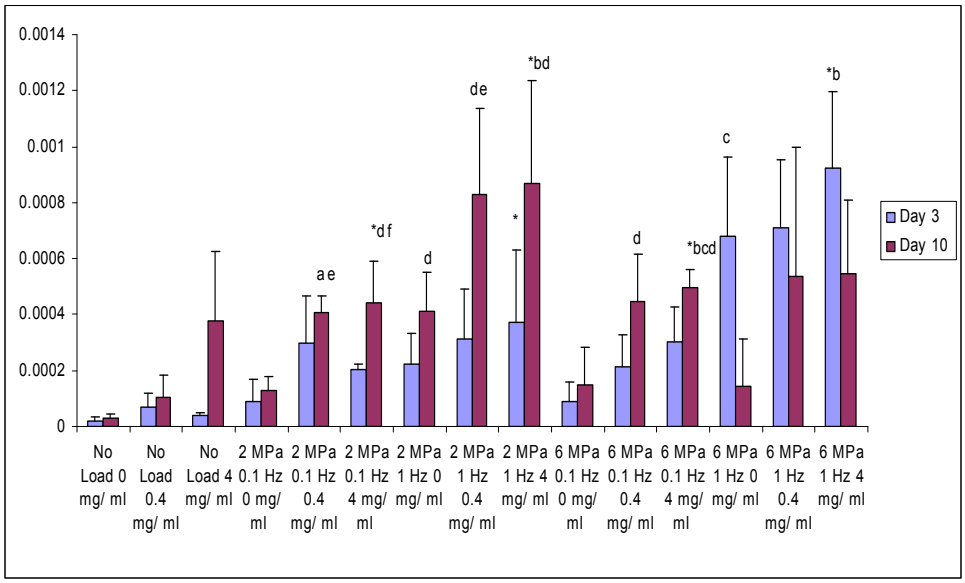
a significantly different from day 3 no load
b significantly different form no load day 3 4 mg/ ml
c significantly different form 2 MPa 0.1 Hz 0 mg day 3.
* significantly different form the corresponding no MPA loaded group.

Figure 4.8 Relative gene expression for MMP 1.



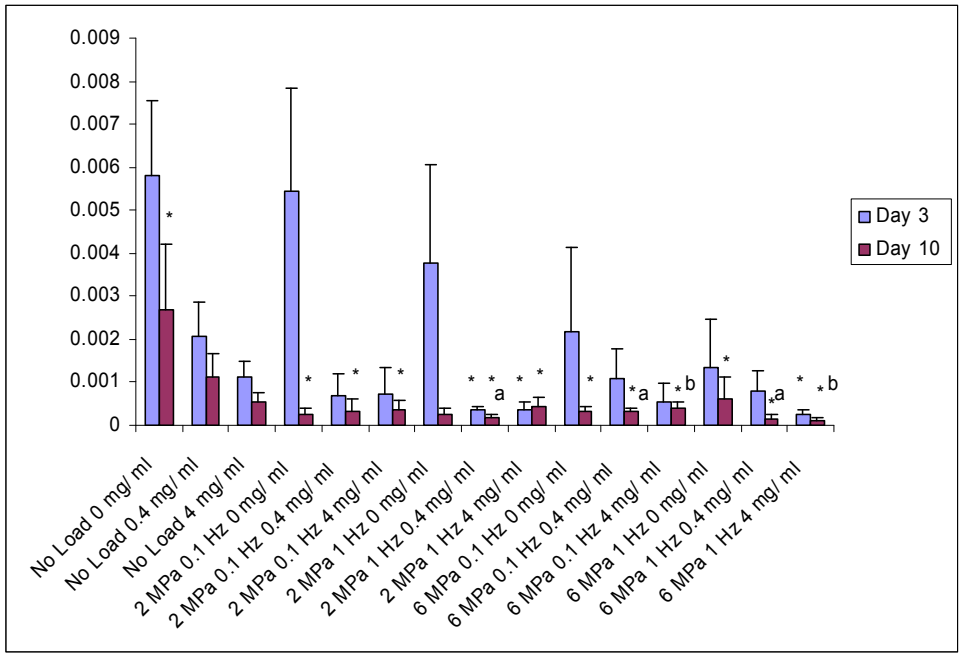
a Sigificantly higher than corresponding no load 0 mg/ ml.
b Sigificantly higher than corresponding no load sample.

Fig 4.9 Relative gene expression for MMP 13



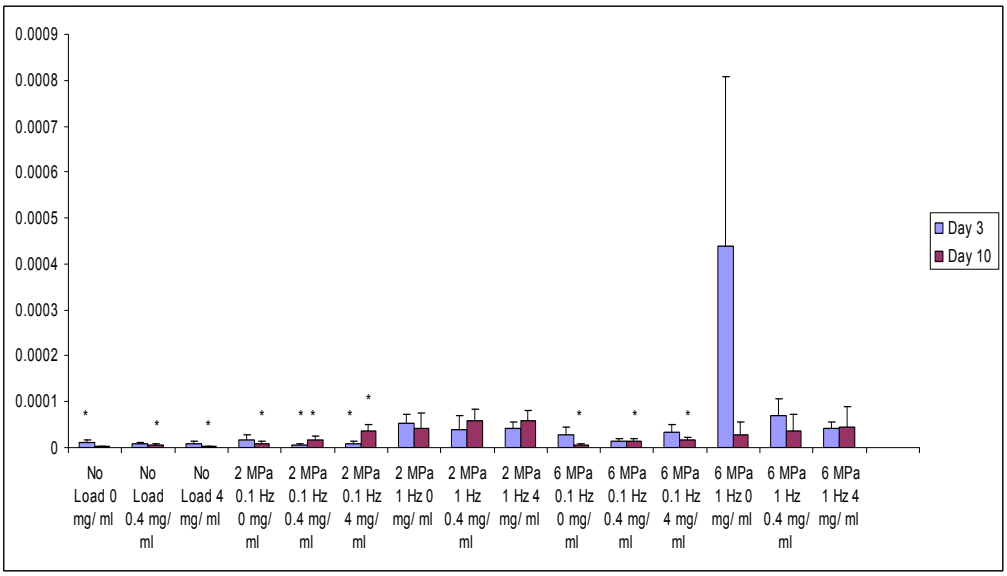
* significantly different from no load 0 mg/ ml day 3
a significantly different from no load 0.4 mg/ ml day 3
b significantly different from no load 4 mg/ ml day 3
c significantly different from 6 MPa 0.1 Hz 0 mg/ ml day 3
d significantly different from no load 0 mg/ ml day 10
e significantly different from no load 0.4 mg/ ml day 10
f significantly different from 2 MPa 0.1 Hz 0 mg/ ml day 3.

Fig 4.10 Relative gene expression for TIMP 1



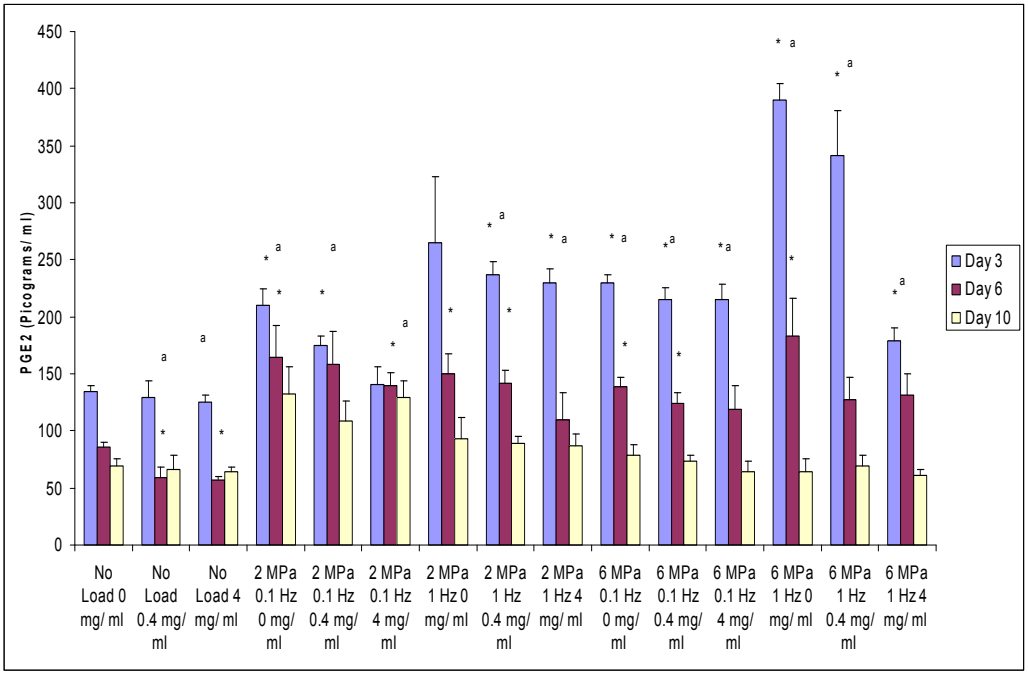
* significantly different form no load 0 mg/ ml day 3.
a significantly different form no load 0.4 mg/ ml day 3.
b significantly different from no load 4 mg/ ml day 3.

Figure 4.11 Relative gene expression for COX 2



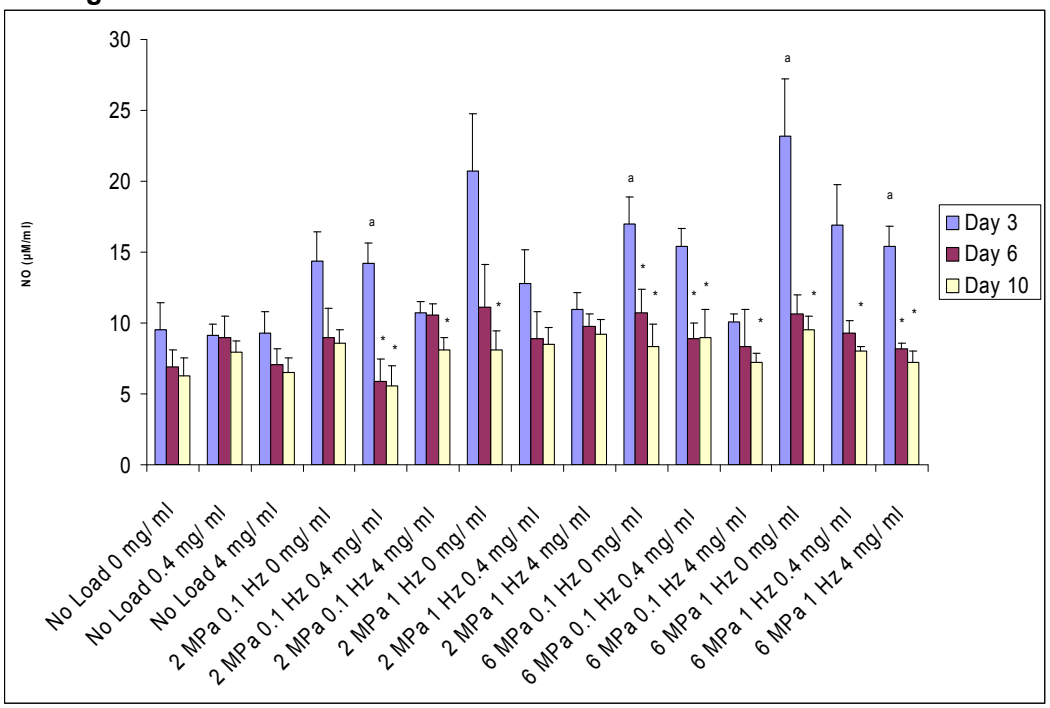
* Significantly different from 6 MPa 1 Hz 0 mg/ ml day 3

Figure 4. 12 Media PGE_2



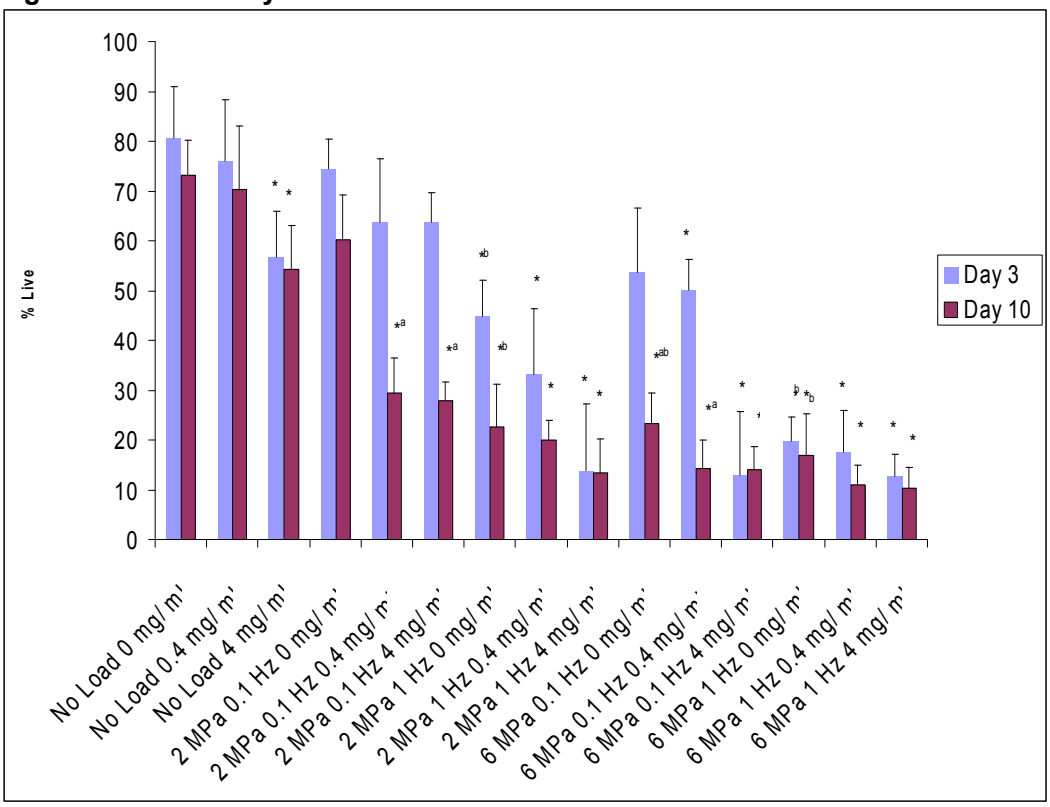
* Significantly different from corresponding no treatment sample
a Significant differences between days.

Fig 4. 13 Media NO content.



* Significantly different from corresponding day 3 sample
a Significantly different from corresponding no load sample.

Fig. 4.14 Cell viability



* Significantly lower than corresponding no treatment
a Significantly lower than corresponding day 3
b Significantly lower than corresponding 2 MPa 0.1 Hz.

An in vitro model of osteoarthritis a comparison of recombinant human and equine IL-1β with compressive loads.

Osteoarthritis is a complex interactive, degradative and repair process of cartilage, bone and synovium characterized by progressive and irreversible articular cartilage extracellular matrix (ECM) degeneration .[1-5]

OA is a major problem in both animals and humans. It is believed that some form of arthritis affects more than 70 million people (1 in 3 adults) in the United States and up to 90% of individuals older than 65 years of age.[6] The cost of arthritis is thought to exceed $90 billion dollars annually in humans. In horses lameness is estimated to cost 1.4 billion dollars annually.[7] A survey of racing thoroughbreds showed that 55% were lame at some point of time during their two to three year old careers, many due to problems associated with the joint.[8] In 20% of these cases, the problem was of sufficient severity to prevent further training.[8] Traditionally, OA has been thought to be caused by wear and tear resulting in noninflammatory arthrosis. It is now increasingly being seen as a mechanically driven, chemically mediated process, where various factors such as mechanical loads, hydrostatic pressures, and soluble mediators from the subchondral bone, synovium, synovial fluid, and cartilage influence the activity of the chondrocytes, resulting in ultimate cartilage breakdown.

Though various factors are involved in the initiation and progression of OA, it has been capsulized as “the application of abnormal stress to normal cartilage or the application of normal stress to abnormal cartilage”.[9,10] Biomechanical stress plays a

major role in cartilage health by enhancing fluid movement into and out of the cartilage matrix thus allowing distribution of matrix components synthesized by the chondrocytes and also nutrient/waste exchange.[11] Biomechanical forces also directly influence chondrocyte ECM through mechanotransduction.[12,13] The magnitude and frequency of loading also play an important role in influencing *in vitro* chondrocyte response. Application of relatively physiologic loads elicit an ECM sparing response by chondrocytes including decreased nitric oxide (NO) and prostaglandin E_2 (PGE_2) synthesis,[14,15]and increased glycosaminoglycan (GAG), total protein and DNA synthesis.[16] Application of supraphysiologic (excessive frequency/magnitude) loads result in increased expression by chondrocytes of matrix metalloproteinase (MMP)-1, MMP-3, MMP-9, interleukin 1β (IL- 1 β), tumor necrosis factor α, cycloxygenase-2, increased chondrocyte cell death and increased synthesis of NO and PGE_2, reduced expression of type II collagen and aggrecan, and inhibited synthesis of DNA, proteoglycan, collagen and protein thus exerting net ECM catabolic response.[17-21]

Various *in vitro* models have been based on chondrocyte monolayer, chondrocyte suspension and cartilage cultured with or without biochemical agents and or traumatic force used to initiate a simulated OA-like response.[22-24,21,25] The advantages of these *in vitro* systems over *in vivo* models are reduced cost, reduction of confounding variables, reduced biological variability, increased experimental control, the use of methodologies which are not feasible *in vivo*, their basic simplicity and above all the need for fewer animals. Although it is possible to isolate large numbers of chondrocytes from normal equine cartilage, growth in an adherent monolayer culture results in dedifferentiation to a more fibroblast-like phenotype characterized by change from round to spindle-shaped

cells, increased proliferative capacity, decreased expression of collagen type II and aggrecan and increased expression of collagen type I and III.[26,22]In contrast, chondrocytes in cartilage explant culture have low mitotic activity, maintain their differentiated phenotype, and the ECM is similar to that observed in vivo.[22] However explants isolated from different locations within a joint exhibit significant differences in biochemical characteristics [total collagen, Glycosaminoglycan (GAG) and DNA concentration] and chondrocyte ECM metabolism (aggrecan, decorin and biglycan synthesis). Therefore, the number of explants available per animal for critical evaluation is somewhat limited because of the desire to compare explants from specific sites within a specific joint.[27-30]

Current models for equine OA have ignored the role of biomechanical stress in the maintenance of chondrocyte homeostasis and the role it plays in initiation and or progression of disease. To the authors knowledge no studies have been reported describing the application of dynamic compressive load to equine cartilage explants. A similar study was done using equine chondrocytes seeded in agarose and subjected to 15% peak cyclic strain for 48 hours. In this study they found reduced NO synthesis; though differences in cell proliferation and PG synthesis were not observed.[15] Though various models have been proposed including single impact models,[25] an ideal model should accurately reflect the course of naturally occurring disease. IL-1 is known to be one of the principal cytokines involved in cartilage catabolism, [31]and is thought to play an important role in the initiation and progression of the disease. IL-1 which could be released by either the synovium or chondrocytes themselves decreases synthesis of collagens and proteogylgans and increases their degradation by increasing the

expression of metalloproteinases and decreasing cell viability due to increased cell death. [31]

Biochemical responses of cartilage in culture can be measured and the response of cells to IL-1β can be examined. IL-1β has been reported to play a central role in the pathophysiology of cartilage damage and degradation in arthritis [32] IL-1β stimulates various enzymes (particularly MMPs) that result in cartilage degradation, [33-36] and also inhibits the compensatory synthesis pathways used by chondrocytes to restore the integrity of the degraded ECM.[37] IL-1 injected one time into mouse joints produced only mild inflammation, but resulted in substantial inhibition of proteoglycan synthesis and enhanced breakdown. Repeated injections however had very severe degradative effects on the cartilage and produced profound inflammation.[38,39] An increase in release of IL-1β in osteoarthritic cartilage has also been reported. [32]

Most studies use evaluating the effects of IL-1β on articular cartilage have used rh IL-1β., [40,41] more recently, re IL-1β has been cloned and used in evaluating its effects on articular cartilage.[42-44]In a study done by Tung et al.,[45] where chondrocyte and explant cultures were exposed to various doses of equine and human IL-1β, the results indicated a apparent increase in the gene expression of MMPs 1, 3,13, and Cox 2 and an increase in the release of NO, MMP 3, and 13 to the media of re IL-1β treated groups when compared to the rh IL-1β group indicating a potential species specific response. The main advantage of using re IL-1β on equine tissues and cells would be that re IL-1β would be more effective in smaller doses than rh IL-1β; however the lack of commercial availability of re IL-1β limits its use to only a few researchers. It is therefore essential to compare the differential effect of re and rh IL-1β on equine tissues and

identify specific dose regimens that produce similar effects for the study of orthopaedic disease.

In naturally occurring OA, the cartilage matrix molecules are exposed to both IL-1 β and load during movement. Therefore, in an *in vitro* model of OA, it is important to incorporate these factors into the model. This study was designed to develop an *in vitro* model to better mimic the initiation and progression of OA in the living horse. The primary goal of this study was to develop an *in vitro* model of equine OA based on cultured cartilage subjected to dynamic compressive loads and stimulated with IL-1β.

Hypothesis: We hypothesized that 1)subjecting articular cartilage to dynamic compressive loads will influence chondrocyte gene expression, tissue PGE_2 and NO production, and ECM metabolism, and these effects will be related to the magnitude and frequency of applied load, 2) subjecting articular cartilage to IL-1β will influence chondrocyte gene expression, tissue PGE_2 and NO production, and ECM metabolism and these effects will be related to the concentration of IL-1β, 3) equine recombinant (re) IL-1β will be more effective than human recombinant (rh) IL-1β at producing degradative changes in equine articular cartilage.

Our **overall goal** was to develop an *in vitro* model for the study of equine OA. The following experiments were conducted to achieve this goal; 1) determine the effect of IL-1β on articular cartilage subjected to dynamic compressive load compared to unloaded and untreated controls, 2) determine the optimum loading frequency needed to initiate OA like changes, 3) determine the effect of various

doses of IL-1β on loaded and unloaded articular cartilage, and 4) determine the effects of various doses of re IL-1β to rh IL-1β on loaded and unloaded articular cartilage.

Materials and methods.

Equine articular cartilage was collected aseptically within 24 hrs of death from grossly normal femoropatellar joints with grossly normal articular cartilage of 6 adult horses that died or were euthanized for reasons unrelated to joint disorders. The cartilage was collected from the medial and lateral trochlear ridges to avoid biochemical and ECM metabolism variations in cartilage due to weight bearing. Cartilage explants were prepared, cultured, and subjected to treatment with rh IL-1β, re IL-1β and dynamic compressive stress after their formation. Twenty five treatment groups (Table 5.1.1) were formed based on concentration of IL-1β { (re 0,10 ng/ml and 100 ng/ml)and (rh 100 and 200 ng/ml)} as well as, level (0, 2 MPa and 6 MPa) and frequency (0.1, and 1 Hz) of sinusoidal peak dynamic compressive stress . Three explants per horse were cultured in each treatment group for either 3 or 10 days At the end of testing, 1 explant each will be subjected to evaluation as follows: 1 explant was subjected to digestion for sulfated glycosaminoglycan (GAG), and hydroxyproline; 1 explant was processed for PCR analysis; and 1 explant was prepared for cell viability assay. The media from each explant was collected at the time of each media replacement and explants harvested and stored at -20^0C for subsequent biochemical analysis for PGE_2 and NO.

Selection of doses of rh IL-1β was based on data from our laboratory [31] where dose levels of 100 ng/ml significantly increased gene expression of MMP 13 and TIMP 1 and 2, and 200 ng/ ml of IL-1β caused a significant decrease in GAG content in canine cartilage. The dose of re IL-1β was based on the results of previous studies. In an earlier study done on equine chondrocytes and explant cultures using re IL-1β,[46] an increase in gene expression was found for MMP 1, MMP 3, MMP 13, TIMP 1, and COX 2 and saturation was reached at 10ng/ml with no significant increase from 10 to 100 ng/ml. The selection of load values was to some extent empirical. The compression bioreactor in our laboratory is configured to deliver defined forces up to 14 lbs and applied stress is calculated (1 MPa = 145.038 psi). The daily contact stress on human joints ranges from approximately 3 to 10 MPa.[47] Although no data is available for the stresses on equine stifle joints, the stress in the equine distal interhalangeal joint at a trot has been estimated to be as high as 5 MPa.[48]

Cartilage explants: – Near full thickness articular cartilage were aseptically removed from the femoropatellar joints of 6 horses aged less than 2 years within 24 hours of death at the University of Missouri Veterinary Medical Teaching Hospital. Cartilage slices were washed in Earle's balanced salt solution (EBSS) and transferred to tissue culture dishes containing Dulbeco's Modified Eagle Media (DMEM). Tissue samples were cut into cylindrical plugs using sterile 3 mm diameter skin biopsy punches and cultured in DMEM with 10 % fetal bovine serum (FBS), 4mM L- glutamine, penicillin (57 U/ml), streptomycin (57 µg/ml) and ascorbic acid (50 ug/ml), and pH adjusted to 7.2 to

which was added re IL-1β (10 or 100 ng/ ml) or rh IL-1β (100 or 200 ng/ml). The media was replenished on days 3, 6 and 10.

Application of cyclic compression: - Explants were subjected to cyclic compressive load using FX- 400 C^{TM} Flexercell Compression Plus unit (Flexcell international, Hillsborough, North Carolina). Cyclic compression driven by air pressure was monitored by an inline manometer and controlled by solenoid valves using Flexsoft^{TM} software. The application of biomechanical force to the explants while in culture is enabled by flexible- bottom culture plates. Explants were subjected to cyclic compression over a range of frequencies and peak magnitudes for 20 minutes three times a day for 3 and 10 days. No load and no treatment control groups were subjected to the described culture conditions but without cyclic compressive loading and/or treatment with rh/re IL-1β.

Chondrocyte viability:- explants (~ 1.5 mm thick) were prepared with a scalpel blade and stained with ethidium homodimer-1 (13 µl/ml phosphate buffered saline (PBS)) and calcein acetoxymethylester (AM) (0.4 µl/ml PBS) fluorescent stain (LIVE/DEAD Viability/Cytotoxicity kit), Molecular Probes, Eugene, Oregon). Cell viability was determined by confocal microscopy. Sections were incubated for 30 minutes at room temperature, placed on glass slides, and moistened with several drops of PBS. A confocal laser microscope (BioRad Radiance 2000 confocal system coupled to an Olympus I X 70 inverted microscope) equipped with Krypton-Argon and red diode lasers were used with a triple labeling technique. The method of determining the location of surviving cells was based on the knowledge that viable and non-viable cells differ in

their ability to exclude fluorescent dyes.[49] The cell membranes of dead, damaged or dying cells were penetrated by ethidium homodimer-1 to stain their nuclei red. Living cells with intact plasma membranes and active cytoplasm metabolize calcein AM and showed green fluorescence.

RNA extraction:- Total RNA was extracted using a modified TRIspin method.[50] Snap frozen explant samples were powdered by crushing, transferred to 1ml of Trizol (Invitrogen, Carlsbad, CA), and homogenized using 3.2mm steel beads (BioSpec Products) and a mini-bead beater (BioSpec Products) set at 5000 rpm for 30 seconds. Homogenates will be chloroform extracted and separated by centrifugation. The RNA was precipitated using isopropanol, and the pellet will be resuspended in 100µl of DEPC water. The RNA was then further cleaned using the RNeasy Minelute cleanup kit (Qiagen Inc., Valencia, CA) following the manufactures protocol. Total RNA was eluted with 14µl of water and contaminating DNA was digested using the Turbo DNase kit (Ambion). Isolated RNA was stored at -80^0C following determination of concentration and purity.

***Reverse Transcription (RT)*:** 500 ng of total RNA was reverse transcribed in 20 µl reactions using 0.5µM of random hexamers and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For each sample a No-RT control was run in parallel to assess DNA contamination. The RT profile will be: 42^0C for 2 hours, 68^0C for 10 minutes, 4^0C hold. The cDNA was diluted

with 180µl of water, and 4µl of the diluted cDNA was used for subsequent real-time Polymerase Chain Reaction (PCR).

Polymerase Chain Reaction (PCR):- An assessment of steady state mRNA concentrations corresponding to genes of interest was made using Real-Time PCR. Primer pairs have been designed (PrimerSelect, DNASTAR, Madison WI) for amplification of the following gene sequences: collagen types I and II matrix metalloproteinases (MMPs) 1 and 13, tissue inhibitor of metalloproteinases (TIMP) - 1, Aggrecan, cyclooxygenase (COX) 2 and glyceraldehyde 3- phosphate dehydrogenase (GAPDH). Real–Time PCR was performed with the Rotor-Gene RG- 3000 (Corbett Research, Sydney, Australia) using the Quantitect SYBR green PCR kit (Qiagen) following the manufacturers guidelines. The PCR profile for all tests consisted of an initial incubation of 94^0C for 15 minutes, followed by 55 cycles of 5 seconds at 94^0C, 10 seconds at 57^0C and 20 seconds at 72^0C. After the PCR profile, a melt curve analysis was done to ensure specific amplification for each sample. SYBR green fluorescence was monitored during the extension step of the PCR profile, and take off values and amplification efficiencies were determined using the Rotor–Gene software. Target gene expression were normalized to GAPDH expression and determined using Q-gene.[51] No-RT controls were tested for each primer set utilized to ensure there was no contamination genomic DNA in the sample.

Glycosaminoglycan Analysis: Total sulfated GAG was quantified using 1-9-dimethlymethylene blue (DMMB) spectrophotometeric assay.[52] Stored media and

explants were thawed and digested in solutions of 2.8 unit/ml papain (Sigma Chemical Co., St. Louis, MO). A 10 µl aliquot of the digested solution was mixed with 240 µl of DMMB solution and absorbance was determined at 525 nm spectrophotometrically (Beckman DU-65 spectrophotometer, Beckman Instruments, Inc., Fullerton, CA). A standard curve was constructed using bovine tracheal chondroitin sulfate A. The results were corrected for differences in sample weight and normalized to dry weight of the sample. Total GAG content in cartilage explants is reported as GAG/weight (µg/g). GAG concentrations in the liquid media were reported as a percentage of total GAG in the cartilage explant.

Hydroxyproline Assay: Total collagen content in cartilage explants were determined by measuring the HP content using a colorimetric procedure, as previously described.[53]-[54] HP content was reported in HP/weight (µg/g).

PGE_2 Analysis: - Total PGE_2 was determined in conditioned media by an enzyme immunoassay system (Amersham International, PLC, Buchinghamshire, England). The stored media was thawed and assayed for PGE_2 content according to the manufacturer's instructions. All samples were run in duplicate. Sample concentrations were determined by comparison with the manufacturer supplied standard curves.

NO Analysis: - Nitric oxide (NO) content in media was determined by measuring nitrite concentration, which is one of the two stable products from the breakdown of NO. Stored samples were thawed and nitrite concentrations determined using the Griess

Reaction (Promega, Madison WI) and evaluation of spectrophotometric (Beckman DU-65, Beckman Instruments, Inc., Fullerton CA) absorbance at 520-550 nm. Briefly, the standard was prepared according to the manufacturers' recommendation from the provided standard. 50 µl of sample was added to each of the test wells followed by the addition of 50 µl of sulfanilamide solution to all wells and incubation for 10 minutes. Fifty µl of N-1-naphthylethylenediamine dihydrochloride (NED) solution was then added to all wells and incubated for 10 minutes at room temperature and absorbance was read at 520-550 nm.

Statistical Analysis: - All statistical analyses was performed using a computer software program (SAS 9.1, SAS institute, Cary, North Carolina). One way ANOVA and paired student T tests were performed to determine differences among treatment groups with respect to each assay at each collection time. When significant differences among groups were detected, an all pair-wise multiple comparison (Tukey test) was performed. Differences compared to day 0 controls with respect to each assay at different collection times were analyzed similarly. Significance was established at $p < 0.05$.

Results

Hydroxyproline: Though a general decrease in hydroxyproline content was observed with increasing load, frequency and IL-1β, the differences were not significant. No significant differences were observed between re and rh treated groups. Figure 5.1

Tissue GAG: Tissue GAG content decreased with increasing dose load and frequency reaching significance (P<0.030) at frequencies of 1 Hz and IL-1β doses of 100 ng/ml and 200 ng/ml of re and rh IL-1β, respectively on day 3. Although there was a significant (P< 0.030) decrease in tissue GAG content with just IL-1β or load, combinations of either 2 or 6 MPa at 1 Hz and IL-1β doses of 100 ng/ml and 200 ng/ml of re and rh IL-1β respectively, caused the greater decrease in GAG content. A moderate increase in GAG content was observed in all groups loaded at 2 MPA 0.1 Hz with the exception of the no IL-1β group, when compared to the unloaded group. Figure 5.2

Media GAG: Release of GAG to media varied among tested samples. Samples treated with 10 ng/ml of reIL-1β and 100 ng/ml of rhIL-1β did not differ form either the untreated controls or the respective groups subjected to the same loading regimens. Whereas the groups treated with 100 ng/ml of reIL-1β and 200 rhIL-1β, a significant (P< 0.036) increase in GAG loss to the media was observed when compared to the unloaded groups on day 3 and a similar increase was noticed in the groups loaded at 2 MPa at a frequency of 0.1 Hz on day 3 and was significant (P=0.035) at 200 ng/ml rhIL-1β, but not on days 6 and 10. A significant (P< 0.012) decrease in media GAG content was seen on day 3 in the samples treated with 100 and 200 ng/ml rhIL-1β in the 6 MPa 1 Hz group when compared to their corresponding untreated controls. Loss of GAG to media decreased over time in all samples and was signicantly (P<0.036) decreased in the unloaded group and the group loaded at 2 MPa 0.1 Hz when compared to their respective untreated controls on days 6 and 10. Figure 5. 3.

Gene expression: Collagen (COL) I gene expression decreased progressively with increasing load. Gene expression of COLI was significantly (P< 0.034) decreased by all doses and types of IL-1β on day 3. COL I gene expression decreased with increasing load. The decreases were significant in the groups treated with IL-1β and the greatest decreases (P<0.011) were observed in the groups treated with reIL-1β in the unloaded control groups. Col I gene expression was also significantly decreased in the load only groups loaded at 1 Hz (P< 0.035). Table 5.4, Figure 5.4

In the loaded groups, the levels of COL II gene expression was significantly (P< 0.036) reduced, but not in the 0.1 Hz groups. No significant differences were seen in the load groups treated with 100 ng/ ml rhIL-1β except at 6 MPa 1 Hz. A greater decrease was seen in the group loaded at 6 MPa 1 Hz when compared to the other groups. An interesting finding here is that in all groups loaded at 2 MPa 0.1 Hz, 2 MPa 1 Hz and 6 MPa 0.1 Hz, the gene expression of COL II was higher than in the no load IL-1β groups when compared to the day 3 groups treated with only IL-1β, also where as all unloaded groups treated with IL-1β were significantly (P<0.036) lower on day 10 than the corresponding no load control no such decrease was observed the groups loaded at 0.1 Hz and treated with IL-1β. Though a general trend of further decrease in collagen gene expression was seen in all groups where combinations of load and IL-1β were used, these were not significant when compared to their respective load only groups except at 6 MPa 1 Hz. Figure 5.5.

Aggrecan gene expression was significantly (P<0.012) reduced by 100 ng/ml reIL-1β or 200ng/ml rhIL-1β in the unloaded group. Aggrecan gene expression progressively decreased with increasing load. A significant (P< 0.036) decrease was seen in aggrecan gene expression in all load and IL-1β combinations. In the load only groups a significant (P< 0.021) decrease was seen only at 1 Hz on day 3. Figure 5.6.

MMP 1 gene expression was significantly (P= 0.007) increased in the group loaded at 6 MPa 1 Hz and the groups treated at 200 ng/ml rhIL-1β on day 3, but was significantly (P= 0.017) below the no load group treated at a similar concentration of rhIL-1β. Though a marked increase was seen in both the re and rh groups only the 10 ng/ml re group was significant (P=0.012) when compared the day 3 controls in the unloaded group, but when compared to the day 10 all the groups were significantly (P<0.034) higher than the controls. MMP 1 gene expression was relatively decreased by day 10 in all samples where an increase was seen at day 3. The greatest increases in MMP 1 gene expression occurred in the no load groups which were significantly (P<0.043) higher than the groups where a combination of load and IL-1β were used. Figure 5.7

MMP 13 gene expression in the unloaded group showed a significant (P<0.011) increase when exposed to IL-1β but this was more with either 100 ng/ml or 200 ng/ml rhIL-1β on day 3 and was further elevated at day 10 in these groups, but on day 10 MMP 13 gene expression in the 100 ng/ml re IL-1β was higher than a similar

concentration of rh IL-1β. In the groups where a combination of IL-1β and load was used, an increase was observed on day 10 in the groups treated with IL-1β and loaded at 0.1 Hz. Such increases were also observed at other combinations of load and IL-1β. However these levels remained below ($P<0.036$) the no load groups only treated with IL-1β. The groups loaded at 1 Hz with IL-1β showed an increase in MMP gene expression on day 3, whereas in the 0.1 Hz groups, this increase was seen on day 10 indicating the effects of frequency. Figure 5.8

TIMP -1 gene expression decreased with increased dose and load levels, when compared to the untreated controls and the respective no IL-1β controls used in each group. An exception to this trend were the groups loaded at 6 MPa 1 Hz and exposed to 100 ng/ml reIL-1β or 200 ng/ml rhIL-1β, where there was a significant ($P<0.013$) increase when compared to the untreated control group subjected to similar loading regimen. A strong but insignificant increase in TIMP 1 gene expression was also seen in the unloaded group treated with 200 ng/ml of rhIL-1β. Figure 5.9

Gene expression patterns of COX 2 showed a significant increase in the 200ng/ml rhIL-1β group in the unloaded samples. A significant ($P<0.013$) increase was seen in the groups loaded at 6 MPa 1 Hz and treated with 100 ng/ml reIL-1β, 100 ng/ml rh IL-1β and 200 ng/ml rh IL-1β on day 3, but this was not seen on day 10. In the unloaded groups this increase ($P< 0.036$) was sustained through day 10. Though the levels of COX 2 gene expression were elevated in all treated groups when compared to the untreated controls at both time points this difference was not significant. Figure 5.10.

Nitric oxide: Although an increase in release of NO was seen in the treated groups when compared to the controls, these were not significant.. (Data not shown).

PGE2 Assay: The release of PGE 2 to the media as measured by the PGE2 assay showed a significantly ($P<0.036$) higher release at all doses of re and rhIL-1β in the unloaded group except at re 10 ng/ml. In the groups where both load and IL-1β were used, though not significant the levels were above their respective only load groups, but were still below their respective IL-1β treated no load groups. However the levels of PGE 2 remained below their corresponding no load IL-1β group at all times. Figure 5.11

Cell Viability: Cell viability was significantly ($P< 0.037$)decreased in all the groups loaded at 6 MPa 1 Hz and in groups exposed to IL-1β in the unloaded groups except the 10 ng/ml re IL-1β($P=0.067$) unloaded group. Although significantly ($P=0.037$) decreased when compared to the untreated controls at day three cell viability was better with a similar dose of rh IL-1β than with 100 ng/ml re IL-1β. In the load only groups loading frequencies of 0.1 Hz did not cause a significant ($P>0.059$) decrease in cell viability on day 3 when compared to the day 3 controls. A combination of 2 and 6 MPa at frequency of 1 Hz and does of 100 ng/ml reIL-1β or 200 ng/ml rhIL-1β showed the maximum cell death even at day 3. Cell death progressively increased in all groups from day 3 to 10 when compared to the untreated controls. Figure 5.12

Discussion:

The most interesting finding of this study was the ability of load to moderate the delitirious effects of IL-1β in an *in vitro* situation. Traditionally, OA has been thought to be caused by wear and tear resulting in noninflammatory arthrosis. It is now increasingly being seen as a mechanically driven, chemically mediated process, where various factors such as mechanical loads, hydrostatic pressures, and soluble mediators from the subchondral bone, synovium, synovial fluid, and cartilage influence the activity of the chondrocytes, resulting in ultimate cartilage breakdown. Cyclic loading can cause an increase in MMP expression and activity in cartilage, [55] and inflammatory cytokines such as TNF-α, IL-1β, and IL-6 further disrupt cartilage homeostasis resulting in progressive, MMP-mediated digestion of cartilage matrix in OA.[56]

A transient increase in collagen denaturatation of 50-100% was seen in bovine cartilage exposed to repeated compressive stresses of 3.5–6.5 MPa.[57] An increase in denatured collagen was also reported by other investigators.[58] The decrease in collagen content in our tissues was not significant in any of the treated groups, but gene expression patterns varied widely indicating a potential decrease in the synthesis of new collagen. Although collagen content is maintained initially in the early stages of OA, its organization is severely perturbed in more advanced stages of OA. [59] This could explain the increased hydration seen in other studies,[60,61] and even though we did not study this parameter, we did notice an increase in the tissue size in some of the treated groups where load was applied. It is possible that since the collagen network prevents the tissue from swelling to more than 20% of its volume,[60] a breakdown in the collagen network would result in a failure of this restriction, thus resulting in tissue swelling.

Collagen type I is produced by hypertrophic and osteoarthritic chondrocytes, as well as fibroblasts.[62] The decrease in the expression of COL-I needs to be further investigated as one would expect to see an increase in its expression in osteoarthritic tissue. Type-I collagen is known to be present early in chondrogenesis but later becomes undetectable by standard methods.[63] However, type-I collagen was detectable in the cartilage of adult pigs after prolonged digestion procedures. [64] The samples in our study were collected from horses with a mean age of less than 2 years. The fact that horses of this age are still growing and maturing could explain the presence of collagen type I in the untreated samples. In other studies from our laboratory, we have observed a decrease in COL I expression during culture even with IL-1β and feel this may be an artifact of culturing.

Moderate exercise has been shown to be beneficial for cartilage.[65,66] We saw an increase in gene expression of collagen II in all groups treated at 2 MPa and in the 6 MPa 0.1 Hz group where a combination of IL-1β and load were used. These results are in agreement with a previous study, where they found that cyclic tensile strain abrogates the affects of IL-1β on chondrocytes,[67] but these beneficial effects of loading were not seen in the group loaded at 6 MPa 1 Hz where the two seemed to have a synergistic effect. Patients diagnosed with OA have also reported decreased pain and improved mobility after exercise.[68] The increased COL-II gene expression and GAG content noted in our study could indicate that moderate levels of load may help overcome some of the deleterious effects of IL-1β or it is possible that these increases were a compensatory response of cartilage to degradation caused by IL-1β.

Aggrecans are the most widely studied proteoglycans. Because of their high negative charge and water-binding capacity, aggrecan molecules provide the mechanical properties of compressibility and elasticity to the cartilage and have also been shown to have a protective effect on collagen II.[69] In a previous study,[70] a decrease in Aggrecan expression in OA cartilage was reported in the superficial layer. We found an overall decrease in aggrecan gene expression in our samples with increasing frequency and dose combinations. These data agree with our GAG assay data. However, further investigation is needed to determine if the decrease in aggrecan was primarily associated with the superficial layer or the entire explant. Since we saw increased chondrocyte death in all layers of samples treated with these combinations of load, frequency, duration and various formulations and concentrations of IL-1β, the decrease in aggrecan was probably throughout the tissue.

Various studies have shown that proteoglycan is rapidly depleted in models of articular cartilage degradation.[71-73] A similar trend in the declining GAG content in the tissue was observed in all our treatment groups. We also saw an increased release of GAG to the media in all groups treated with either re or rh IL-1β without load, or a load of 2 MPa or 6 MPa at a frequency of 0.1 HZ, and high dose of re or rh IL-1β. These results go well with the results of tissue GAG content in the unloaded group as it was these groups which saw the maximum decrease in GAG content. In the loaded group the maximum decrease in tissue GAG content was seen in the groups loaded at either 2 or 6 MPa or a frequency of 1 Hz. Therefore, we might expect to see an increase in release of GAG to the media in these groups. A possible explanation could be that since

cell death was rapid in the group loaded at 6 MPa 1 Hz, there was no production of GAG and what was being released to the media was what was in the cartilage. The size of our explants was only 3 mm, and therefore the quantity of GAG produced may have been below the detectable range of our assay. It could also be that in the groups where we saw an increased release of GAG to the media, it was due to a compensatory increase in GAG synthesis by the cells. But, given that aggrecan gene expression was not increased in these tissues, this is an unlikely explanation.

MMPs are responsible for digestion of cartilage ECM. MMPs, particularly MMP-1 and 13, are thought to play a major role in the degradation of type II collagen OA.[74,56] MMP-3 has been reported to be involved in the degradation of proteoglycans [75] TIMPs are the inhibitors of MMPs and in the normal joint the levels of MMPs and TIMPs are tightly regulated. In OA the levels of TIMPs and MMPs become imbalanced. [56] Cyclic loading can increase MMP expression and activity in cartilage.[55] Inflammatory cytokines such as TNF-α, IL-1β, and IL-6 further disrupt cartilage homeostasis resulting in progressive, MMP-mediated digestion of cartilage matrix in OA.[56] In our study, a similar trend was noted with an increase in MMP gene expression on day 3 but not at day 10 in the groups with a combination of load and IL-1β and a continued increase in groups treated with only IL-1β. The decrease in MMP levels on day 10 could be due to increased cell death. Although MMPs appear to be the principle molecules responsible for matrix catabolism, the molecular mechanism of MMP regulation remains unclear. In a previous study done in our laboratory, increasing frequency and load caused an

increase in MMP gene expression. When evaluated separately, an increase in MMP gene expression was still seen in the loaded groups in this study, but the increase was much greater in the IL-1β only groups. In the loaded groups also treated with IL-1β these levels are significantly below the IL-1β only groups. Xu et al.,[67] showed that cyclic tensile strains of 0.05 Hz reduced MMP expression in IL-1β stimulated chondrocytes. Similar results were seen in synovial cells stimulated with IL-1β and exposed to tensile strains of 1 Hz.[76] Our results were in agreement with these studies as we saw a marked increase in the IL-1β only groups, but this response significantly decreased in the groups where both load and IL-1β were used in combination. Additionally, an increased degradation of collagen and proteoglycans was seen in our study when load and IL-1β were used in combination. Further studies are required to determine why MMP expression is reduced in the presence of increased degradation of collagen and proteoglycans when exposed to the combination of load and IL-1β. Both load [72,66] and IL-1β[56] have been reported to increase MMP synthesis individually and we expected to see a more pronounced and severe effect of these in combination. Although mechanical overload may stimulate deregulation of MMP expression in chondrocytes, inflammatory cytokines are the main promoters of this response or may represent an alternative pathway in the development of OA.

IL-1β has been shown to alter chondrocyte metabolism to increase MMP synthesis, and decrease the synthesis of collagen, proteoglycans, and inhibitors of MMP. [56] A similar trend was noted in our samples loaded at higher frequencies and exposed to higher doses of IL-1β. Others have reported a species specific response to

re Vs rh IL- 1β. [45] Our results were consistent with this provious report in that equine articular cartilage is more sensitive to re IL-1β than rh IL-1β.

The increase in TIMP-1 gene expression was observed mostly in samples where there was a simultaneous increase in MMP gene expression on day 3. Previous studies have also reported an increase in TIMP expression with high magnitudes of cyclic load. [77] This could be a compensatory mechanism whereby an increased production of TIMPs was attempted to control the rising levels of MMPs in those samples at day 3.

The expression of COX 2 and its main product PGE 2 showed a similar trend. COX 2 gene expression and PGE 2 release were significantly increased in the IL-1β only group and the load + IL-1β group at 6 MPa 1 Hz. It is possible that dynamic compression may suppress the effects of IL-1β, but when an over load occurs these have a synergistic effect. Another possibility could be that OA chondrocytes have a higher sensitivity to the stimulation of metalloprotease synthesis by IL-1β than do normal cells and since the cells exposed to these loads were probably damaged, they could be more sensitive to stimulation by IL-1β.

Previous studies have demonstrated that mechanical loading can have effects on chondrocyte viability and matrix breakdown, which are more pronounced in the superficial zone.[78,79,66] In our study, superficial cell death was observed in all loaded samples. Similar results have been reported in other studies done in our laboratory and by other investigators.[78,79,66] One of the reasons for superficial cell death could be that it is due to contact of tissue with our loading platen. The platens used in our study are relatively smooth and non porous, but are rigid and hard and may be the cause for

superficial cell death. Similar patterns of cell death have been reported in other loading studies[66] and also in studies where two cartilage pieces were placed opposing each other.[78] It is possible that synovial fluid, which is present between the opposing cartilage surfaces, in an *in vivo* environment, provides a cushion to the cartilage and in *in vitro* studies; cell death could be increased due to the absence of synovial fluid. Another possible explanation could be the absence of subchondral bone, which would provide further support *in vivo*. Further the compositional differences among the different layers of the cartilage could also play a role in cell death.[66] Previous studies have also reported an increase in cell death due to mechanical trauma and it is likely the cells in the superficial zone of the cartilage are directly exposed to the force and hence could result in the increased cell death observed in this zone.[79,66]

Another factor for death in the superficial zone involves the compressive modulus, which is lowest at the superficial zone and deforms up to 25 times more than the middle or deep zones.[80] This deformation could lead to membrane rupture and eventual cell death. Further, we see a pattern of increasing cell death even in the deeper zones with increased frequency over a period of 10 days in our explants. This indicates that higher frequency and prolonged loading may cause cartilage damage. This may not be true in the live animal where other factors are involved which support the cartilage and weight probably gets distributed over a wider surface area. Also one could argue that in an *in vivo* situation the likelihood of the same section of cartilage being exposed to loads at a frequency of 1 Hz for 20 minutes would be unlikely, if not impossible. However even in an *in vivo* situation, long term strenuous exercise has been shown to cause osteoarthritis like changes in cartilage.[81,82] Also, the exact

mechanism leading from trauma/ excessive load to cell death needs to be investigated and it could well be that certain other factors such as caspases[83] which are also activated by IL-1β [84] are activated and involved in the actual process of initiating cell death rather than the trauma itself.

Recently Thomas et al.,[85] reported an incidence of apoptosis as high as 44% in the most severely degenerate cartilage from horses. To survive and function properly, chondrocytes need an attachment to either the ECM or another cell. In cartilage degradation there is extensive loss of proteoglycans and a breakdown of the collagen network, resulting in a loss of chondrocyte anchorage to the ECM.[86] This could also be an explanation for the cell death noted in our study as we observed a decrease in proteoglycan content in our samples. Further investigation needs to determine if cell death is due to apoptosis or necrosis and if it precedes ECM degradation or is a sequel of this process. Though it is possible the cell death noted in our study was due to necrosis rather than apoptosis, apoptosis is generally seen in post-loading incubation.[79]

The results of this study indicate that moderate loads mitigate some of the deleterious effects of IL-1β, whereas at higher loads, where direct tissue damage occurs, the effects of load and IL-1β are synergistic. An ideal model for the study of OA should be one which causes tissue damage in a situation similar to the joint. This study demonstrates that exposing tissue to IL-1β or load alone causes increased tissue damage and degradation. However, the combination of IL-1β and load may prove to be a better model for the development of OA. This study has further shown the effects of

species specificity, as lower doses of re IL-1β were equally if not more effective than rh IL-1β on equine tissue. The ideal model for the *in vitro* study of OA should include intermittent loading in the presence of IL-1β.

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Table 5.1.: Description of treatment groups based on concentration of re IL-1β and rh IL-1β, and the level and frequency of load applied

	IL-1β (ng/ml)									
	re			rh		Load (MPa)			Frequency (Hz)	
Group	0	10	100	100	200	0	2	6	0.1	1
1	X					X				
2		X				X				
3			X			X				
4				X		X				
5					X	X				
6	X						X		X	
7		X					X		X	
8			X				X		X	
9				X			X		X	
10					X		X		X	
11	X						X			X
12		X					X			X
13			X				X			X
14				X			X			X
15					X		X			X
16	X							X	X	
17		X						X	X	
18			X					X	X	
19				X				X	X	
20					X			X	X	
21	X							X		X
22		X						X		X
23			X					X		X
24				X				X		X
25					X			X		X

Note: E= recombinant equine IL-1, H = recombinant human IL-1

Figure 5.1 Tissue HP content

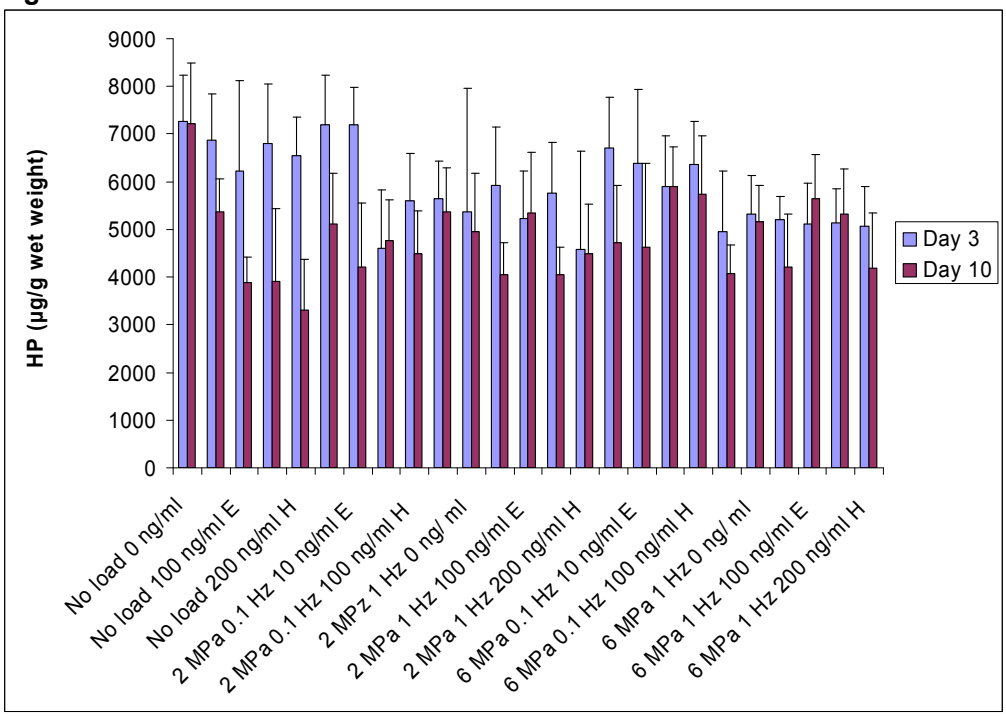
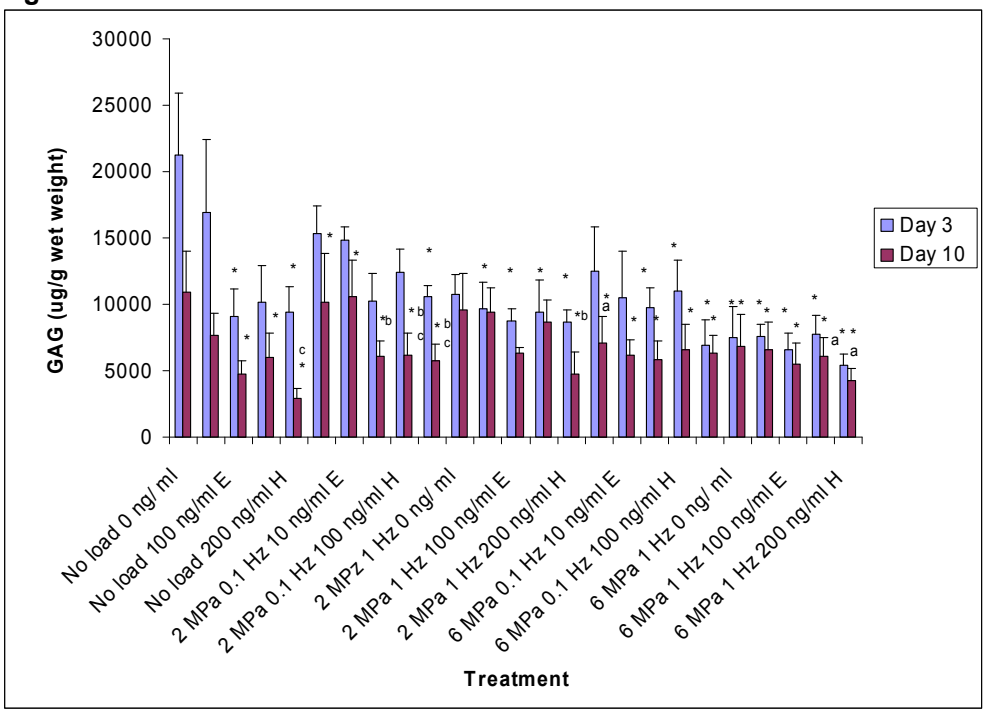
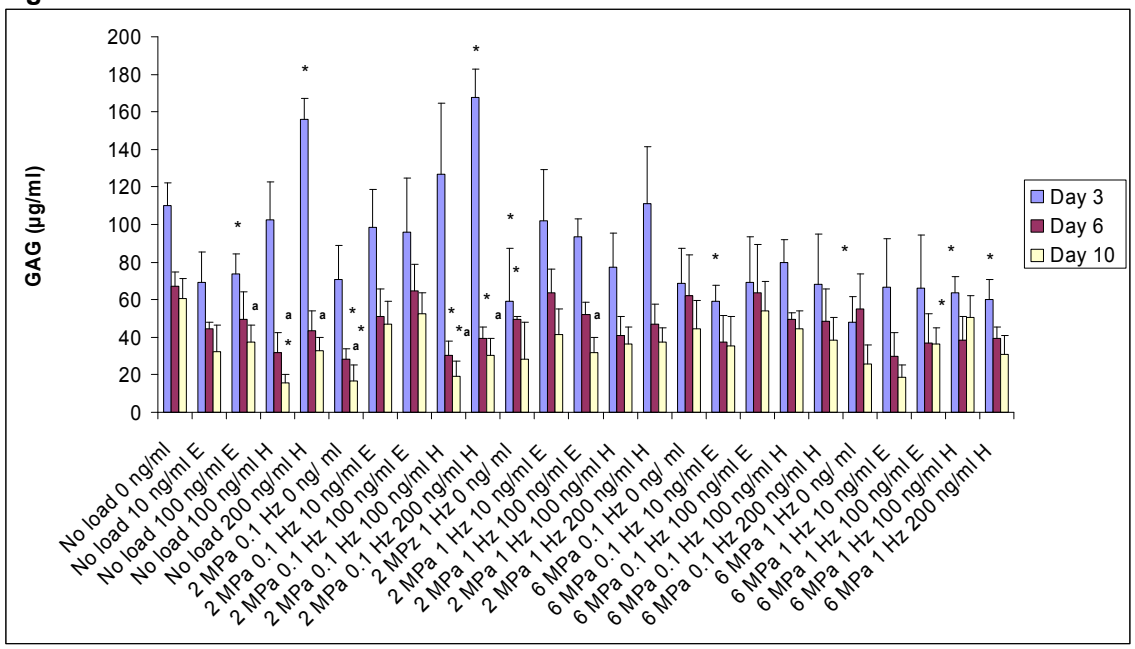


Fig 5.2 Tissue GAG content.



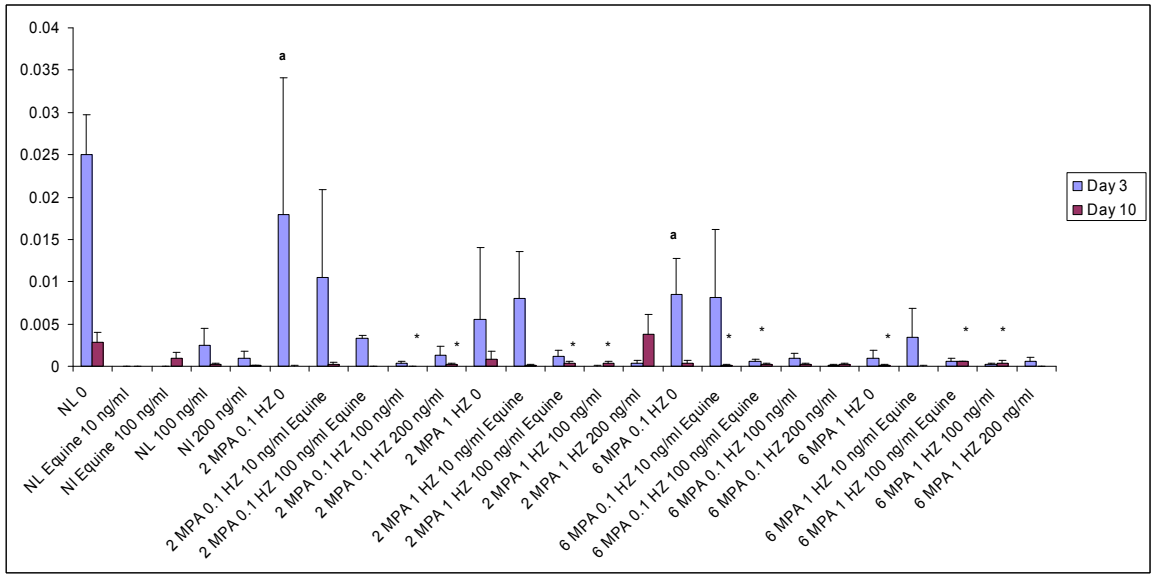
* significantly different from untreated day 3 control.
a significantly different from 2 MPa 0.1 Hz day 3
b significantly different form its no IL 1 group on day 3.
c significantly different form corresponding day 3 group.

Fig 5.3 Media GAG.



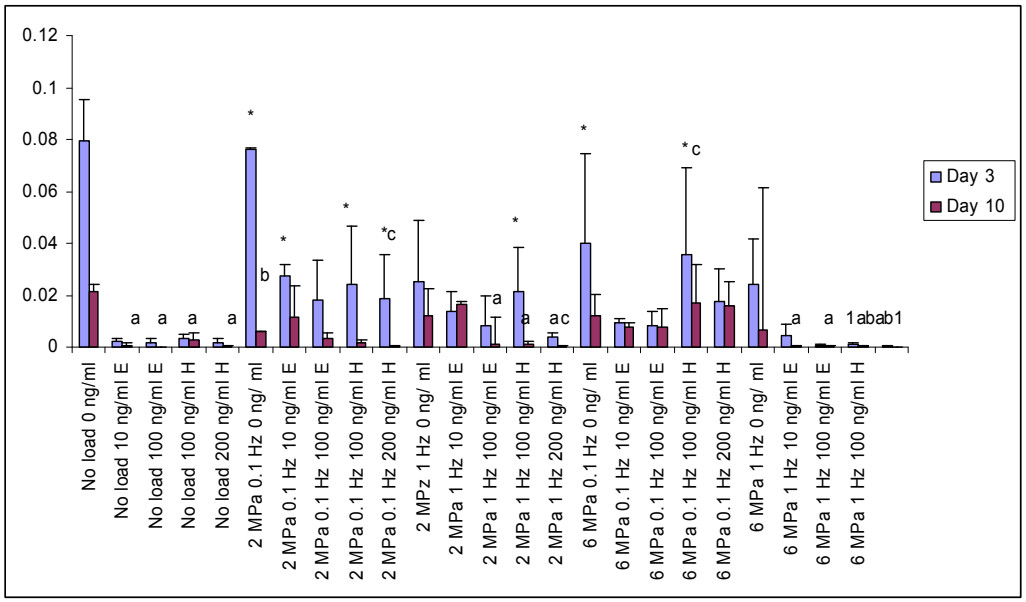
* Significantly different from corresponding no treatment control.
a Significantly different from corresponding day 3.

Fig 5.4 Col I Relative gene expression.



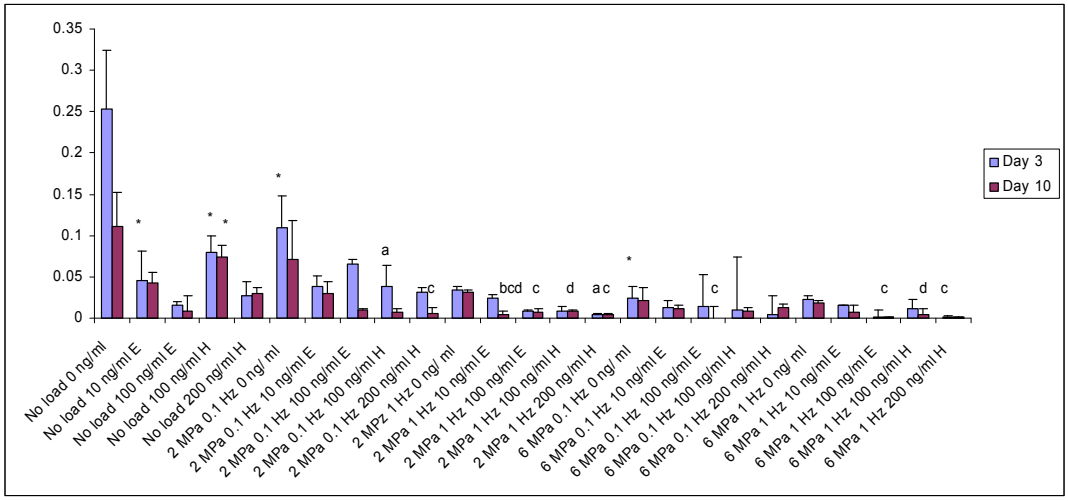
a Not significantly different from no load 0 ng/ ml day 3
* Significantly different from no load 0 ng/ ml day 10

Figure 5.5 Relative gene expression collagen II



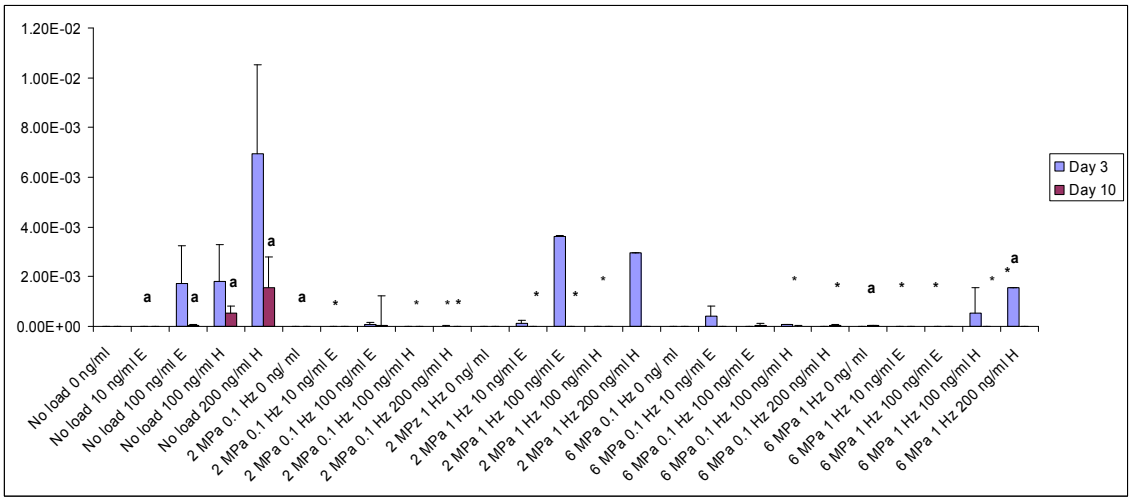
* not significantly different from no treatment control day 3
a significantly different from no treatment control day 10
b significantly different from its respective no IL 1 Control
1 significantly different from similar treatment at day 3
c significantly higher than 6 MPa 1 Hz 200 ng/ ml H day 10

Figure 5.6 Relative gene expression aggrecan.



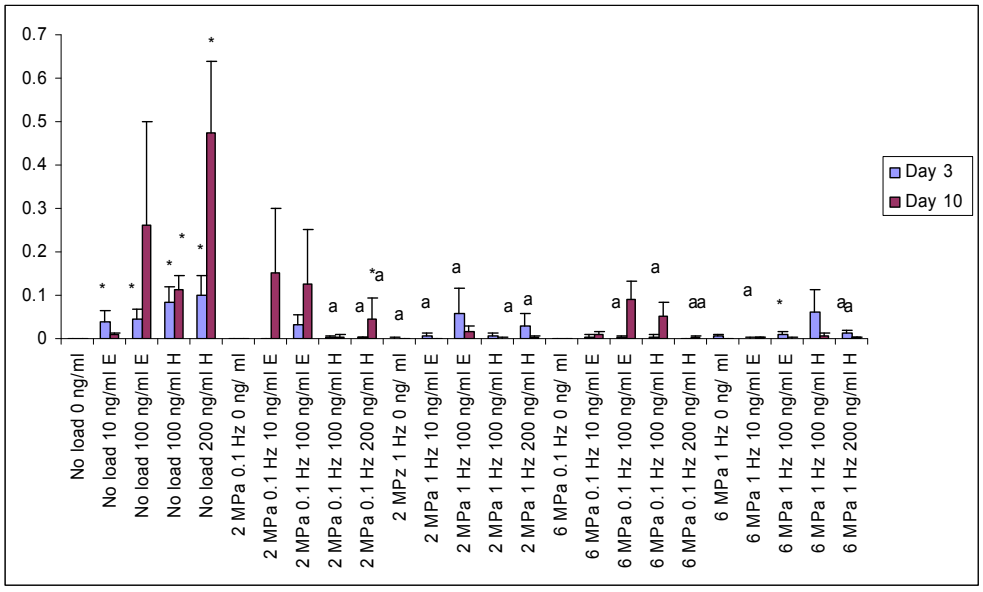
* not significantly different from no load 0 mg/ ml day 3.
a significantly different from 2 MPa 0.1 Hz 0 ng/ ml.
b significantly different from 10 ng/ ml E.
c significantly different from no load 0 mg/ ml day 10.
d significantly different from 2 MPa 0.1 Hz 100 ng/ ml H.
e significantly different from 2 MPa 0.1 Hz 10 ng/ ml E.

Figure 5.7 Relative gene expression MMP 1



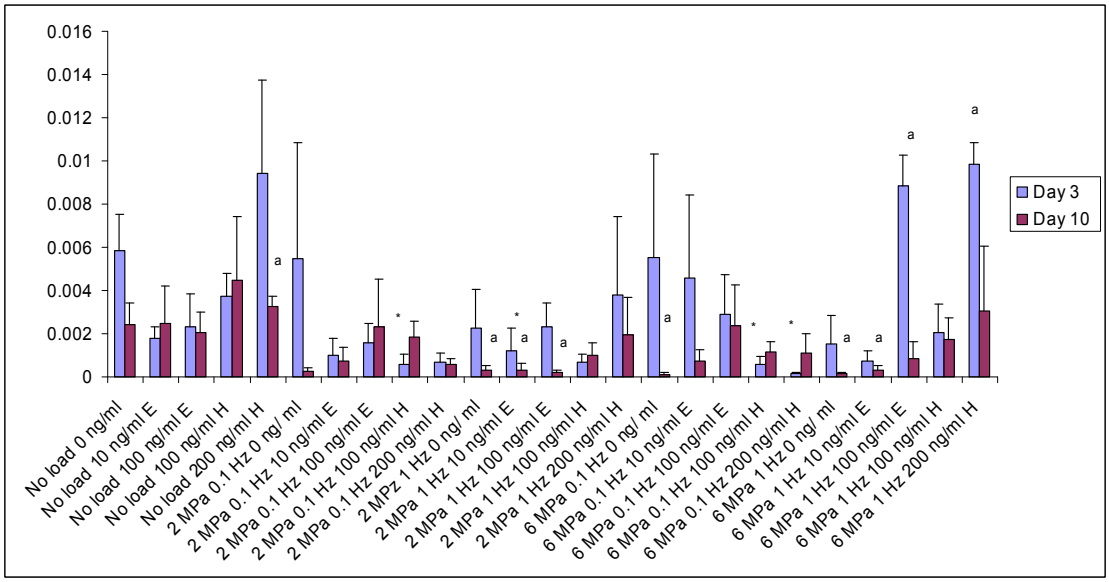
* Significantly different from corresponding no load group.
a Significantly different from corresponding control.

Figure 5.8 MMP 13 relative gene expression.



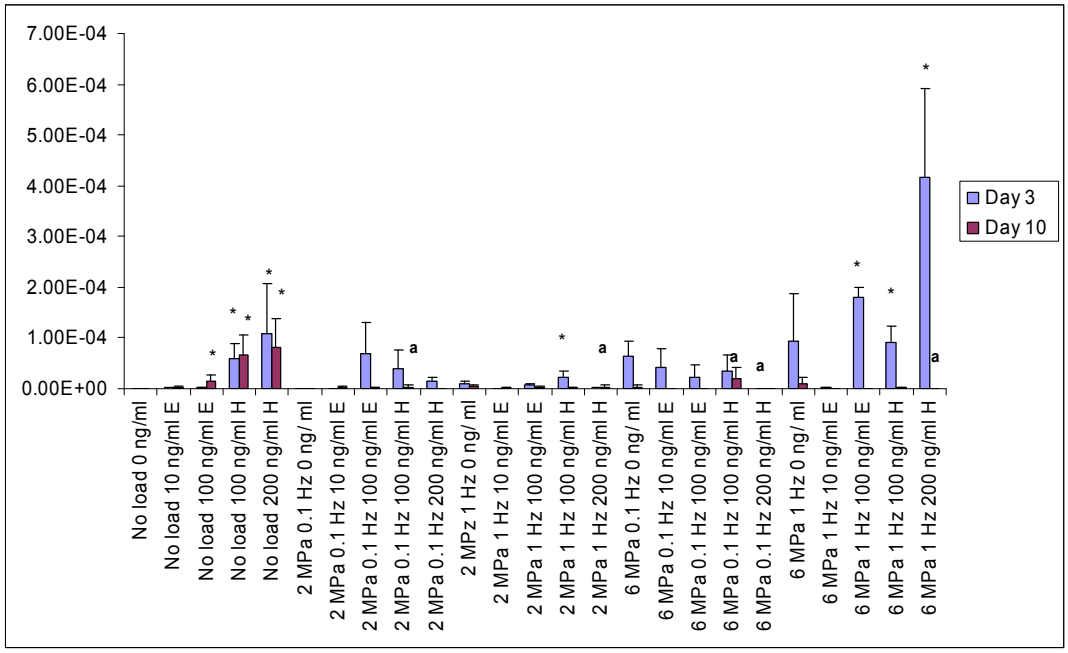
* Significantly different from corresponding control.
a Significantly different from corresponding no load group.

Figure 5.9 TIMP 1 relative gene expression.



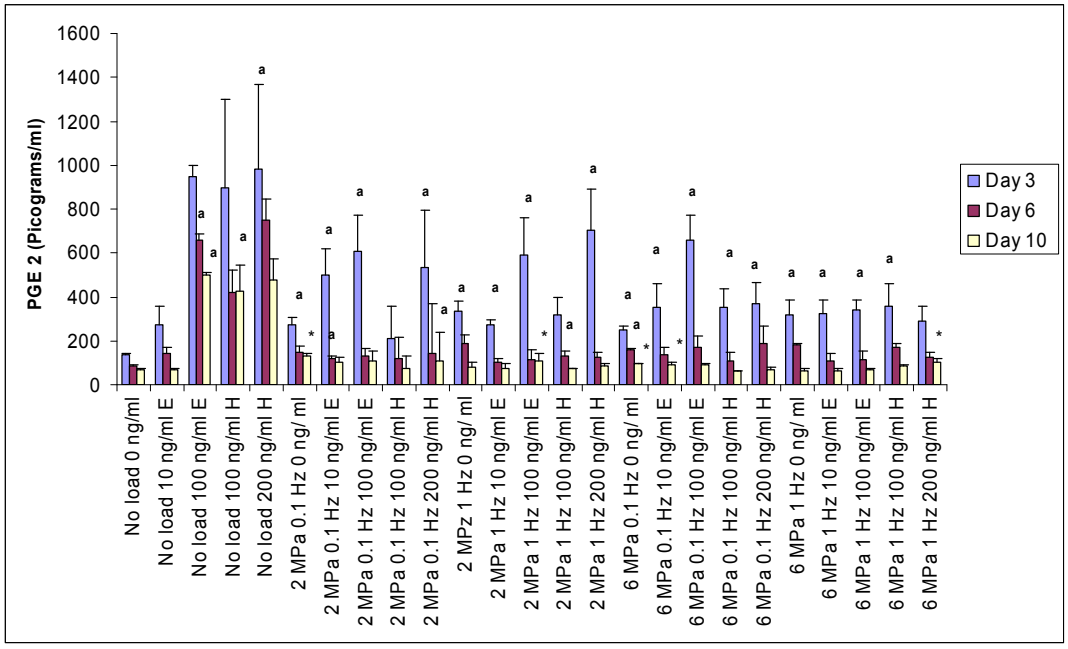
* Significantly different from corresponding no load group.
a Significantly different from corresponding control

Figure 5.10 COX 2 relative gene expression.



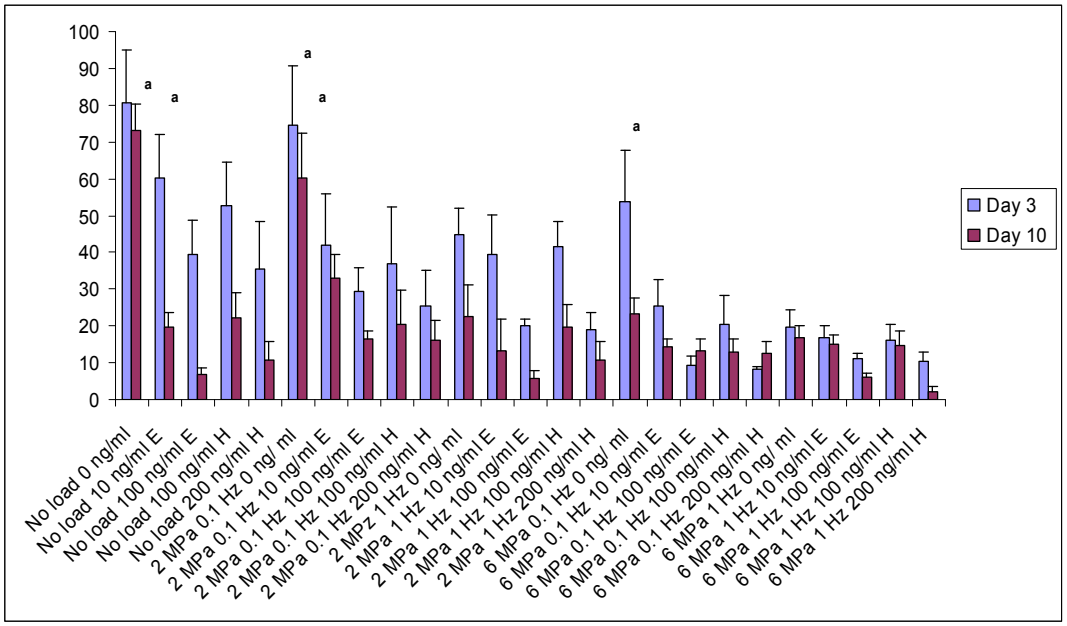
* Significantly different from corresponding control.
a Significantly different from corresponding no load group.

Figure 5.11 Media PGE_2.



* Significantly different from corresponding no load group.
a Significantly different from corresponding no treatment group.

Figure 5.12 Cell viability assay.



a Not significantly different from no load 0 ng/ml day 3.

Vita

Sree Sai Satish Adusumilli was born on 20 August 1978 in Vijayawada, India. After studying in various private and public schools in India. He received the following degrees Bachelor of Veterinary Science and Animal Husbandry for ANGR Agricultural University, Hyderabad, India in 1999 and a Masters in Veterinary Science from Indian Veterinary Research Institute, Izathnagar, Bareilly, India in 2002.